(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 December 2020 (17.12.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/249769 A1

(51) International Patent Classification:
C12Q 1/6883 (2018.01) *G01N 33/68* (2006.01)

(21) International Application Number:
PCT/EP2020/066364

(22) International Filing Date:
12 June 2020 (12.06.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
19305764.3 14 June 2019 (14.06.2019) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: METHODS AND COMPOSITIONS FOR TREATING OCULAR DISEASES RELATED TO MITOCHONDRIAL DNA MAINTENANCE

(57) Abstract: Inventors report heterozygous mutations in mitochondrial single-stranded DNA-binding protein SSBP1 in multiple unrelated families with non-syndromic dominant optic atrophy, associated in half of the cases by a striking occurrence of a foveopathy. SSBP1 is a key protein for the mtDNA replication machinery. Inventors have identified two mutations in SSBP1 in families with dominant optic atrophy and shown that SSBP1 mutation affects mtDNA replication. Patient fibroblasts displayed unstable formation of SSBP1 dimer/tetramer affecting mtDNA replication leading to a decrease of mtDNA copy-number. They provide evidences that SSBP1 deficiency in human results in a novel mtDNA syndrome resulting in an isolated visual defect. The present invention relates to a method for predicting the risk of having or developing ocular disease related to mtDNA maintenance by measuring at least one mutation in SSBP1. The invention relates also to treat ocular disease related to mtDNA maintenance by administering an inhibitor of SSBP1 gene expression or a vector which comprises a nucleic acid molecule encoding for SSBP1.

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METHODS AND COMPOSITIONS FOR TREATING OCULAR DISEASES RELATED TO MITOCHONDRIAL DNA MAINTENANCE

FIELD OF THE INVENTION:

The invention is in the field of ophthalmology. More particularly, the invention relates to methods and compositions for treating ocular diseases related to mitochondrial DNA maintenance.

BACKGROUND OF THE INVENTION:

Mitochondrial diseases are clinically heterogeneous and can be caused by mutations in either nuclear or mitochondrial DNA (mtDNA) (1). Mutations in genes known to be essential for mtDNA maintenance are recognized to cause mitochondrial DNA depletion syndromes (MDS) and mtDNA multiple deletion syndromes (2). Such genes encode components of the mtDNA replication machinery (POLG, POLG2, TWNK, TFAM, RNASEH1, MGME1 and DNA2), dNTP supply for mtDNA synthesis (TK2, DGUOK, SUCLG1, SUCLA2, ABAT, RRM2B, TYMP, SLC25A4, AGK and MPV17) and mitochondrial dynamics (OPA1, MFN2). Ocular involvement is a well-known clinical feature of mitochondrial disease that may occur as retinal, macular, optic nerve dysfunction, or progressive external ophthalmoplegia (PEO) and ptosis involving the extraocular muscles. In MDS, ocular features are rarely isolated and may be associated with neurological and/or multi-systemic presentation including muscle, liver and kidney.

Autosomal dominant optic atrophy (ADOA) is characterized by progressive visual loss, central scotoma, dyschromatopsia and optic atrophy due to degeneration of the retinal ganglion cells and their axons which form the optic nerve. Mutations in OPA1, which encodes a dynamin-like GTPase involved in mitochondrial fusion and mtDNA maintenance (3-8), is the leading cause of ADOA (9, 10). However, a decent fraction of ADOA still remains unsolved and are likely caused by mutations in other genes.

SUMMARY OF THE INVENTION:

The invention relates to a method for predicting the risk of having or developing ocular disease related to mitochondrial DNA (mtDNA) maintenance in a subject, comprising the steps of: i) identifying at least one mutation in the SSBP1 gene and/or protein; ii) concluding that the subject is at risk of having or developing ocular disease related to mtDNA maintenance when at least one mutation is identified. In particular, the invention is defined by claims.

DETAILED DESCRIPTION OF THE INVENTION:

Here, inventors report heterozygous mutations in mitochondrial single-stranded DNA-binding protein SSBP1 in multiple unrelated families with non-syndromic dominant optic atrophy, associated in half of the cases by a striking occurrence of a foveopathy. SSBP1 is a key protein for the mtDNA replication machinery. Inventors have identified two mutations in SSBP1 in families with dominant optic atrophy and shown that SSBP1 mutation affects mtDNA replication. Patient fibroblasts displayed unstable formation of SSBP1 dimer/tetramer affecting mtDNA replication leading to a decrease of mtDNA copy-number. They provide evidences that SSBP1 deficiency in human results in a novel mtDNA syndrome resulting in an isolated visual defect.

Method for predicting the risk of having or developing ocular disease related to mtDNA maintenance

Accordingly, in a first aspect, the invention relates to a method for predicting the risk of having or developing ocular disease related to mtDNA maintenance in a subject comprises following steps: i) determining the expression level of SSBP1 gene, ARN or protein in a biological sample obtained from said subject, ii) comparing the expression level of SSBP1 gene, ARN or protein determined at step i) with a predetermined reference value and iii) concluding that the subject is at risk of having or developing ocular disease related to mtDNA maintenance when the expression level of SSBP1 gene, RNA or protein determined at step i) is lower than the predetermined reference value, or concluding that the patient is not at risk of having or developing ocular disease related to mtDNA maintenance when the expression level of SSBP1 gene, RNA or protein determined at step i) is higher than the predetermined reference value.

As used herein, the term "predicting" means that the subject to be analyzed by the method of the invention is allocated either into the group of subjects who will have or develop ocular disease related to mtDNA maintenance, or into a group of subjects who will not have or develop ocular disease related to mtDNA maintenance. Having or developing ocular disease related to mtDNA maintenance referred to in accordance with the invention, particularly, means that the subject will have higher risk to have or develop ocular disease related to mtDNA maintenance. Typically, said risk is elevated as compared to the average risk in a cohort of subjects suffering from ocular disease related to mtDNA maintenance.

In the context of the invention, the risk of having the ocular disease related to mtDNA maintenance in a subject susceptible to suffer from ocular disease related to mtDNA maintenance shall be predicted. The term "predicting the risk", as used herein, refers to

assessing the probability according to which the patient as referred to herein will have or develop ocular disease related to mtDNA maintenance.

As will be understood by those skilled in the art, such an assessment is usually not intended to be correct for 100% of the subjects to be investigated. The term, however, requires that prediction can be made for a statistically significant portion of subjects in a proper and correct manner. Whether a portion is statistically significant can be determined without further ado by the person skilled in the art using various well known statistic evaluation tools, e.g., determination of confidence intervals, p-value determination, Student's t-test, Mann- Whitney test etc. Details are found in Dowdy and Wearden, Statistics for Research, John Wiley & Sons, New York 1983. Preferred confidence intervals are at least 90%, at least 95%, at least 97%, at least 98% or at least 99 %. The p-values are, preferably, 0.1, 0.05, 0.01, 0.005, or 0.0001. Preferably, the probability envisaged by the invention allows that the prediction of an increased risk will be correct for at least 60%, at least 70%, at least 80%), or at least 90% of the subjects of a given cohort or population.

As used herein, the term "ocular disease" refers to diseases in the eye. Typically, the ocular disease is selected from the group consisting of but not limited to: cataract, age-related macular degeneration (AMD), glaucoma, retinal diseases etc. In the context of the invention, the ocular disease is related to mtDNA maintenance. Typically, ocular involvement is a well-known clinical feature of mitochondrial disease that may occur as retinal, macular, optic nerve dysfunction, or progressive external ophthalmoplegia (PEO) and ptosis involving the extraocular muscles.

As used herein, the term "mtDNA" refers to mitochondrial DNA. Typically, mutations in genes encoding components of the mitochondrial DNA (mtDNA) replication machinery are causing mtDNA depletion syndromes (MDS), which associate ocular features to severe neurological syndromes. Inventors have shown that mutation in SSBP1 gene and/or protein level affects the fidelity of the mtDNA replication, but does not promote mtDNA recombination. For the first time, inventors have shown that ocular diseases caused by pathogenic variants in the nuclear genes involved in mtDNA maintenance resulting in impaired mtDNA synthesis leading to quantitative (mtDNA depletion) and qualitative (multiple mtDNA deletions) defects in mtDNA. Defective mtDNA leads to eye dysfunction due to insufficient mtDNA-encoded protein synthesis, resulting in an inadequate energy production to meet the needs of affected ocular system.

In a particular embodiment, the method according to the invention, wherein the ocular disease related to mtDNA maintenance is selected from the group consisting of but not limited to: optic neuropathy, Autosomal dominant optic atrophy (ADOA), Foveopathy, Leber's hereditary optic neuropathy (LHON), glaucoma, retinal macular dystrophy, optic nerve dysfunction, or progressive external ophthalmoplegia (PEO), or ptosis involving the extraocular muscles.

In a particular embodiment, the ocular disease related to mitochondrial DNA (mtDNA) maintenance is optic neuropathy. Typically, the optic nerve contains axons of nerve cells that emerge from the retina, leave the eye at the optic disc, and go to the visual cortex where input from the eye is processed into vision. As used herein, the term “optic neuropathy” refers to damage to the optic nerve due to any cause. Damage and death of these nerve cells, leads to characteristic features of optic neuropathy.

The optic neuropathy can result from various reasons, such as, Ischemic optic neuropathy, Optic neuritis, Compressive optic neuropathy, Infiltrative optic neuropathy, Traumatic optic neuropathy, mitochondrial optic neuropathy, Nutritional optic neuropathies, toxic optic neuropathies, hereditary optic neuropathies.

In some embodiments, the optic neuropathy condition may be selected from such conditions as, but not limited to: traumatic neuropathy (that may result from any type of trauma to the optic nerve); ischemic neuropathy (such as, for example, Nonarteritic Anterir Ischemic Optic neuropathy (NAION), Anterior ischemic optic neuropathy (AION), Posterior ischemic optic neuropathy); Radiation optic neuropathy (RON)), Glaucoma, Optic neuritis, Compressive optic neuropathy, Infiltrative optic neuropathy, Mitochondrial optic neuropathy, Nutritional optic neuropathies, toxic optic neuropathies, Hereditary optic neuropathy and the like; or combinations thereof. Each possibility is a separate embodiment.

In a particular embodiment, the ocular disease related to mitochondrial DNA (mtDNA) maintenance is Autosomal dominant optic atrophy (ADOA). ADOA is characterized by progressive visual loss, central scotoma, dyschromatopsia and optic atrophy due to degeneration of the retinal ganglion cells and their axons which form the optic nerve.

In a particular embodiment, the ocular disease related to mitochondrial DNA (mtDNA) maintenance is foveopathy. As used herein, the term “foveopathy” refers to an atrophy of fovea. Fovea is a small, central pit composed of closely packed cones in the eye. It is located in the center of the macula lutea of the retina.

In a further embodiment, the ADOA is combined with a singular foveopathy.

In a particular embodiment, the ocular disease related to mitochondrial DNA (mtDNA) maintenance is non-syndromic dominant optic atrophy. In a further embodiment, the non-syndromic dominant optic atrophy is combined with a singular a foveopathy.

In a particular embodiment, the ocular disease related to mitochondrial DNA (mtDNA) maintenance is inherited optic neuropathy.

As used herein, the term “inherited optic neuropathy” covers a spectrum of clinically and genetically heterogenic conditions. In the context of the invention, the inherited optic neuropathy is Leber hereditary optic neuropathy (LHON). LHON is mainly characterized by bilateral, painless subacute loss of central vision during young adult life. In the context of the invention, the inherited optic neuropathy is dominant optic atrophy OPA1.

As used herein, the term “subject” denotes a mammal, such as a rodent, a feline, a canine, and a primate. Particularly, the subject according to the invention is a human. More particularly, the subject according to the invention has or is susceptible to have an ocular disease related to mtDNA maintenance. In particular embodiment, the subject has or is susceptible to have Autosomal dominant optic atrophy (ADOA). In particular embodiment, the subject has or is susceptible to have ADOA accompanied with a foveopathy. In particular embodiment, the subject has or is susceptible to have dominant optic atrophy OPA1.

In a particular embodiment, the subject has or is susceptible to have dominant optic atrophy. In a particular embodiment, the subject has or is susceptible to have foveopathy.

In particular embodiment, the subject has or is susceptible to have dominant optic neuropathy accompanied with foveopathy

As used herein, the term "SSBP1” refers to single-stranded DNA-binding protein. SSBP1 is a housekeeping gene involved in mitochondrial biogenesis. The gene SSBP1 encodes mitochondrial single-stranded DNA-binding protein and interacts with POLG and TWINKLE at the mitochondrial replication fork to stimulate synthesis of mtDNA (25).

During replication, SSBP1 binds to single-stranded mtDNA (ssmtDNA) as a tetramer and prevents re-annealing and nucleolytic attacks.

The naturally occurring human SSBP1 gene has a nucleotide sequence as shown in Genbank Accession numbers NM_001256510, NM_001256511, NM_001256512, NM_001256513 and NM_003143. The naturally occurring human SSBP1 protein has an aminoacid sequence as shown in Genbank Accession numbers NP_001243439, NP_001243440, NP_001243441, NP_001243442 and NP_003134.

As used herein, the term "gene" has its general meaning in the art and refers to means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed.

As used herein the "allele" has its general meaning in the art and refers to an alternative form of a gene (one member of a pair) that is located at a specific position on a specific chromosome which, when translated result in functional or dysfunctional (including nonexistent) gene products.

As used herein, the term “protein” has its general meaning in the art and refers to one or more long chains of amino acid residues which comprise all or part of one or more proteins or enzymes. Typically, SSBP1 protein is involved in mtDNA replication and binds to single-stranded mtDNA (ssmtDNA) as a tetramer and prevents re-annealing and nucleolytic attacks.

As used herein, the term “RNA” for “Ribonucleic acid” has its general meaning in the art and refers to a polymeric molecule essential in various biological roles in coding, decoding, regulation and expression of genes. Like DNA, RNA is assembled as a chain of nucleotides, but unlike DNA, RNA is found in nature as a single strand folded onto itself, rather than a paired double strand. RNA includes messenger RNA and non-coding RNAs such as transfer RNA, ribosomal RNA. Messenger RNA (mRNA) is the RNA that carries information from DNA to the ribosome, the sites of protein synthesis (translation) in the cell.

In some embodiment, the RNA expression level determined in step i) is messenger RNA (mRNA).

As used herein, the term “biological sample” refers to any sample obtained from a subject, such as a serum sample, a plasma sample, a urine sample, a blood sample, a lymph sample, or a tissue biopsy. In a particular embodiment, biological sample for the determination of an expression level include samples such as a blood sample or a urine sample, lymph sample, or a biopsy.

In a particular embodiment, the biological sample is a blood sample, more particularly, peripheral blood mononuclear cells (PBMC). Typically, these cells can be extracted from whole blood using Ficoll, a hydrophilic polysaccharide that separates layers of blood, with the PBMC forming a cell ring under a layer of plasma. Additionally, PBMC can be extracted from whole blood using a hypotonic lysis, which will preferentially lyse red blood cells. Such procedures are known to the experts in the art.

Inventors have shown that missense mutations in SSBP1, leading to the R38Q and R107Q amino-acid changes in the mitochondrial single-stranded DNA-binding protein, a crucial protein involved in mtDNA replication.

In a particular embodiment, the method according to the invention, wherein the mutation is c.113G>A and/or c.320G>A in the SSBP1 gene.

In a particular embodiment, the method according to the invention, wherein the mutation is R38Q and/or R107Q in the SSBP1 protein.

Accordingly, the present invention also relates to a method for predicting the risk of having or developing ocular disease related to mtDNA maintenance in a subject in need thereof, comprising the step of detecting SSBP1 single nucleotide polymorphism (SNP) in a biological sample obtained from said subject.

In a further aspect, the present invention relates to a method for predicting the risk of having or developing ocular disease related to mtDNA maintenance in a subject in need thereof, comprising the step of determining the expression level of SSBP1 and/or detecting SSBP1 SNP in a biological sample obtained from said subject.

In a particular embodiment, the invention relates to a method for predicting the risk of having or developing ocular disease related to mtDNA maintenance in a subject in need thereof, comprising the steps of: i) determining the expression level of SSBP1 protein and/or detecting SSBP1 SNP in a biological sample obtained from said subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the subject is at risk of having or developing ocular disease related to mtDNA maintenance when the expression level determined at step i) is lower than the predetermined reference value and/or when the SSBP1 SNP is detected, or concluding that the patient is not at risk of having or developing ocular disease related to mtDNA maintenance when the expression level determined at step i) is higher than the predetermined reference value and/or when the SNP is not detected.

In a particular embodiment, the method according to the invention, further comprising the steps of: i) identifying at least one mutation in the SSBP1 gene and/or protein; ii) concluding that the subject is at risk of having or developing ocular disease related to mtDNA maintenance when at least one mutation is identified.

As used herein, the term "mutation" has its general meaning in the art and refers to any detectable change in genetic material, e.g. DNA, RNA, cDNA, or any process, mechanism, or result of such a change. This includes gene mutations, in which the structure (e.g. DNA sequence) of a gene is altered, any gene or DNA arising from any mutation process, and any expression product (e.g. protein or enzyme) expressed by a modified gene or DNA sequence.

Mutations include deletion, insertion or substitution of one or more nucleotides. The mutation may occur in the coding region of a gene (i.e. in exons), in introns, or in the regulatory regions (e.g. enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, promoters) of the gene. Generally a mutation is identified in a subject by comparing the sequence of a nucleic acid or polypeptide expressed by said subject with the corresponding nucleic acid or polypeptide expressed in a control population. Where the mutation is within the gene coding sequence, the mutation may be a "missense" mutation, where it replaces one amino acid with another in the gene product, or a "non sense" mutation, where it replaces an amino acid codon with a stop codon. A mutation may also occur in a splicing site where it creates or destroys signals for exon-intron splicing and thereby lead to a gene product of altered structure. A mutation in the genetic material may also be "silent", i.e. the mutation does not result in an alteration of the amino acid sequence of the expression product.

As used herein, the term "homozygous" refers to an individual possessing two copies of the same allele. As used herein, the term "homozygous mutant" refers to an individual possessing two copies of the same allele, such allele being characterized as the mutant form of a gene.

As used herein, the term "heterozygous" refers to an individual possessing two different alleles of the same gene, i.e. an individual possessing two different copies of an allele, such alleles are characterized as mutant forms of a gene. In a particular embodiment, the mutation allows to a truncated protein. Typically, truncated protein refers to a protein shortened by a mutation which specifically induces premature termination of messenger RNA translation.

As used herein, the term “single nucleotide polymorphism (SNP)” refers to is a single basepair variation in a nucleic acid sequence of SSBP1 gene. Polymorphisms can be referred to, for instance, by the nucleotide position at which the variation exists, by the change in amino acid sequence caused by the nucleotide variation, or by a change in some other characteristic of the nucleic acid molecule that is linked to the variation {e.g., an alteration of a secondary structure such as a stem-loop, or an alteration of the binding affinity of the nucleic acid for associated molecules, such as polymerases, RNases, and so forth). For example, the SNP in the context of the invention is missense mutation in exon 1 and exon 4 leading to the G113A in exon 4 and/or G320A substitution in SSBP1.

In the methods according to the present the invention, the presence or absence of a SNP can be determined by nucleic acid sequencing, PCR analysis or any genotyping method known in the art such as the method described in the example. Examples of such methods include, but are not limited to, chemical assays such as allele specific hybridization (DASH),

pyrosequencing, molecular beacons, SNP microarrays, restriction fragment length polymorphism (RFLP), flap endonuclease (FEN), single strand conformation polymorphism, temperature gradient gel electrophoresis (TGGE), denaturing high performance liquid chromatography (DHPLC), high-resolution melting of the entire amplicon, and DNA mismatch-binding proteins. primer extension, allele specific oligonucleotide ligation, sequencing, enzymatic cleavage, flap endonuclease discrimination; and detection methods such as fluorescence, chemiluminescence, and mass spectrometry.

For example, the presence or absence of said polymorphism may be detected in a DNA sample, preferably after amplification. For instance, the isolated DNA may be subjected to couple reverse transcription and amplification, such as reverse transcription and amplification by polymerase chain reaction (RT-PCR), using specific oligonucleotide primers that are specific for the polymorphism or that enable amplification of a region containing the polymorphism. According to a first alternative, conditions for primer annealing may be chosen to ensure specific reverse transcription (where appropriate) and amplification; so that the appearance of an amplification product be a diagnostic of the presence of the polymorphism according to the invention. Otherwise, DNA may be amplified, after which a mutated site may be detected in the amplified sequence by hybridization with a suitable probe or by direct sequencing, or any other appropriate method known in the art.

Currently numerous strategies for genotype analysis are available (Antonarakis et al., 1989; Cooper et al., 1991; Grompe, 1993). Briefly, the nucleic acid molecule may be tested for the presence or absence of a restriction site. When a base polymorphism creates or abolishes the recognition site of a restriction enzyme, this allows a simple direct PCR genotype the polymorphism. Further strategies include, but are not limited to, direct sequencing, restriction fragment length polymorphism (RFLP) analysis; hybridization with allele-specific oligonucleotides (ASO) that are short synthetic probes which hybridize only to a perfectly matched sequence under suitably stringent hybridization conditions; allele specific PCR; PCR using mutagenic primers; ligase-PCR, HOT cleavage; denaturing gradient gel electrophoresis (DGGE), temperature denaturing gradient gel electrophoresis (TGGE), single-stranded conformational polymorphism (SSCP) and denaturing high performance liquid chromatography (Kuklin et al., 1997). Direct sequencing may be accomplished by any method, including without limitation chemical sequencing, using the Maxam-Gilbert method; by enzymatic sequencing, using the Sanger method; mass spectrometry sequencing; pyrosequencing; sequencing using a chip-based technology and real-time quantitative PCR.

Preferably, DNA from a patient is first subjected to amplification by polymerase chain reaction (PCR) using specific amplification primers. However several other methods are available, allowing DNA to be studied independently of PCR, such as the rolling circle amplification (RCA), the InvaderTMassay, or oligonucleotide ligation assay (OLA). OLA may be used for revealing base polymorphisms. According to this method, two oligonucleotides are constructed that hybridize to adjacent sequences in the target nucleic acid, with the join sited at the position of the polymorphism. DNA ligase will covalently join the two oligonucleotides only if they are perfectly hybridized to one of the allele. Oligonucleotide probes or primers may contain at least 10, 15, 20 or 30 nucleotides. Their length may be shorter than 400, 300, 200 or 100 nucleotides.

According to the invention, the determination of the presence or absence of said SNP may also be determined by detection or not of the mutated protein by any method known in the art. The presence of the protein of interest may be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labelled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation, etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith. Labels are known in the art that generally provide (either directly or indirectly) a signal. As used herein, the term “labelled” with regard to the antibody or aptamer, is intended to encompass direct labelling of the antibody or aptamer by coupling (i.e., physically linking) a detectable substance, such as a radioactive agent or a fluorophore (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or indocyanine (Cy5), to the antibody or aptamer, as well as indirect labelling of the probe or antibody (e.g., horseradish peroxidise, HRP) by reactivity with a detectable substance. An antibody or aptamer may be also labelled with a radioactive molecule by any method known in the art. For example, radioactive molecules include but are not limited radioactive atom for scintigraphic studies such as I123, I124, In111, Re186 and Re188. The aforementioned assays generally involve separation of unbound protein in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which may be used in the practice of the invention include substrates such as nitrocellulose (e.g., in membrane or microtiter well form); polyvinylchloride (e.g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, etc.

More particularly, an ELISA method may be used, wherein the wells of a microtiter plate are coated with an antibody against the protein to be tested. A biological sample containing or suspected of containing the marker protein is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate (s) can be washed to remove unbound moieties and a detectably labelled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

Alternatively, an immunohistochemistry (IHC) method may be used. IHC specifically provides a method of detecting a target in a biological sample or tissue specimen in situ. The overall cellular integrity of the sample is maintained in IHC, thus allowing detection of both the presence and location of the target of interest. Typically a biological sample is fixed with formalin, embedded in paraffin and cut into sections for staining and subsequent inspection by light microscopy. Current methods of IHC use either direct labeling or secondary antibody-based or hapten-based labeling. Examples of known IHC systems include, for example, EnVision™ (DakoCytomation), Powervision® (Immunovision, Springdale, AZ), the NBA™ kit (Zymed Laboratories Inc., South San Francisco, CA), HistoFine® (Nichirei Corp, Tokyo, Japan).

In one embodiment of the present invention, direct sequencing of the whole genome is used to detect the SNP locus SSBP1. The whole genome sequencing may be achieved by use of the next generation sequencing (NGS) assay. In NGS, a single genomic DNA is first fragmented into a library of small segments that can be uniformly and accurately sequenced in millions of parallel reactions. The newly identified strings of bases, called reads, are then reassembled using a known reference genome as a scaffold (resequencing), or in the absence of a reference genome (de novo sequencing). The full set of aligned reads would reveal the entire sequence of each chromosome of the genomic DNA.

In another embodiment of the present invention, primer extension assay is used to detect the SNP locus SSBP1. The primer extension assay may be achieved by use of Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Mass spectrometry is an experimental technique used to identify the components of a heterogeneous collection of biomolecules, by sensitive discrimination of their molecular masses. In MALTI-TOF MS, the sample to be analyzed is placed in a UV-absorbing matrix pad and exposed to a short laser pulse. The ionized molecules are accelerated off the matrix pad (i.e., desorption) and move into an electric field towards a detector. The “time of flight” required to reach the detector

depends on the mass/charge (m/z) ratio of the individual molecules. To use MALTI-TOF MS for DNA sequencing, the DNA sequence to be sampled is first transcribed into RNA in vitro in 4 separate reactions, each with three rNTP bases and one specific dNTP. The incorporated dNTP in the transcribed RNA will prevent cleavage from occurring at that dNTP position by RNAse, and therefore generate distinct fragments. Each fragment has a characteristic m/z ratio that appears as a peak in MALTI-TOF spectrum. The MALTI-TOF mass signal pattern obtained for the DNA sample is then compared with the expected m/z spectrum of the reference sequence, which includes the products of all 4 cleavage reactions. Any SNP differences between the sample DNA and the reference DNA sequences will produce predictable shifts in the spectrum, and their exact nature can be deduced.

In still another embodiment of the present invention, quantitative polymerase chain reaction (qPCR) is used to detect the desired SNP locus. In qPCR, DNA sample that includes the SNP locus is amplified and simultaneously detected and quantitated with different primer sets that target each allele separately. Well-designed primers will amplify their target SNP at a much earlier cycle than the other SNPs. This allows more than two alleles to be distinguished, although an individual qPCR reaction is required for each SNP. To achieve high enough specificity, the primer sequence may require placement of an artificial mismatch near its 3′-end, which is an approach generally known as Taq-MAMA. This artificial mismatch induces a much greater amplification delay for non-target alleles than a single mismatch would alone, yet does not substantially affect amplification of the target SNP.

In still another embodiment of the present invention, the SNP locus is detected by direct sequencing of a specified DNA segment containing the SNP locus of SSBP1.

As used herein, the term “expression level” refers to the expression level of SSBP1 with further other values corresponding to the clinical parameters. Typically, the expression level of the gene may be determined by any technology known by a person skilled in the art. In particular, each gene expression level may be measured at the genomic and/or nucleic and/or protein level. In a particular embodiment, the expression level of SSBP1gene is measured. The expression level of SSBP1 is assessed by analyzing the expression of the protein translated from said gene. Said analysis can be assessed using an antibody (e.g., a radio-labelled, chromophore-labelled, fluorophore-labelled, or enzyme-labelled antibody), an antibody derivative (e.g., an antibody conjugate with a substrate or with the protein or ligand of a protein of a protein/ligand pair (e.g., biotin-streptavidin)), or an antibody fragment (e.g., a single-chain antibody, an isolated antibody hypervariable domain, etc.) which binds specifically to the protein translated from the gene encoding for SSBP1.

Methods for measuring the expression level of SSBP1 in a sample may be assessed by any of a wide variety of well-known methods from one of skill in the art for detecting expression of a protein including, but not limited to, direct methods like mass spectrometry-based quantification methods, protein microarray methods, enzyme immunoassay (EIA), radioimmunoassay (RIA), Immunohistochemistry (IHC), Western blot analysis, ELISA, Luminex, ELISPOT and enzyme linked immunosorbent assay and indirect methods based on detecting expression of corresponding messenger ribonucleic acids (mRNAs). The mRNA expression profile may be determined by any technology known by a man skilled in the art. In particular, each mRNA expression level may be measured using any technology known by a man skilled in the art, including nucleic microarrays, quantitative Polymerase Chain Reaction (qPCR), next generation sequencing and hybridization with a labelled probe.

Said direct analysis can be assessed by contacting the sample with a binding partner capable of selectively interacting with the biomarker present in the sample. The binding partner may be an antibody that may be polyclonal or monoclonal, preferably monoclonal (e.g., a isotope-label, element-label, radio-labelled, chromophore- labelled, fluorophore-labelled, or enzyme-labelled antibody), an antibody derivative (e.g., an antibody conjugate with a substrate or with the protein or ligand of a protein of a protein/ligand pair (e.g., biotin-streptavidin)), or an antibody fragment (e.g., a single-chain antibody, an isolated antibody hypervariable domain, etc.) which binds specifically to the protein translated from the gene encoding for the biomarker of the invention. In another embodiment, the binding partner may be an aptamer.

The binding partners of the invention such as antibodies or aptamers, may be labelled with a detectable molecule or substance, such as an isotope, an element, a fluorescent molecule, a radioactive molecule or any others labels known in the art. Labels are known in the art that generally provide (either directly or indirectly) a signal.

As used herein, the term "labelled", with regard to the antibody, is intended to encompass direct labelling of the antibody or aptamer by coupling (i.e., physically linking) a detectable substance, such as an isotope, an element, a radioactive agent or a fluorophore (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)) to the antibody or aptamer, as well as indirect labelling of the probe or antibody by reactivity with a detectable substance. An antibody or aptamer of the invention may be produced with a specific isotope or a radioactive molecule by any method known in the art. For example radioactive molecules include but are not limited to radioactive atom for scintigraphy studies such as I123, I124, In111, Re186, Re188, specific isotopes include but are not limited to 13C, 15N, 126I, 79Br, 81Br.

The aforementioned assays generally involve the binding of the binding partner (ie. antibody or aptamer) to a solid support. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, silicon wafers.

In a particular embodiment, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies which recognize SSBP1 protein. A sample containing or suspected of containing said biomarker is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labelled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art such as Singulex, Quanterix, MSD, Bioscale, Cytof.

In one embodiment, an Enzyme-linked immunospot (ELISpot) method may be used. Typically, the sample is transferred to a plate which has been coated with the desired anti-SSBP1 protein capture antibodies. Revelation is carried out with biotinylated secondary Abs and standard colorimetric or fluorimetric detection methods such as streptavidin-alkaline phosphatase and NBT-BCIP and the spots counted.

In one embodiment, when multi-biomarker expression measurement is required, use of beads bearing binding partners of interest may be preferred. In a particular embodiment, the bead may be a cytometric bead for use in flow cytometry. Such beads may for example correspond to BD™ Cytometric Beads commercialized by BD Biosciences (San Jose, California). Typically cytometric beads may be suitable for preparing a multiplexed bead assay. A multiplexed bead assay, such as, for example, the BD(TM) Cytometric Bead Array, is a series of spectrally discrete beads that can be used to capture and quantify soluble antigens. Typically, beads are labelled with one or more spectrally distinct fluorescent dyes, and detection is carried out using a multiplicity of photodetectors, one for each distinct dye to be detected. A number of methods of making and using sets of distinguishable beads have been described in the literature. These include beads distinguishable by size, wherein each size bead is coated with a different target-specific antibody (see e.g. Fulwyler and McHugh, 1990, Methods in Cell Biology 33:613-629), beads with two or more fluorescent dyes at varying concentrations, wherein the beads are identified by the levels of fluorescence dyes (see e.g. European Patent

No. 0 126,450), and beads distinguishably labelled with two different dyes, wherein the beads are identified by separately measuring the fluorescence intensity of each of the dyes (see e.g. U.S. patent Nos. 4,499,052 and 4,717,655). Both one-dimensional and two-dimensional arrays for the simultaneous analysis of multiple antigens by flow cytometry are available commercially. Examples of one-dimensional arrays of singly dyed beads distinguishable by the level of fluorescence intensity include the BD(TM) Cytometric Bead Array (CBA) (BD Biosciences, San Jose, Calif.) and Cyto-Plex(TM) Flow Cytometry microspheres (Duke Scientific, Palo Alto, Calif.). An example of a two-dimensional array of beads distinguishable by a combination of fluorescence intensity (five levels) and size (two sizes) is the QuantumPlex(TM) microspheres (Bangs Laboratories, Fisher, Ind.). An example of a two-dimensional array of doubly-dyed beads distinguishable by the levels of fluorescence of each of the two dyes is described in Fulton et al. (1997, Clinical Chemistry 43(9):1749-1756). The beads may be labelled with any fluorescent compound known in the art such as e.g. FITC (FL1), PE (FL2), fluorophores for use in the blue laser (e.g. PerCP, PE-Cy7, PE-Cy5, FL3 and APC or Cy5, FL4), fluorophores for use in the red, violet or UV laser (e.g. Pacific blue, pacific orange). In another particular embodiment, bead is a magnetic bead for use in magnetic separation. Magnetic beads are known to those of skill in the art. Typically, the magnetic bead is preferably made of a magnetic material selected from the group consisting of metals (e.g. ferrum, cobalt and nickel), an alloy thereof and an oxide thereof. In another particular embodiment, bead is bead that is dyed and magnetized.

In one embodiment, protein microarray methods may be used. Typically, at least one antibody or aptamer directed against SSBP1 protein is immobilized or grafted to an array(s), a solid or semi-solid surface(s). A sample containing or suspected of containing SSBP1 protein is then labelled with at least one isotope or one element or one fluorophore or one colorimetric tag that are not naturally contained in the tested sample. After a period of incubation of said sample with the array sufficient to allow the formation of antibody-antigen complexes, the array is then washed and dried. After all, quantifying SSBP1 protein may be achieved by using any appropriate microarray scanner like fluorescence scanner, colorimetric scanner, SIMS (secondary ions mass spectrometry) scanner, maldi scanner, electromagnetic scanner or any technique allowing to quantify said labels.

In another embodiment, the antibody or aptamer grafted on the array is labelled.

In another embodiment, reverse phase arrays may be used. Typically, at least one sample is immobilized or grafted to an array(s), a solid or semi-solid surface(s). An antibody or aptamer against the suspected biomarker is then labelled with at least one isotope or one element or one

fluorophore or one colorimetric tag that are not naturally contained in the tested sample. After a period of incubation of said antibody or aptamer with the array sufficient to allow the formation of antibody-antigen complexes, the array is then washed and dried. After all, detecting quantifying and counting by D-SIMS said biomarker containing said isotope or group of isotopes, and a reference natural element, and then calculating the isotopic ratio between the biomarker and the reference natural element. may be achieve using any appropriate microarray scanner like fluorescence scanner, colorimetric scanner, SIMS (secondary ions mass spectrometry) scanner, maldi scanner, electromagnetic scanner or any technique allowing to quantify said labels.

In one embodiment, said direct analysis can also be assessed by mass Spectrometry. Mass spectrometry-based quantification methods may be performed using either labelled or unlabelled approaches (DeSouza and Siu, 2012). Mass spectrometry-based quantification methods may be performed using chemical labeling, metabolic labelingor proteolytic labeling. Mass spectrometry-based quantification methods may be performed using mass spectrometry label free quantification, LTQ Orbitrap Velos, LTQ-MS/MS, a quantification based on extracted ion chromatogram EIC (progenesis LC-MS, Liquid chromatography-mass spectrometry) and then profile alignment to determine differential expression of the biomarker.

In another embodiment, the SSBP1 expression level is assessed by analyzing the expression of mRNA transcript or mRNA precursors, such as nascent RNA, of SSBP1 gene. Said analysis can be assessed by preparing mRNA/cDNA from cells in a sample from a subject, and hybridizing the mRNA/cDNA with a reference polynucleotide. The prepared mRNA/cDNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, polymerase chain reaction analyses, such as quantitative PCR (TaqMan), and probes arrays such as GeneChip(TM) DNA Arrays (AFFYMETRIX).

Advantageously, the analysis of the expression level of mRNA transcribed from the gene encoding for biomarkers involves the process of nucleic acid amplification, e. g., by RT-PCR (the experimental embodiment set forth in U. S. Patent No. 4,683, 202), ligase chain reaction (Barany, 1991), self-sustained sequence replication (Guatelli et al., 1990), transcriptional amplification system (Kwoh et al., 1989), Q-Beta Replicase (Lizardi et al., 1988), rolling circle replication (U. S. Patent No. 5,854, 033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers. As used

herein, amplification primers are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or vice-versa) and contain a short region in between. In general, amplification primers are from about 10 to 30 nucleotides in length and flank a region from about 50 to 200 nucleotides in length. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule comprising the nucleotide sequence flanked by the primers.

As used herein, the term “predetermined reference value” refers to a threshold value or a cut-off value. The setting of a single “reference value” thus allows discrimination between a subject at risk of having or developing IA and a subject not at risk of having or developing IA with respect to the overall survival (OS) for a subject. Typically, a "threshold value" or "cut-off value" can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary skilled in the art. The threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. Preferably, the person skilled in the art may compare the expression level (obtained according to the method of the invention) with a defined threshold value. In one embodiment of the present invention, the threshold value is derived from the expression level (or ratio, or score) determined in a biological sample derived from one or more subjects at risk of having or developing IA. Furthermore, retrospective measurement of the expression level (or ratio, or scores) in properly banked historical subject samples may be used in establishing these threshold values.

Predetermined reference values used for comparison may comprise “cut-off” or “threshold” values that may be determined as described herein. Each reference (“cut-off”) value for SSBP1 may be predetermined by carrying out a method comprising the steps of

a) providing a collection of samples from subjects at risk of having or developing ocular disease related to mtDNA maintenance;

b) determining the expression level of SSBP1protein for each sample contained in the collection provided at step a);

c) ranking the biological samples according to said expression level;

d) classifying said samples in pairs of subsets of increasing, respectively decreasing, number of members ranked according to their expression level,

e) providing, for each sample provided at step a), information relating to the risk of having or developing ocular disease related to mtDNA maintenance or the actual clinical outcome for the corresponding subject (i.e. the duration of the overall survival (OS));

f) for each pair of subsets of samples, obtaining a Kaplan Meier percentage of survival curve;

g) for each pair of subsets of samples calculating the statistical significance (p value) between both subsets;

h) selecting as reference value for the expression level, the value of expression level for which the p value is the smallest.

For example the expression level of SSBP1 has been assessed for 100 samples of 100 patients. The 100 samples are ranked according to their expression level. Sample 1 has the best expression level and sample 100 has the worst expression level. A first grouping provides two subsets: on one side sample Nr 1 and on the other side the 99 other samples. The next grouping provides on one side samples 1 and 2 and on the other side the 98 remaining samples etc., until the last grouping: on one side samples 1 to 99 and on the other side sample Nr 100. According to the information relating to the actual clinical outcome for the corresponding patient, Kaplan Meier curves are prepared for each of the 99 groups of two subsets. Also for each of the 99 groups, the p value between both subsets was calculated.

The reference value is selected such as the discrimination based on the criterion of the minimum p value is the strongest. In other terms, the expression level corresponding to the boundary between both subsets for which the p value is minimum is considered as the reference value. It should be noted that the reference value is not necessarily the median value of expression levels.

In routine work, the reference value (cut-off value) may be used in the present method to discriminate samples and therefore the corresponding patients.

Kaplan–Meier curves of percentage of survival as a function of time are commonly to measure the fraction of patients living for a certain amount of time after treatment and are well known by the man skilled in the art.

The man skilled in the art also understands that the same technique of assessment of the expression level of SSBP1 should of course be used for obtaining the reference value and thereafter for assessment of the expression level of a biomarker of a patient subjected to the method of the invention.

In one embodiment, the reference value may correspond to the expression level of SSBP1 determined in a sample associated with subject at risk of having or developing ocular

disease related to mtDNA maintenance. Accordingly, a lower expression level of SSBP1 than the reference value is indicative of a subject at risk of having or developing ocular disease related to mtDNA maintenance, and a higher or equal expression level of SSBP1 than the reference value is indicative of a subject not at risk of having or developing acute ocular disease related to mtDNA maintenance.

In another embodiment, the reference value may correspond to the expression level of SSBP1 determined in a sample associated with subject not at risk of having or developing ocular disease related to mtDNA maintenance. Accordingly, a higher or equal expression level of SSBP1 than the reference value is indicative of a subject not at risk of having or developing ocular disease related to mtDNA maintenance, and a lower expression level of SSBP1 than the reference value is indicative of a subject at risk of having or developing ocular disease related to mtDNA maintenance.

Method for treating ocular disease related to mtDNA maintenance

In a second aspect, the invention relates to a method for treating ocular disease related to mtDNA maintenance in a subject in need thereof comprising a step of administering to said subject a therapeutically effective amount of an inhibitor of mutations in SSBP1 gene.

In a particular embodiment, the method according to the invention, wherein the mutations lead to the R38Q and R107Q amino-acid changes in the mitochondrial single-stranded DNA-binding protein.

In other word, the invention relates to a method for treating ocular disease related to mtDNA maintenance in a subject in need thereof comprising a step of administering to said subject a therapeutically effective amount of an inhibitor of mutated SSBP1 gene.

As used herein, the terms “treating” or “treatment” refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of subject at risk of contracting the disease or suspected to have contracted the disease as well as subject who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction

period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a subject during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a subject during treatment of an illness, e.g., to keep the subject in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., pain, disease manifestation, etc.]).

As used herein, the term “inhibitor” refers to a natural or synthetic compound able to inhibit the activity and/or expression of mutated SSBP1.

As used herein the terms "mutated SSBP1 activity" refers to unstable formation of SSBP1 dimer/tetramer affecting mtDNA replication leading to a decrease of mtDNA copy-number.

As used herein, the term “mutated SSBP1 expression” refers to SSBP1 gene comprising a missense mutation leading to the R38Q and R107Q amino-acid changes in the mitochondrial single-stranded DNA-binding protein.

In a particular embodiment, the missense mutation is c.113G>A and/or c.320G>A in the SSBP1 gene.

In a particular embodiment, the missense mutation is R38Q and/or R107Q in the SSBP1 protein.

In a particular embodiment, the inhibitor of SSBP1 is an inhibitor of mutated SSBP1 expression.

An "inhibitor of mutated SSBP1 expression" refers to a natural or synthetic compound that has a biological effect to inhibit or significantly reduce the expression of the mutated gene encoding for a truncated and inactive SSBP1. Typically, the inhibitor of mutated SSBP1 gene expression has a biological effect on one or more of the following events: (1) production of an RNA template from a DNA sequence (e.g., by transcription); (2) processing of an RNA transcript (e.g., by splicing, editing, 5' cap formation, and/or 3' end formation); (3) translation

of an RNA into a polypeptide or protein; and/or (4) post-translational modification of a polypeptide or protein.

In a particular embodiment, the method according to the invention, wherein the inhibitor of mutated SSBP1 gene expression is siRNA, shRNA, miRNA, antisense oligonucleotide, or a ribozyme.

In some embodiments, the inhibitor of mutated SSBP1 expression is an antisense oligonucleotide. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of SSBP1 mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of SSBP1 proteins, and thus activity, in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding SSBP1 can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous, subcutaneous or intravitreal injection. Methods for using antisense techniques for specifically alleviating gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

In a particular embodiment, the inhibitor of mutated SSBP1 expression is a shRNA. shRNA is generally expressed using a vector introduced into cells, wherein the vector utilizes the U6 promoter to ensure that the shRNA is always expressed. This vector is usually passed on to daughter cells, allowing the gene silencing to be inherited. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which is then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs that match the siRNA to which it is bound.

In some embodiments, the inhibitor of mutated SSBP1 expression is a small inhibitory RNAs (siRNAs). SSBP1 expression can be reduced by contacting the subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that SSBP1 expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836). In a particular embodiment, the siRNA is ALN-PCS02 developed by Alnylam (phase 1 ongoing).

In some embodiments, the inhibitor of mutated SSBP1 expression is a ribozyme. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of SSBP1 mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

In some embodiments, the inhibitor of mutated SSBP1 expression is an endonuclease. The term “endonuclease” refers to enzymes that cleave the phosphodiester bond within a polynucleotide chain. Some, such as Deoxyribonuclease I, cut DNA relatively nonspecifically (without regard to sequence), while many, typically called restriction endonucleases or restriction enzymes, and cleave only at very specific nucleotide sequences. The mechanism behind endonuclease-based genome inactivating generally requires a first step of DNA single or double strand break, which can then trigger two distinct cellular mechanisms for DNA repair, which can be exploited for DNA inactivating: the errorprone nonhomologous end-joining (NHEJ) and the high-fidelity homology-directed repair (HDR). In a particular embodiment, the endonuclease is CRISPR-cas. As used herein, the term “CRISPR-cas” has its general meaning in the art and refers to clustered regularly interspaced short palindromic repeats associated which are the segments of prokaryotic DNA containing short repetitions of base sequences. In some embodiment, the endonuclease is CRISPR-cas9 which is from Streptococcus pyogenes. The CRISPR/Cas9 system has been described in US 8697359 B1 and US 2014/0068797. In some embodiment, the endonuclease is CRISPR-Cpf1 which is the more recently characterized CRISPR from Provotella and Francisella 1 (Cpf1) in Zetsche et al. (“Cpf1 is a Single RNA-guided Endonuclease of a Class 2 CRISPR-Cas System (2015); Cell; 163, 1-13).

As used herein the terms "administering" or "administration" refer to the act of injecting

or otherwise physically delivering a substance as it exists outside the body (e.g., an inhibitor of SSBP1expression/activity) into the subject, such as by, intravitreal, intravenous, subcutaneous, and/or any other method of physical delivery described herein or known in the art. When a disease, or a symptom thereof, is being treated, administration of the substance typically occurs after the onset of the disease or symptoms thereof. When a disease or symptoms thereof, are being prevented, administration of the substance typically occurs before the onset of the disease or symptoms thereof.

By a "therapeutically effective amount" is meant a sufficient amount of an inhibitor of SSBP1 expression and/or activity for use in a method for the treatment of ocular disease related to mtDNA maintenance at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well known within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Typically, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic 20 adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, typically from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

In a further embodiment, the invention relates to a method of treating ocular disease related to mtDNA maintenance in a subject in need thereof comprising a step of administering to said subject a therapeutically effective amount of a vector which comprises a nucleic acid molecule encoding for SSBP1.

In a particular embodiment, the method according to the invention, wherein the nucleic acid molecule encoding for a SSBP1 polypeptide comprising an amino acid sequence having at least 90% of identity with the sequence as set forth in SEQ ID NO:1:

MFRRPVLQVL RQFVRHESET TTSLVLERSL NRVHLLGRVG QDPVLRQVEG
5 KNPVTIFSLA TNEMWRSGDS EVYQLGDVSQ KTTWHRISVF RPGLRDVAYQ
YVKKGSRIYL EGKIDYGEYM DKNNVRRQAT TIIADNIIFL SDQTKEKE.

In a particular embodiment, the method according to the invention wherein the nucleic acid molecule is operatively linked to a promoter sequence.

In a particular embodiment, the method according to the invention, wherein the vector
10 is a viral vector.

In a particular embodiment, the method according to the invention, wherein the viral vector is lentivirus (LV).

As used herein, the term “lentivirus” refers to enveloped RNA particles measuring approximately 120 nm in size are efficient drug delivery tools and more particularly gene
15 delivery tools. The LV binds to, and enters into target cells through its envelope proteins which confer its pseudotype. Once the LV has entered into the cells, it releases its capsid components and undergoes reverse transcription of the lentiviral RNA before integrating the proviral DNA into the genome of target cells. Non-integrative lentiviral vectors have been generated by modifying the properties of the vector integration machinery and can be used for transient gene
20 expression. Virus-like particles lacking a provirus have also been generated and can be used to deliver proteins or messenger RNA. LV can be used for example, for gene addition, RNA interference, exon skipping or gene editing. All of these approaches can be facilitated by tissue or cell targeting of the LV via its pseudotype.

Lentivirus-like particles are described for example in Muratori et ah, Methods Mol.
25 Biol., 2010, 614, 111-24; Burney et al, Curr. HIV Res., 2006, 4, 475-484; Kaczmarczyk et al, Proc Natl Acad Sci U S A., 2011, 108, 16998-17003; Aoki et al., Gene Therapy, 2011, 18, 936-941. Examples of lentivirus-like particles are VLPs generated by co-expressing in producer cells, a syncytin protein with a gag fusion protein (Gag fused with the gene of interest). The drug and/or syncytin may be, either displayed on the surface of the particles, or enclosed
30 (packaged) into the particles. The syncytin protein is advantageously displayed on the surface of the particles, such as coupled to the particles or incorporated into the envelope of (enveloped) virus particles or virus-like particles to form pseudotyped enveloped virus particles or virus-like particles. The drug is coupled to the particles or packaged into the particles. For example, the drug is coupled to viral capsids or packaged into viral capsids, wherein said viral capsids

may further comprise an envelope, preferably pseudotyped with syncytin. In some preferred embodiments, the drug is packaged into the particles pseudotyped with syncytin protein. The drug which is packaged into particles is advantageously a heterologous gene of interest which is packaged into viral vector particles, preferably retroviral vector particles, more preferably lenti viral vector particles.

In a particular embodiment, the method according to the invention, wherein the viral vector is adenovirus.

As used herein, the term “adenovirus” refers to medium-sized (90–100 nm), nonenveloped (without an outer lipid bilayer) viruses with an icosahedral nucleocapsid containing a double stranded DNA genome.

In a particular embodiment, the method according to the invention, wherein the viral vector is an adeno-associated virus (AAV) vector.

As used herein the term "AAV" has its general meaning in the art and is an abbreviation for adeno-associated virus, and may be used to refer to the virus itself or derivatives thereof. The term covers all serotypes and variants both naturally occurring and engineered forms. According to the invention the term "AAV" refers to AAV type 1 (AAV-1), AAV type 2 (AAV-2), AAV type 3 (AAV-3), AAV type 4 (AAV-4), AAV type 5 (AAV-5), AAV type 6 (AAV-6), AAV type 7 (AAV-7), and AAV type 8 (AAV-8) and AAV type 9 (AAV9). The genomic sequences of various serotypes of AAV, as well as the sequences of the native terminal repeats (TRs), Rep proteins, and capsid subunits are known in the art. Such sequences may be found in the literature or in public databases such as GenBank. See, e.g., GenBank Accession Numbers NC_001401 (AAV-2), AF043303 (AAV¬2), and NC_006152 (AAV-5). As used herein, a "rAAV vector" refers to an AAV vector comprising the polynucleotide of interest (i.e the polynucleotide encoding for the SSBP1 polypeptide). The rAAV vectors contain 5' and 3' adeno-associated virus inverted terminal repeats (ITRs), and the polynucleotide of interest operatively linked to sequences, which regulate its expression in a target cell.

The AAV vector of the present invention typically comprises regulatory sequences allowing expression and, secretion of the encoded polypeptide (i.e. the SSBP1 polypeptide), such as e.g., a promoter, enhancer, polyadenylation signal, internal ribosome entry sites (IRES), sequences encoding protein transduction domains (PTD), and the like. In this regard, the vector comprises a promoter region, operably linked to the polynucleotide of interest, to cause or improve expression of the protein in infected cells. Such a promoter may be ubiquitous, tissue-specific, strong, weak, regulated, chimeric, inducible, etc., to allow efficient and suitable production of the protein in the infected tissue. The promoter may be homologous to the

encoded protein, or heterologous, including cellular, viral, fungal, plant or synthetic promoters. Examples of such regulated promoters include, without limitation, Tet on/off element-containing promoters, rapamycin-inducible promoters and metallothionein promoters. Examples of ubiquitous promoters include viral promoters, particularly the CMV promoter, CAG promoter (chicken beta actin promoter with CMV enhancer), the RSV promoter, the SV40 promoter, etc. and cellular promoters such as the PGK (phosphoglycerate kinase) promoter. The promoters may also be neurospecific promoters such as the Synapsin or the NSE (Neuron Specific Enolase) promoters (or NRSE (Neuron restrictive silencer element) sequences placed upstream from the ubiquitous PGK promoter), or promoters specific for RPE cell types such as the RPE65, the BEST1, the Rhodopsin or the cone arrestin promoters. The vector may also comprise target sequences for miRNAs achieving suppression of transgene expression in non¬-desired cells. In some embodiments, the vector comprises a leader sequence allowing secretion of the encoded protein. Fusion of the polynucleotide of interest with a sequence encoding a secretion signal peptide (usually located at the N-terminal end of secreted polypeptides) will allow the production of the therapeutic protein in a form that can be secreted from the transduced cells. Examples of such signal peptides include the albumin, the β-glucuronidase, the alkaline protease or the fibronectin secretory signal peptides.

The recombinant AAV vector of the present invention is produced using methods well known in the art. In short, the methods generally involve (a) the introduction of the rAAV vector into a host cell, (b) the introduction of an AAV helper construct into the host cell, wherein the helper construct comprises the viral functions missing from the rAAV vector and (c) introducing a helper virus into the host cell. All functions for rAAV virion replication and packaging need to be present, to achieve replication and packaging of the rAAV vector into rAAV virions. The introduction into the host cell can be carried out using standard virological techniques simultaneously or sequentially. Finally, the host cells are cultured to produce rAAV virions and are purified using standard techniques such as CsCl gradients. Residual helper virus activity can be inactivated using known methods, such as for example heat inactivation. The purified rAAV vector is then ready for use in the method of the present invention.

In a particular embodiment, the method according to the invention, wherein the AAV vector is selected from vectors derived from AAV serotypes having tropism for and high transduction efficiencies in ocular cells.

In a particular embodiment, the method according to the invention, wherein the AAV vector is an AAV1, AAV2, AAV3, AAV4, AAV 5, AAV 6, AAV7, AAV 8 or AAV9.

In a particular embodiment, the method according to the invention, wherein the AAV vector is an AAV1, AAV 2, AAV 5, AAV 7, 8 or AAV 9.

By a "therapeutically effective amount" of AAV vector as above described is meant a sufficient amount of the AAV vector for the treatment of ocular disease related to mtDNA maintenance. It will be understood, however, that the total dosage of the AAV vector of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. Typically, from 10^8 to 10^{10} viral genomes (vg) are administered *per dose* in mice. Typically, the doses of AAV vectors to be administered in humans may range from 10^{10} to 10^{12} vg.

Administering the recombinant AAV vector of the present invention to the subject is preferably performed by intravenous, intravitreal, subcutaneous delivery. In some embodiments, the recombinant AAV vector of the present invention is administered to the subject by the intravitreous injection.

In a particular embodiment, the method according to the invention, wherein the vector is delivered by intravitreous, subcutaneous or intravenous delivery.

In a particular embodiment, the method according to the invention, wherein the vector is delivered in retinal ganglion cells, photoreceptors or pigmented epithelium.

Kit

In a third aspect, the invention relates to a kit for performing the methods of the present invention, wherein said kit comprises means for measuring the expression level of SSBP1 protein and/or detecting SSBP1 SNP that is indicative of subject at risk of having or developing ocular disease related to mtDNA maintenance.

Typically the kit may include antibodies, primers, probes, macroarrays or microarrays as above described. For example, the kit may comprise a set of antibodies, primers, or probes as above defined, and optionally pre-labelled. Alternatively, antibodies, primers, or probes may be unlabelled and the ingredients for labelling may be included in the kit in separate containers.

The kit may further comprise hybridization reagents or other suitably packaged reagents and materials needed for the particular hybridization protocol, including solid-phase matrices, if applicable, and standards. The kit may further comprise amplification reagents and also other suitably packaged reagents and materials needed for the particular amplification protocol.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: SSBP1 protein expression in human retina and fibroblasts. A) Quantification of SSBP1 transcripts level in cultured skin fibroblasts from controls (C) and affected individuals (P1, P2 and P3). The mRNA levels were normalized to the reference gene L27. Western blot in lysates from controls (C1 and C2) and patient fibroblasts (P1, P2 and P3) and densitometric analysis of SSBP1 protein abundance in lysates from controls (C) and patient fibroblasts (P1, P2 and P3). GAPDH was used as a loading control. Data represent mean ± SEM. *P<0.05. (B) Representative ultrastructure of the mitochondria from control (C1) and patient fibroblasts (P1, P2 and P3) by TEM. Scale bar = 1 µm. Quantification analyses of abnormal mitochondria (large vacuoles, disturbed cristae) in fibroblasts from controls (C) and patients (P1, P2, P3). Data are presented as mean percentage of abnormal mitochondria ± SEM in total examined mitochondria. *P<0.05. All data are representative of 3 independent experiments.

Figure 2: Structural effects of SSBP1 mutations. Densitometric quantification of SSBP1 monomer (left) and dimer (right) in control (C1) and patient fibroblasts (P1, P2, P3). Data represent mean ± SEM. **P<0.01 and ***P<0.001, using unpaired t-test. All data are representative of 3 independent experiments.

Figure 3: Functional effect of SSBP1 mutation on mtDNA replication. (A) Quantification of mtDNA. mtDNA content was measured by qPCR and normalised to a nuclear gene (β-hemoglobin) in control (C) and patient fibroblasts (P1, P2, P3). (B) Quantification of area and volume of mtDNA nucleoids in controls (C) and patient fibroblasts (P1, P2, P3). (C) Quantification of mtDNA replication rate. Data represent mean ± SEM. *P<0.05, **P<0.01 using unpaired ttest. Mutation rates in mtDNA from control (C) and patient (P) blood. All data are representative of 3 independent experiments.

Figure 4: Zebrafish Knockdown of SSBP1 causes abnormalities in zebrafish development. (A) Gross developmental characteristics of zebrafish embryos injected with MO1 morpholino (MO SSBP1-1), or non injected at 72 hpf. (B) Percentage of morpholino-

injected embryos with normal phenotype or abnormal developmental characteristics at 72 hpf. NI: non injected, Std: standard control oligo, MO1: MO SSBP1-1, MO2: MO SSBP1-2, MO3: MO SSBP1-3.

EXAMPLE:

5 **Material & Methods**

Patients

Informed consent was obtained for clinical examination and genetic analysis from all patients. All methods were carried out in accordance with approved protocols of Montpellier University Hospital, Tuebingen University Hospital and in agreement with the Declaration of 10 Helsinki. The Ministry of Public Health accorded approval for biomedical research under the authorization number 11018S.

Age of onset, initial symptoms, best-corrected visual acuity, Goldmann visual fields, multimodal imaging (Nidek non mydriatic camera and Heidelberg Spectralis OCT device, full-fied (Ff-ERG) and multifocal electroretinography (Mf-ERG) were reviewed in affected 15 members of families.

Targeted exome sequencing

Genomic DNA is captured using Agilent enrichment solution method with their biotinylated oligonucleotide bank probes (Human All Exon V5, Agilent). The paired-end high-throughput sequencing of 75bp Illumina HiSeq 2000. For a detailed explanation of the protocol, 20 see the publication in Nature Methods(31). Sequence capture, enrichment and elution are performed according to the supplier's protocol and recommendations (SureSelect, Agilent) without modification. Briefly, 3 µg of each genomic DNA is fragmented by sonication and purified to obtain fragments of 150-200 bp. The oligonucleotide adapters for sequencing both ends of the fragments are ligated and repaired with adenine, added to the ends and then purified 25 and enriched by 4-6 PCR cycles. 500 ng of purified libraries are then hybridized to SureSelect capture oligonucleotides bank for 24 hours. After hybridization, washing and elution, the eluted fraction is amplified by 10 to 12 PCR cycles, purified and quantified by quantitative PCR to obtain sufficient DNA template for subsequent downstream processes. Each DNA sample was eluted and enriched then sequenced on an Illumina HiSeq 2000 for 75 b sequences from each 30 end. Image analysis and determination of the bases are made by Illumina RTA software version 1.14 with default settings.

Bioinformatics Analysis

Bioinformatics analysis of sequenced data is based on Illumina CASAVA1.8 pipeline. CASAVA1.8 is a suite of scripts including the sequence alignment to the complete genome

(build37 for human), counting and detection of allelic variants (SNPs and indels). The alignment algorithm used is ELANDv2e (Maloney alignment and multi-seed reducing artifactual mismatches). Note that only the positions included in the coordinates of the target regions are preserved.

5 Annotation of genetic variation is carried out internally, including gene annotation (RefSeq and Ensembl), referenced polymorphisms (dbsnp132, 1000Genomes) followed by a characterization of the mutation (exon, intron, silent, false nonsense, etc). For each position, exomic frequencies are also determined (Homo and HTZ) taking into account at least 150 Exomes sequenced in IntegraGen. The results are made per sample, as tabulated text files. We 10 also provide the control quality results of sequencing targets (depth/coverage).

Cell cultures

Fibroblasts were cultured from skin biopsies taken after obtaining informed consent from two controls and three affected patients carrying mutations in SSBP1 gene (p.R38Q). Fibroblasts were cultured in two-thirds of RPMI 1640 Medium (Thermo Fisher Scientific) 15 supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin–amphotericin B (Thermo Fisher Scientific) and one third of AmnioMax-C100 basal media (Thermo Fisher Scientific) with Amniomax C100 supplement (Thermo Fisher Scientific).

Reverse Transcription and qPCR studies

RT-qPCR was used to analyse the expression of SSBP1 mRNA in fibroblasts. RNA was 20 isolated using the QiaShredder and RNeasy mini kits (QIAGEN) according to the manufacturer's instructions. 1 µg was reverse transcribed using the SuperScript III First Strand Synthesis System Kit (Invitrogen). qPCR amplification was performed using SSBP1 specific primers (forward: TCAGGACCCTGTCTTGAGA (SEQ ID NO: 2) and reverse: GATATTCTGTGCCATGTTGTC (SED ID NO: 3)) and the Light Cycler 480 SYBR Green I 25 Master mix on a Light Cycler 480 II thermal cycler (Roche). Results were normalised to the ribosomal protein L27 gene expression and were analysed using LightCyclerVR 480 software and the Microsoft Excel program. n=3 independent experiments.

Mitochondrial DNA measurements

Total DNA purifications were performed using the DNeasy kit (QIAGEN) and 30 quantified by spectrophotometry (Nano-Drop 1000). Nuclear and mitochondrial DNA respective abundances were quantified in triplicate by qPCR in standard conditions using the LightCycler FastStart DNA Master SYBR Green I kit (Roche), with the human primers, HunuclS (ACACAACTGTGTTCACTAGC (SEQ ID NO: 4)), HunuclAS (CCAACTTCATCCACGTTCA SEQ ID NO: 5)), HumitS (TTCAGACCGGAGTAATCCAG

(SEQ ID NO: 6)) and HumitAS (AGTAGAACAGCGATGGTGAG (SEQ ID NO: 7)). The ratios between mtDNA and nuclear DNA concentrations are reported on graphs. n=3 independent experiments.

Mitochondrial respiration measurements

5 Controls and patients fibroblasts were seeded at 7000 cells/well in 100 µl of medium (RPMI/Amniomax) in Seahorse XF96 Cell Culture Microplates coated with 1/100 dilution Corning Matrigel hESC-qualified matrix (Dominique Dutscher) and using 8 replicates. Cells were incubated for 48 hours at 37°C in 5% CO2 atmosphere.

Cellular oxygen consumption (OCR) was assayed using the Seahorse XF96 10 Extracellular Flux Analyser with sequential addition of oligomycin (0,5µM), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 1µM), rotenone (1µM) and antimycin (0.05µM) to measure basal and maximal respiration.

Microscopy

Cells were seeded on glass coverslips (n=4 per conditions) and then fixed in 4% 15 paraformaldehyde in PBS for 10 min at room temperature, then washed three times with PBS and permeabilized in 0.1% Triton X-100 in PBS containing 5% FBS for 30 min at room temperature (PBSF), followed by washing three times with PBS. Primary anti-DNA mouse IgM antibodies (PROGEN, 61014) were diluted 1 µg/mL in PBSF, anti ATP synthase rabbit Ig antibodies (ATP5A, ab121229, Abcam) were diluted 1/500 in PBSF and incubated with cells 20 overnight at 4°C. After washing three times with PBSF, the cells were incubated for 1 h at room temperature with the fluorescent secondary goat anti-mouse IgG-Alexa-Fluor 488 antibody (Molecular Probes) and donkey anti-rabbit IgG-Alexa-Fluor 594 antibody (Molecular Probes), diluted 1/1000 in PBSF. Cells were rinsed three times with PBS for 10 min, and coverslips were mounted using fluorescence Mounting Medium (Dako).

25 The images were acquired using an LSM 700 LIVE DUO Confocal microscope (Carl Zeiss Microscopy). For all imaging, Z-stack images (1024×1024 pixels) were acquired and processed with the Bitplane Imaris 4 software. Two pictures with at least eight cells per pictures were analyzed for each line. To measure colocalization in the whole cell, images were processed using Imaris 4.0 (Bitplane) software using the automatic thresholding feature for colocalization. 30 Nucleoid fluorescence area and volume were obtained after maximal projection of the images using MetaMorph 5.0 software (Molecular Devices).

Protein production, crystal structure analysis and in-silico mutants

Protein production was as previously reported (32). The protein sample was cleaned from glycerol by gel filtration, the eluted fractions incubated with a 35nt poly-cytosine single

strand DNA (ssDNA) purification (4:2.2 Prot:DNA ratio), and dialysed in two steps to 50 mM NaCl, 20 mM TrisHCl pH 7. The final mix was concentrated to 8.6 mg/ml and used for extensive crystallization conditions screening. Crystallization conditions optimization by vapour diffusion method at 20 ºC in 24-well plates sitting drop format (Hampton Research) yielded crystals in 11 % PEG1500, 0.1 M cacodylate pH 6.5, 0.2 M magnesium chloride as reservoir solution. Crystals were incubated in 20% PEG200 and flash frozen in liquid nitrogen. Diffraction data was collected at ALBA synchrotron beamline XALOC in collaboration with ALBA staff, processed with XDS (33), scaled with Aimless and truncated with C-truncate at 2.1 Å (34). A set of 776 (5.2%) reflections were kept aside for cross-validation of the model. Previous human SSBP1 crystal structure (PDB code 3ULL(16)) mutated to poly-alanines was used for molecular replacement searches with MOLREP (34). Our sequence assignment clearly indicated a shift compared to the 3ULL structure, from Asn123 to the C-terminus. Indeed, 3ULL had previously been revised by the same authors by using a structural energy score validation method and taking the E.coli structure as a reference (PDB code 1S3O) (15), which revealed the sequence shift we experimentally found. However, their new sequence assignment revision did not improve the crystallographic phases and the electron density was still ambiguous at the C-terminus (15). Our structure, of higher resolution, unambiguously shows the actual sequence of h-mtSSB, and confirms 1S3O. Two □-hairpins (aa 67-73 in molecule A, and 66-79 in B) could not be fully traced due to high flexibility. An additional short segment of four residues (chain C, not traced in former structures) is sandwiched between □-sheets of symmetry partners, but its side chains were not built due to weak density; its close proximity to the first traced residue in molA suggests it may correspond to the N-terminal end. Stereochemistry validation indicated 100% of traced residues are in allowed Ramachandran regions. 20 models for the R38Q and R107Q mutants and WT controls were automatically generated with the single mutant routine implemented in Modeller (35). Since arginine and glutamine perform electrostatic interactions, we favoured these by setting the electrostatic restraints shell to 15 Å, so that optimization of side chain orientation was not based only in stereochemical clashes.

EdU labeling and detection

For 5-ethynyl-2'-deoxyuridine (EdU) labeling (Invitrogen) to detect and quantify mtDNA synthesis, primary patient and control fibroblasts were grown on coverslips in Dulbecco's modified Eagle's medium (DMEM; Lonza BE12-604F) supplemented with 10 % fetal calf serum (FCS) (GE Healthcare), in a humidified 37 °C incubator at 5 % CO2. All cell lines were routinely tested for mycoplasma contamination and found to be negative. EdU

labeling and detection was done as previously described (36) using 100 μM EdU for a 1 hr labeling period and Alexa Fluor 488 azide for detection. Following EdU labeling cells were incubated with an SSB1 antibody (Sigma, HPA002866, 1:100) detected with anti-rabbit Alexa Fluor 647 and a DNA antibody (Progen, 61014, Mouse monoclonal, IgM, 1:400) detected with an anti-mouse IgM AlexaFluor 568 antibody. For quantification, EdU and mtDNA foci were manually counted for 3 individual images for each patient cell line and control cell lines, as described previously (36).

Western Blot analyses

Levels of proteins were detected by immunoblot using commercially available antibodies, revealed using chemiluminescence. Pellets were mixed with 100 μl of RIPA lysis buffer (Sigma) with 1X Protease Inhibitor Cocktail (Roche). The cellular protein content was determined with the BCA kit (Thermo Fisher Scientific). Thirty μg of total fibroblasts protein were mixed with 2x Laemmli's sample buffer (Biorad) containing 1/20 dilution of b-mercaptoethanol (Sigma). The samples were heated 5 min at 95°C and loaded onto a 10% polyacrylamide precast MiniProtean TGX gel. The separated proteins were electrotransferred using a Trans-BlotVR TurboTM PVDF Transfer Pack and System. Membrane were saturated with 5% non-fat milk dissolved in 0.1% Tween-TBS for 2h at room temperature then incubated overnight at 4°C with polyclonal sheep anti-SSBP1 (1μg/ml AF6588 Bio-techne), monoclonal mouse anti-Grim19 (1:1000 ab110240 Abcam), anti-SDHA (1:1000 ab14715 Abcam), anti-UQCRCII (1:1000 ab14745 Abcam), anti-MTCO1 (1:1000 ab14705 Abcam), anti-GAPDH (1:2000 G8795 Sigma) and polyclonal rabbit anti-ATP synthase (1:1000 ab151229 Abcam).

Membranes were washed three times in 0.1% Tween-TBS and incubated with anti-sheep IgG horseradish peroxidase conjugated antibody (1:1000 Bio-techne), anti-rabbit IgG, or anti-mouse IgG HRP linked antibody (1:10 000 Sigma) for 2h at room temperature. The immunoreactive proteins were visualized with enhanced chemiluminescence (ECL+ Western Blotting Detection Reagents, Amersham Biosciences). Band intensities were quantified with ImageJ. n=3 independent experiments.

For sensitivity of mutant and wild-type SSBP1 to reducing conditions, three biological repeats of the three patient fibroblast cell lines as well as two control fibroblast lines were collected and lysed for 10 min on ice in lysis buffer (50 mM Tris-HCl pH7.4, 150mM NaCl, 1 mM EDTA, 1% TX-100 and 2.5 mM PMSF) followed by a centrifugation step of 14 000g for 5 min at 4°C. 60 μg of cellular lysates were separated by SDS-PAGE using sample buffer that included freshly added β-mercaptoethanol to obtain a final concentration in the sample of 1%. SDS-PAGE was followed by Western blotting onto supported nitrocellulose membranes.

Membranes were probed with antibodies against proteins of interest and HRP-conjugated secondary antibodies followed by ECL detection. ECL reactions were visualized with a ChemiDoc instrument (Biorad). Antibodies used for Western blot detection were SSBP1 (Sigma, HPA002866); TFAM (kind gift of Dr. R. Wiesner); POLG1 (Santa Cruz, sc5931); GRSF1 (Sigma, HPA036985) and Actin (Novusbio, NB600-532H). Band intensities were determined and corrected for loading based on the actin signal. The results for the three biological repeats of both control cell lines were pooled to obtain single control values, while patient cell lines were treated separately.

Mitochondrial DNA deletion analysis

For Long-Range PCR (LR), control and patient DNA samples (50ng/µl) were prepared. Mitochondrial DNA was amplified using three pairs of primers: LR1: F1:8285-8314 and R1: 15600-15574 (wild-type mtDNA fragment of 7315 bp), LR2: F2: 3485-3519 and R2: 14820-14786 (wild-type mtDNA fragment of 11335 bp) and LR3: F3: 5459-5493 and R3: 735-701 (wild-type mtDNA fragment of 11845 bp). The PCR conditions are: 1 cycle at 94°C for 1 min; 30 cycles at 98°C for 10 s and 68°C for 11 min; a final extension cycle at 72°C for 10 min. The PCR was performed using TaKara LA Taq DNA polymerase for the first pair of primers, and TaKara Ex Taq DNA polymerase for the other two sets of primers (TaKara Shuzo Corp.).

DNA extraction

Total DNA was extracted from the peripheral blood (n=35) using phenol chloroform standard procedures, and from the urine (n=29) using the High Pure PCR Template Preparation Kit (Roche).

MtDNA sequencing and analysis

The entire mtDNA molecule was amplified as two over-lapping 8.5 kilo base (kb) fragments. Library preparation was performed using the Ion Plus Fragment Library Kit (Cat. no. 4471269).

Sample emulsion PCR, emulsion breaking, and enrichment were performed using the Ion 540 Kit–Chef (Cat. No. A27759) and sequenced on the Ion S5 Sequencer.

Sequencing data base calling and mapping were performed using Ion Torrent Suite. Variant analysis was done with a dedicated in-house bioinformatic pipeline including the calling, annotation and prioritization steps. The calling module integrates the prediction of six callers. All of the variant calling formats (VCFs) generated were normalized and decomposed before launching the annotation-prioritization module, which combines the ANNOVAR for variant prioritization. ANNOVAR allowed the inclusion of several databases, i.e. Mitomap and MitImpact2, and prioritization tools, i.e. Polyphen2, SIFT and MutationTaster.

Searching for mtDNA deletions and insertions was performed using the eKLIPSE program which is based on a soft-clipping analysis (37).

Morpholino Injection

The following antisens morpholino-oligonucleotides (MOs) were obtained from Gene Tools LLC: MO SSBP1-1 5'-GGTGCTATAATGTTTACCGATATGT-3' (SEQ ID NO: 8), MO SSBP1-2 5'-CTCAACATCTTCTCTGCTGCGTC-3' (SEQ ID NO: 9) MO SSBP1-3 5'-TGAGAAGGCTGCAATAACCCCACGC-3' (SEQ ID NO: 10). Zebrafish embryos (from WT-AB) were injected at the 1-2 cell stage with antisense morpholino oligonucleotides. Ssbp1 MOs and standard controls oligo were injected at 0,1mM each.

Statistics

Statistical analyzes of the data were carried out using the GraphPad Prism software, version 5.00. For the analysis of mutation and deletion rates in blood and urine mtDNA, a comparison between both groups was carried out using the non-parametric test (Mann-Whitney test). t test was used for quantification of mtDNA synthesis and SSBP1 monomer and dimer. The Mann-Whitney was used to compare the fibroblasts from SSBP1 patients and controls. Differences were considered significant at $p<0.05$ *, $p<0.01$ ** and $p<0.001$ ***.

Results

Identification of mutations in SSBP1 in families with dominant optic atrophy.

In our cohort of unsolved patients with ADOA, we performed Whole Exome Sequencing (WES) to identify the causative gene defect. WES (Human All Exon V5, Agilent followed by Illumina HiSeq2000) was performed on 8 affected individuals IV:4, IV:6, V:3, V:9, V:11, VI:4, VI:8, VI:17 and one unaffected individual VI:18 from family A (data not shown). We confirmed that no mutation in genes previously associated with ADOA or maculopathy was present in these patients. After filtering for variants potentially affecting protein function due to the introduction of missense, nonsense, splicing or frameshift in coding regions, we focussed our analysis on genes that code for mitochondrial proteins. We identified a c.113G>A (p.Arg38Gln) substitution in exon 4 of SSBP1 (MIM: 600439) (data not shown), which encodes the mitochondrial single-stranded DNA-binding protein, a key protein in the regulation of mitochondrial DNA (mtDNA) replication (12). This variant was not found in the sequenced alleles list from the Genome Aggregation Database (gnomAD). The mutation changes an amino acid evolutionary conserved among vertebrates (data not shown) and is

predicted to be pathogenic by SIFT and PolyPhen2. Sanger sequencing demonstrated that the variant segregated with the optic atrophy in all sequenced individuals.

Screening of SSBP1 in a cohort of 174 European probands with inherited optic neuropathy and without genetic diagnosis, identified four additional German families with SSBP1 mutation (data not shown). One (Family B) was also heterozygous for the c.113G>A (p.Arg38Gln) mutation in exon 4. The three other families (Family C, D and E) were heterozygous for a novel mutation c.320G>A causing the amino acid p.Arg107Gln substitution (data not shown). This missense mutation occurs also within a region conserved among species (Figure 1C) and was absent in gnomAD.

Both mutations are located within the DNA binding region, which is required to maintain the structural stability of the mitochondrial genome in mtDNA replication (13) (data not shown).

Clinical phenotype of patients with SSBP1 mutations.

The predominant clinical symptom exhibited by the present cohort of patients with SSBP1 mutations is an optic neuropathy. DNA samples from 36 patients from four generations in family A (Table 1), showed that 28 patients carried the c.113G>A (p.Arg38Glu) mutation in exon 4 of the SSBP1 gene. Three patients sharing the mutation c.133G>A (p.Arg38Glu) were asymptomatic with 20/20 visual acuity, but with some color vision anomalies. All the 18 symptomatic patients had an optic neuropathy with a bilateral pallor of the temporal neuroretinal rim (data not shown). Visual acuity varied from 20/400 to 20/20. Protan or deutan color defects were noted. Central, coeco-central and paracentral scotomas with preserved peripheral isopters were identified in all symptomatic patients. Among these 18 patients, 10 patients also had a foveopathy only discovered by SD-OCT with tiny bilateral small defects of the EZ and IZ restricted to the foveola (data not shown). The 4 other families exhibited isolated optic atrophy, except for family B, in which the 2 sisters (III:1 and III:2) of the last generation had abnormal macula.

SSBP1 expression.

To study the cellular localization of SSBP1 in human retina, human retinal slices were co-stained with mitochondrial marker of complex V, ATP synthase and SSBP1. SSBP1 was preferentially expressed in retinal ganglion cells, photoreceptors and pigmented epithelium (data not shown).

To further investigate the molecular consequences of SSBP1 mutations, we characterized skin fibroblasts from 3 affected patients (V:11, VI:17, V:9, respectively P1, P2, P3) and 2 healthy control individuals (V:14, VI:18, respectively C1, C2). SSBP1 transcript

levels were first examined. No significant difference in the mRNA expression of SSBP1 was found in patients compared to control fibroblasts (Figure 1A). Western blot analysis showed that SSBP1 was markedly reduced in P1, P2 and P3 compared to controls (Figure 1A), indicating that this mutation leads to the loss of SSBP1 stability. We then examined whether mitochondrial morphology were altered in SSBP1 mutant cells. Mitochondrial network remains unchanged but ultrastructural examination of mitochondria with transmission electron microscopy (TEM) indicated that the overall structure was abnormal in the most affected patient P1 showing swelling and disorganized cristae (Figure 1B).

Novel revised SSBP1 crystal structure and structural effects of SSBP1 mutations.

The effect of Arg38Gln and Arg107Gln mutations was elucidated within the three-dimensional structure of SSBP1. In the Protein Data Bank, three SSBP1 crystal structures are available (PDB codes 3ULL and 1S3O (15, 16), and 2DUD, without associated citation). However, these structures show different sequence assignment for the 15 aminoacids at the C-terminus. To clarify this issue, we solved the crystal structure of SSBP1 at higher resolution and calculated the electron density maps. Human SSBP1 consists of a dimer (molecules molA and B) that contacts a second, symmetrically-related dimer (molA' and B') giving rise to the tetrameric biological unit (16, 17) (data not shown).

Strikingly, Arg38 and Arg107 contact each other at the end of neighboring β-strands of the barrel in a monomer (data not shown). Arg38 is close to the surface that in the homologues contacts the DNA (label (DNA), whereas Arg107 is packed against a hydrophobic pocket at the dimer interface. Therefore, both arginines participate in AB (and BA) dimer interactions.

To understand the effect of the mutations, we generated crystal structure-based mutant models employing single-site mutation modelling (20 models per mutant). As a control, we 'mutated' the two arginines 38 and 107 to Arg (R38R and R107R, respectively) (data not shown). The models of these latter showed highly variable orientations for the Arg38 side chain, which sometimes entailed breaking its interaction with Glu27 molB and often reaching the region plausibly contacted by single strand DNA (ssDNA). R38Q mutation showed that the much shorter side chain does not reach the putative DNA binding region (data not shown). Mutation of the Arg107 residue to Gln increases the distance to main-chain carbonyls of either Arg28 molB or Phe139-B' molB', thus the contacts with the other subunits are lost (data not shown). Finally, it is worth noting that the stabilizing van der Waals interactions between arginine side chains observed in the crystal cannot take place in the mutants due to shortening of side chains and the presence of charges at positions were methylenes are found in the WT.

Human SSBP1 tetramers have DNA binding activities. The aforementioned modelling of disease mutations based on the new crystal structure suggested instability of dimer formation. We assessed the effect of R38Q mutation on SSBP1 multimer (dimer, tetramer) stability in patient fibroblasts. Under mild reducing conditions with SDS/PAGE, SSBP1 showed that patient fibroblasts contained an important increase in monomers with respect to dimers, whereas in controls dimers are more prominent than monomers (Figure 2). These findings suggested that the Arg38Gln mutation is sufficient to destabilize the SSBP1 tetramers.

SSBP1 mutation affects mtDNA replication.

To clarify the molecular consequences of SSBP1 mutations, we analyzed patient fibroblasts to detect eventual mtDNA deletion and depletion. MtDNA copy number analysis by qPCR detected significant depletion of mtDNA in all fibroblasts tested (Figure 3A-B). This was also observed by staining the mitochondrial nucleoids with an anti-DNA antibody (data not shown). In line with these findings, we observed that TFAM and POLG1 protein levels were also reduced in patient fibroblasts (data not shown). We further evaluated area and volume of nucleoids in patient fibroblasts, disclosing a 75% reduction of anti-DNA immunofluorescence in patients compared to controls, confirming the depletion observed by qPCR, and a marked decrease in the number of nucleoids in patient cells (Figure 3A-B). To further examine the consequence of SSBP1 mutation on mtDNA replication, we visualized newly synthesized mtDNA using EdU incorporation, a marker for DNA synthesis. EdU incorporation was downregulated in patient fibroblasts compared to controls (Figure 3C). These results clearly indicate perturbed mtDNA replication, similar to that observed in patients with pathogenic mutations in genes coding for proteins that function directly at the replication fork (POLG, TWINKLE) (18). Since SSBP1 stimulates mtDNA replication, destabilization of the replication machinery may cause mutations and promote mispairing, thereby inducing deletions. To test this eventuality, we collected urines from affected and unaffected individuals from the family A (n=29) and sequenced by NGS their mtDNA, together with the ones from the blood samples (n=35). After eliminating variants related to the haplogroup differences, analyses of the sequences revealed a significantly higher number of mutations in the mtDNA from the blood samples from the SSBP1 patients compared to that of controls (Figure 3C), while no difference was found between the urine samples (Supplemental Figure 3), although the levels of mtDNA mutations were 4 times higher in the mtDNA from urine than in blood. Long-range PCR revealed the absence of mtDNA deletion in the skin biopsies (data not shown), blood and urine samples (data not shown) from patients of the family A. These results suggested that SSBP1

R38Q mutation affects the fidelity of the mtDNA replication, but does not promote mtDNA recombination.

We then evaluated the effect of SSBP1 R38Q mutation on mitochondrial respiration using the Agilent Seahorse XF Analyzer. Even if patient's values were somehow lower than controls, basal and maximal respiration were not significantly different between the controls and patient fibroblasts (data not shown). Complex I and complex II driven respiration remained unchanged between controls and patient fibroblasts. Protein level were quantified for each complex and the protein abundance of complex I (GRIM19), complex II (SDHA), complex III (UQCRC2), complex IV (MTCO1) and complex V (ATP synthase) subunits did not differ between control and patient cells (data not shown).

In this work, we identified *SSBP1* as a new gene associated to mitochondrial DNA depletion diseases and with an unexpected dominant phenotype: a non-syndromic optic atrophy with foveopathy. Using Whole Exome sequencing on DNA from 8 affected members from a large family with clinical features of ADOA and following target sequencing of the candidate gene in additional ADOA families we have shown the linkage between mutations in *SSBP1* and familial ADOA in 5 independent families, expanding the number of genes associated with ADOA. The patients have an optic neuropathy with a clinical presentation similar to *OPA1* mutations but also a specific clinical feature, which consists in a singular foveopathy never described in any mitochondriopathy or optic neuropathy.

Our study completes years of investigations on mtDNA maintenance disorders adding to previous known genes, mutations in SSBP1 causing an unexpected form of dominant transmission of mtDNA disease associated with dominant optic neuropathy and foveopathy.

Moreover, injection of fertilized zebrafish eggs with different antisense morpholino oligonucleotides to block the translation or splicing of endogenous *ssbp1* mRNA resulted, in preliminary results, in alteration embryo development with MO1. Abnormal embryos were small, short or stunted. In contrast, the respective mismatch *ssbp1* morpholino controls had no effects (Figure 4A-B).

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method for predicting the risk of having or developing ocular disease related to mtDNA maintenance in a subject comprises following steps: i) determining the expression level of SSBP1 gene, ARN or protein in a biological sample obtained from said subject, ii) comparing the expression level of SSBP1 gene, ARN or protein determined at step i) with a predetermined reference value and iii) concluding that the subject is at risk of having or developing ocular disease related to mtDNA maintenance when the expression level of SSBP1 gene, ARN or protein determined at step i) is lower than the predetermined reference value, or concluding that the patient is not at risk of having or developing ocular disease related to mtDNA maintenance when the expression level of SSBP1 gene, ARN or protein determined at step i) is higher than the predetermined reference value.

2. The method according to claim 1 wherein said method further, comprising the steps of: i) identifying at least one mutation in the SSBP1 gene and/or protein; ii) concluding that the subject is at risk of having or developing ocular disease related to mtDNA maintenance when at least one mutation is identified.

3. The method according to claim 2, wherein the mutation is c.113G>A and/or c.320G>A in the SSBP1 gene.

4. The method according to claim 2, wherein the mutation is R38Q and/or R107Q in the SSBP1 protein.

5. The method according to claims 1 to 4, wherein the ocular disease related to mtDNA maintenance is selected from the group consisting of but not limited to: optic neuropathy, Autosomal dominant optic atrophy (ADOA), Foveopathy, Leber's hereditary optic neuropathy (LHON), glaucoma, retinal macular dystrophy, optic nerve dysfunction, or progressive external ophthalmoplegia (PEO), or ptosis involving the extraocular muscles.

6. A method for treating ocular disease related to mtDNA maintenance in a subject in need thereof comprising a step of administering to said subject a therapeutically amount of an inhibitor of mutated SSBP1 gene expression.

7. The method according to claim 6, wherein the inhibitor of mutated SSBP1 gene expression is siRNA, shRNA, miRNA, antisense oligonucleotide, or a ribozyme.

8. A method of treating ocular disease related to mtDNA maintenance in a subject in need thereof comprising a step of administering to said subject a therapeutically effective amount of a vector which comprises a nucleic acid molecule encoding for SSBP1.

9. The method of claim 8, wherein the nucleic acid molecule encoding for a SSBP1 polypeptide comprising an amino acid sequence having at least 90% of identity with the sequence as set forth in SEQ ID NO:1.

10. The method according to claim 8, wherein the vector is a viral vector.

11. The method of claim 8, wherein the vector is an adeno-associated virus (AAV) vector.

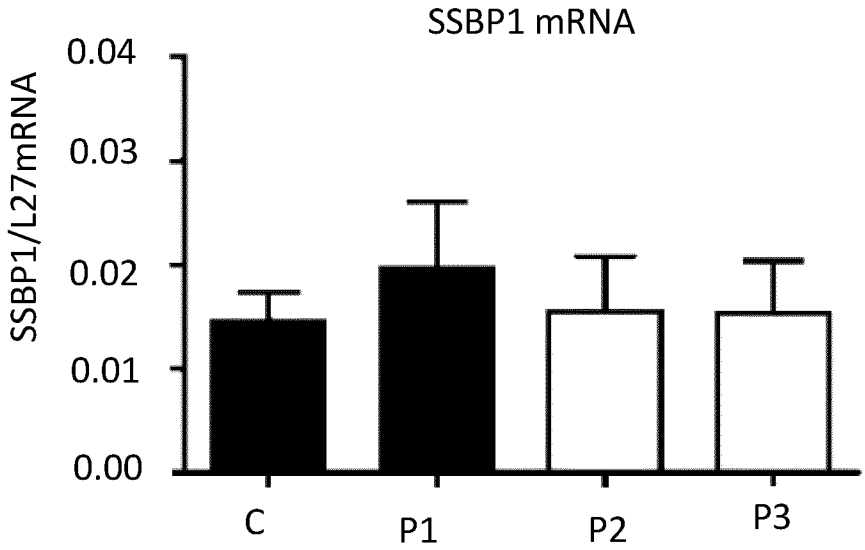
12. The method of claim 8, wherein the AAV vector is selected from vectors derived from AAV serotypes having tropism for and high transduction efficiencies in ocular cells.

13. The method of claim 8, wherein the AAV vector is an AAV1, AAV 2, AAV 5, AAV 7, 8 or AAV 9.

14. The method of claim 8, wherein the vector is delivered by intravitreous, subcutaneous, or intravenous delivery.

15. A kit for performing the methods according to claims 1 to 5, wherein said kit comprises means for measuring the expression level of SSBP1 protein and/or detecting SSBP1 SNP that is indicative of subject at risk of having or developing ocular disease related to mtDNA maintenance.

A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SSBP1/L27mRNA
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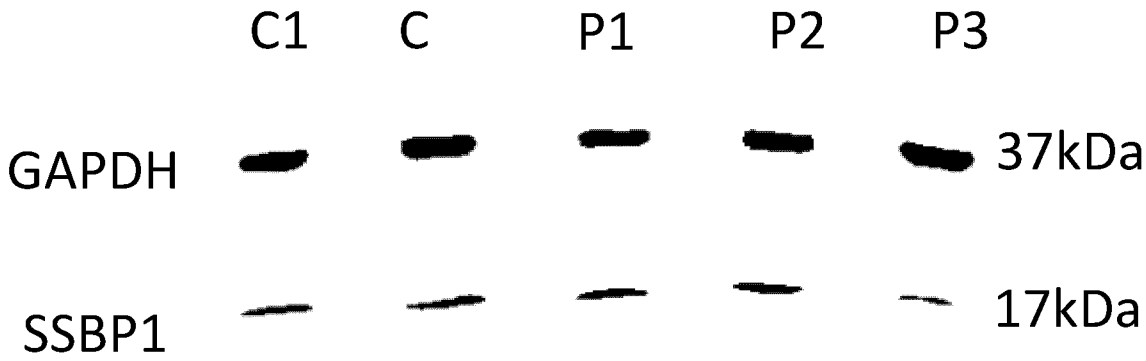
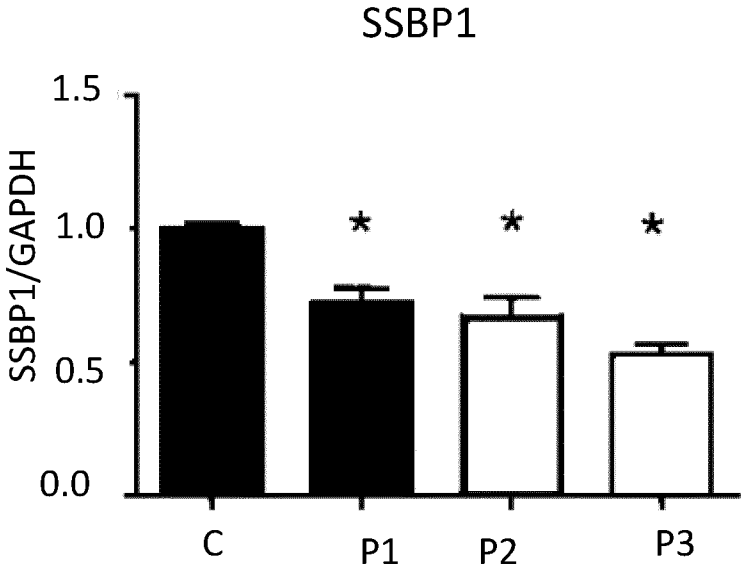
0.03
0.02
0.01
0.00
C
P1
P2
P3


C1
C
P1
P2
P3
GAPDH
37kDa
SSBP1
17kDa


SSBP1
SSBP1/GAPDH
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1.0
0.5
0.0
*
*
*
C
P1
P2
P3

Figure 1A

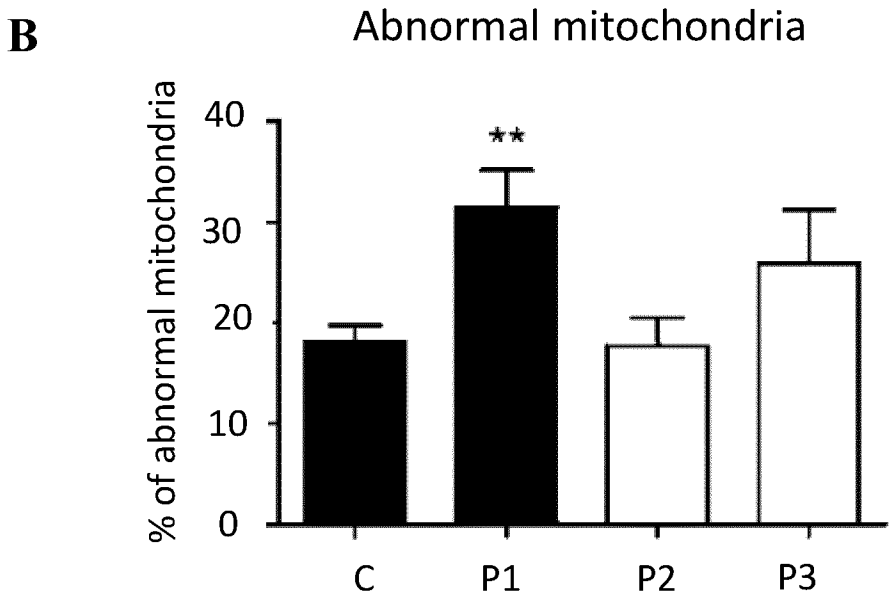


Figure 1B

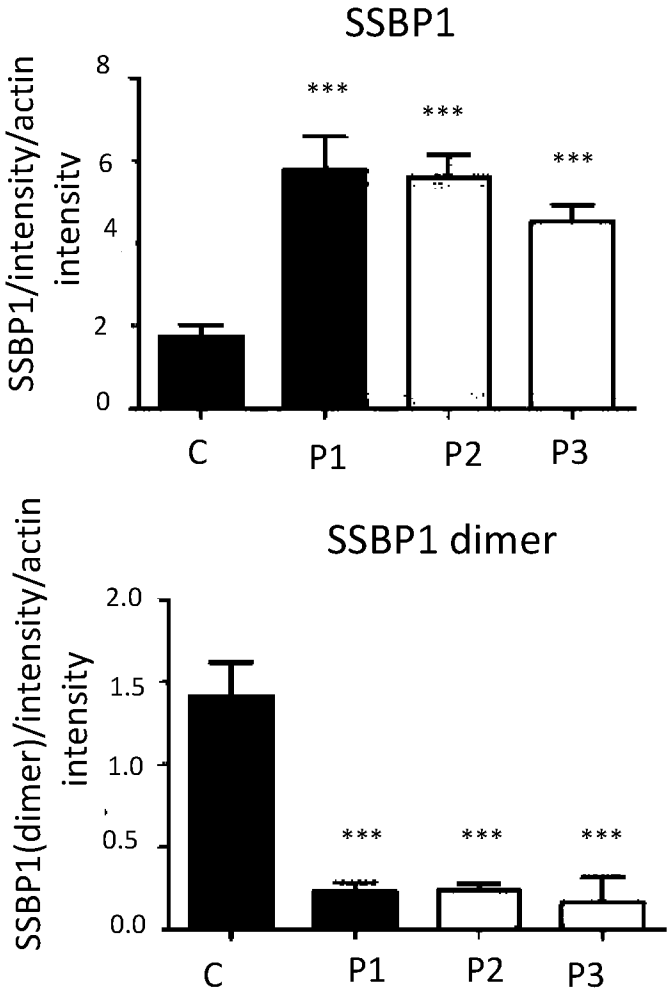


Figure 2

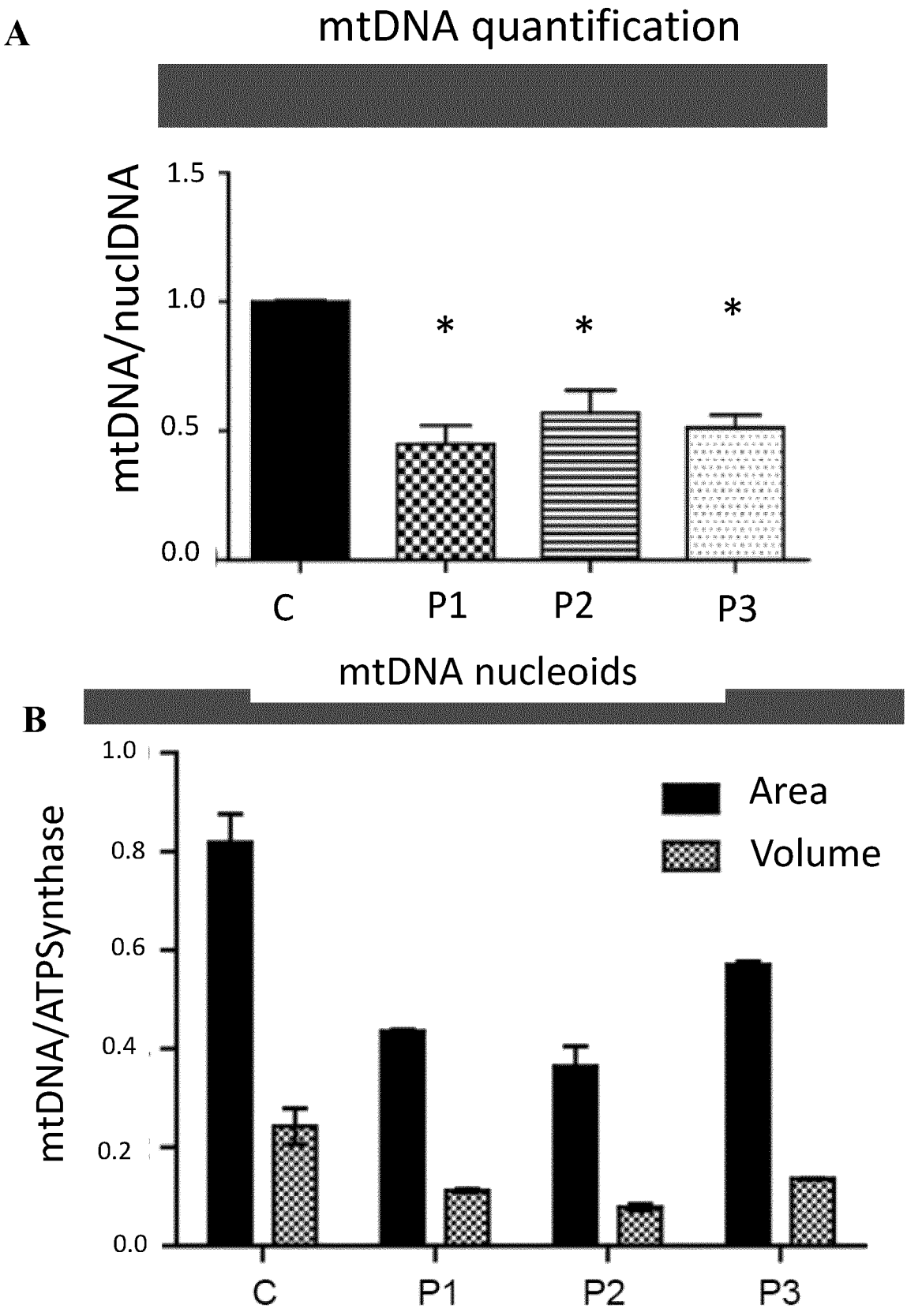
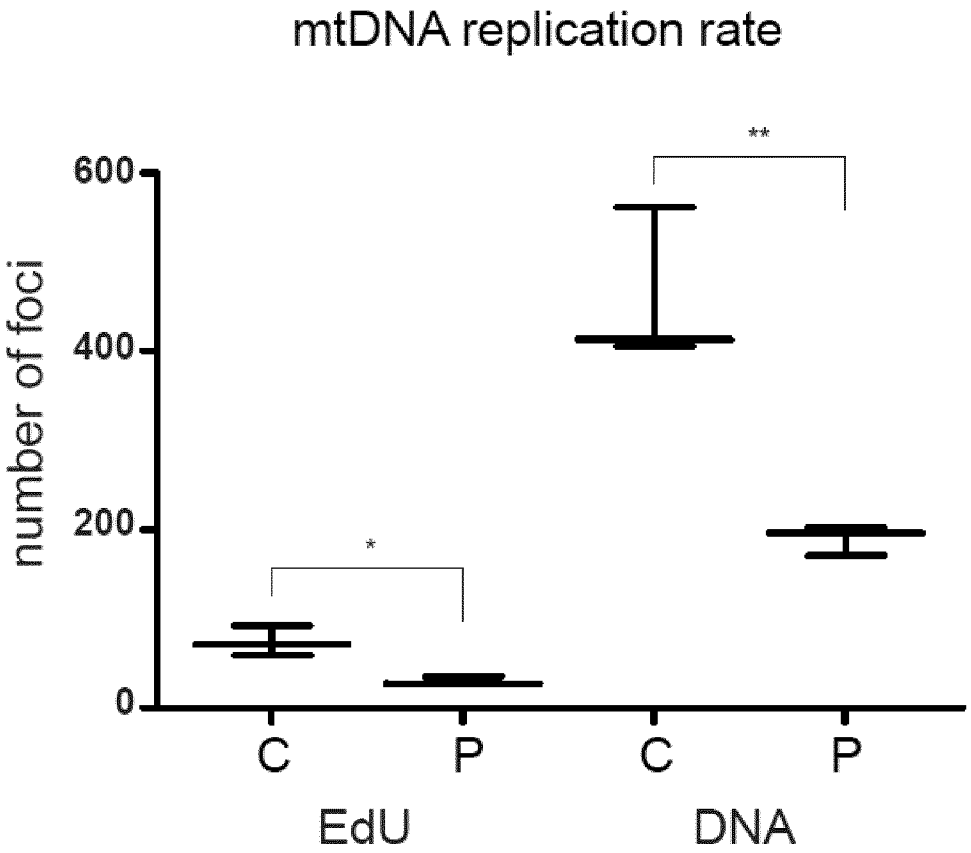

A
mtDNA quantification
mtDNA/nuclDNA
1.5
1.0
0.5
0.0
*
*
*
C
P1
P2
P3
mtDNA nucleoids
B
mtDNA/ATPSynthase
1.0
0.8
0.6
0.4
0.2
0.0
Area
Volume
C
P1
P2
P3

Figure 3A-B

C


mtDNA replication rate
number of foci
600
400
200
0
**
*
C
P
C
P
EdU
DNA

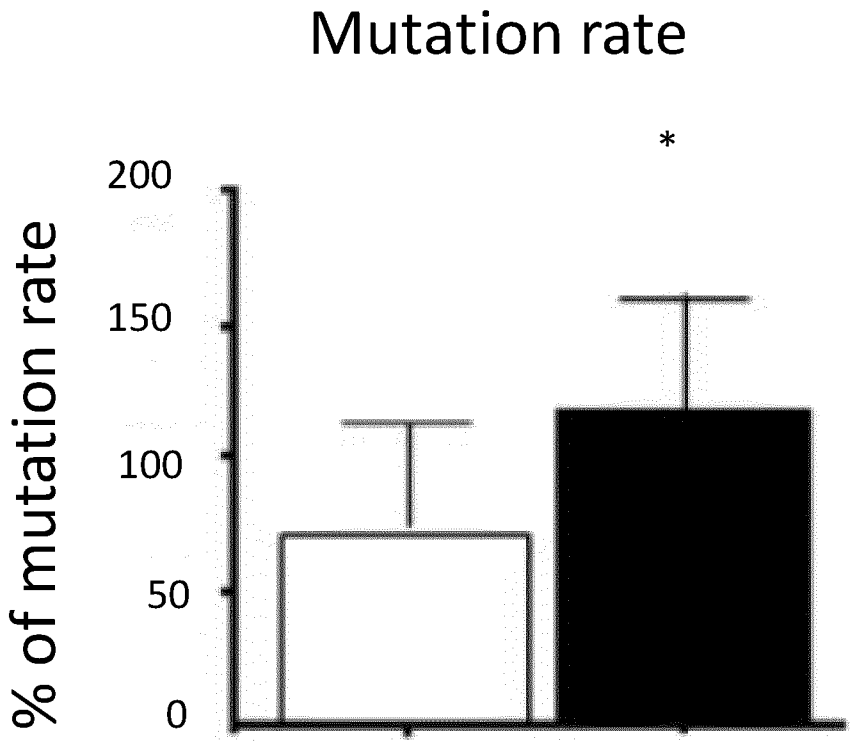

Mutation rate
% of mutation rate
200
150
100
50
0
*

Figure 3C

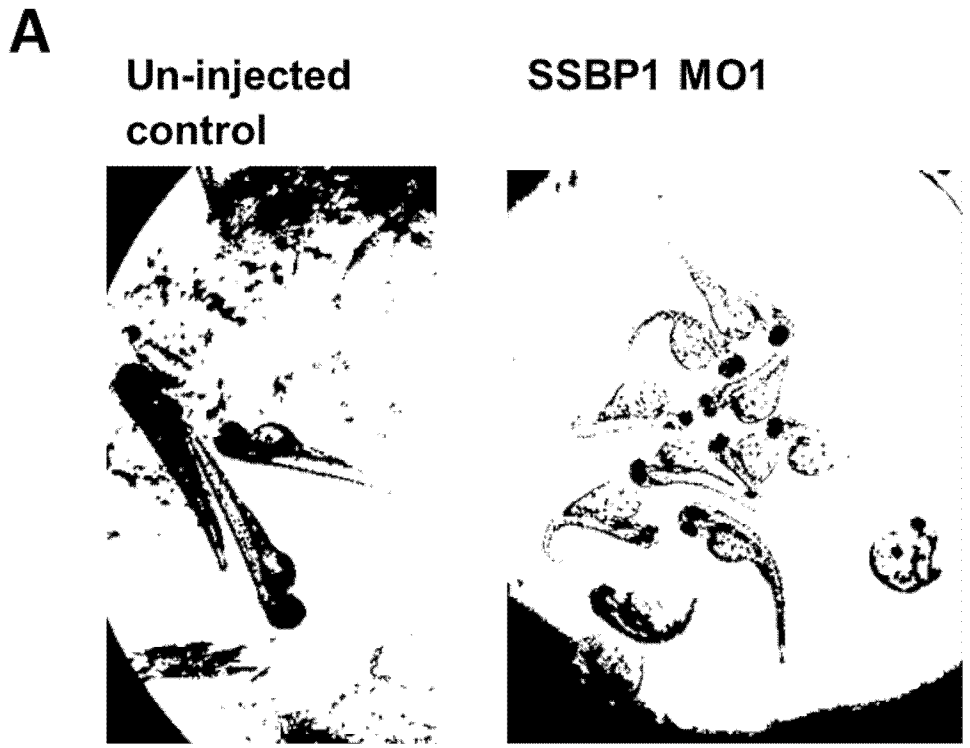


Figure 4A

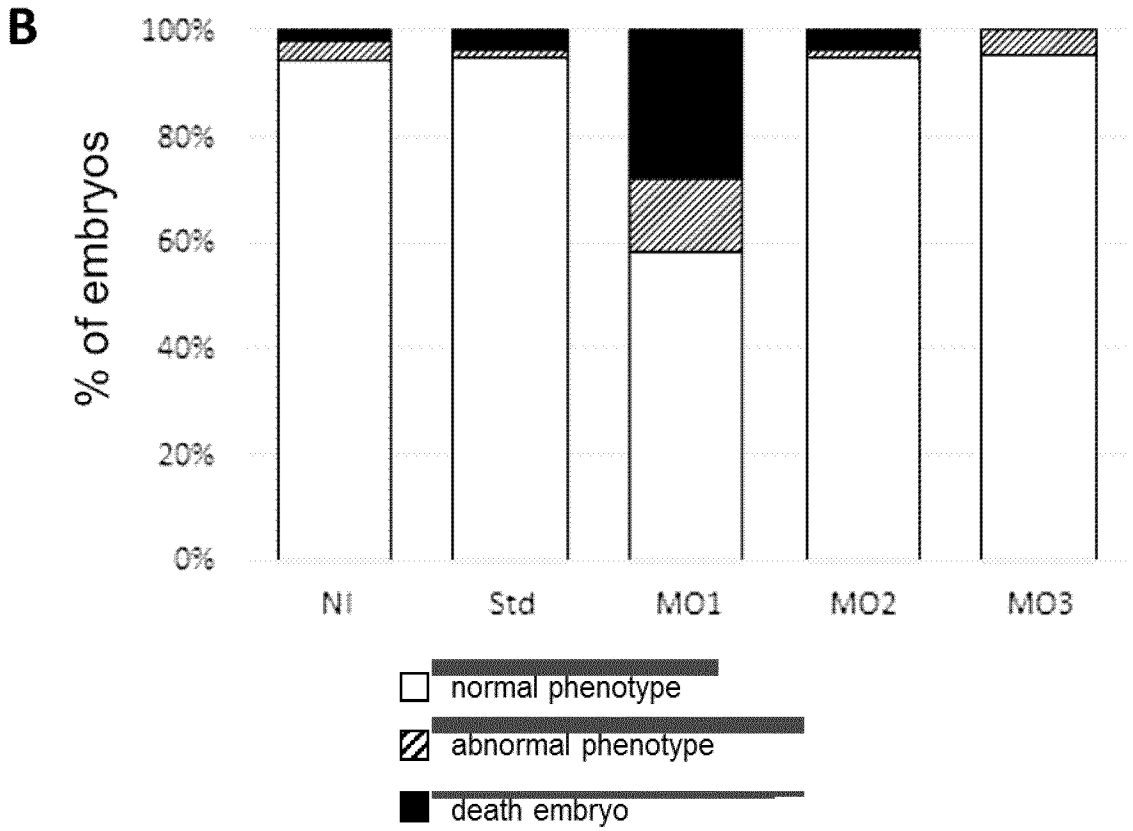


Figure 4B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/066364

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/6883 G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q G01N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YOU WANG ET AL: "Downregulation of Mitochondrial Single Stranded DNA Binding Protein (SSBP1) Induces Mitochondrial Dysfunction and Increases the Radiosensitivity in Non-Small Cell Lung Cancer Cells", JOURNAL OF CANCER, vol. 8, no. 8, 12 May 2017 (2017-05-12), pages 1400-1409, XP055640362, AU ISSN: 1837-9664, DOI: 10.7150/jca.18170 page 1401, column 2, paragraph 3 - page 1402, column 1, paragraph 1 ----- -/--	15

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 August 2020	17/08/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bradbrook, Derek

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/066364

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	VALENTINA DEL DOTTO ET AL: "SSBP1 mutations cause mtDNA depletion underlying a complex optic atrophy disorder", JOURNAL OF CLINICAL INVESTIGATION, 24 September 2019 (2019-09-24), XP055640371, DOI: 10.1172/JCI128514 the whole document	

T	CAMILLE PIRO-MÉGY ET AL: "Dominant mutations in mtDNA maintenance gene SSBP1 cause optic atrophy and foveopathy", JOURNAL OF CLINICAL INVESTIGATION, 24 September 2019 (2019-09-24), XP055640372, DOI: 10.1172/JCI128513 the whole document	

T	NERINGA JURKUTE ET AL: "SSBP1 mutations in dominant optic atrophy with variable retinal degeneration", ANNALS OF NEUROLOGY., vol. 86, no. 3, 12 July 2019 (2019-07-12), pages 368-383, XP055640377, BOSTON, US ISSN: 0364-5134, DOI: 10.1002/ana.25550 the whole document	

A	DELETTRE C ET AL: "NUCLEAR GENE OPA1, ENCODING A MITOCHONDRIAL DYNAMIN-RELATED PROTEINIS MUTATED IN DOMINANT OPTIC ATROPHY", NATURE GENETICS, NATURE PUBLISHING GROUP, NEW YORK, US, vol. 26, no. 2, 1 October 2000 (2000-10-01), pages 207-210, XP000993218, ISSN: 1061-4036, DOI: 10.1038/79936 abstract	1-15

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