

PRENYLATION ASSAY

FIELD OF THE INVENTION

The present invention relates to an assay for use in determining the activity of Rab escort protein 1 (REP1). More specifically, the invention relates to the use of Rab6a in an assay as a substrate for prenylation, in particular wherein the REP1 has been delivered to a cell using a gene therapy vector.

BACKGROUND TO THE INVENTION

Choroideremia is a rare disease which leads to degeneration of the choroid, retinal pigment epithelium and photoreceptors of the eye. Afflicted males typically exhibit nightblindness during teenage years, progressive loss of peripheral vision during the 20's and 30's and complete blindness in the 40's. Female carriers have mild symptoms, most notably nightblindness, but may occasionally have a more severe phenotype.

Choroideremia is caused by mutations in the *CHM* gene, which encodes for Rab escort protein 1 (REP1). Rab escort protein 2 (REP2), which is 75% homologous to REP1, compensates for any REP1 deficiency in most cells of the body. However, REP2 is unable to compensate for REP1 deficiency in the eye. This leads to insufficient Rab escort protein activity to maintain normal prenylation of target Rab GTPases and gives rise to cellular dysfunction and ultimately cell death.

Choroideremia may be successfully treated by providing functional copies of the REP1 transgene to the affected cells of the eye (MacLaren, R.E. *et al.* (2014) *Lancet* 383: 1129-37). Specifically, it has been shown that adeno-associated virus (AAV) gene therapy vectors may be used to deliver a nucleotide sequence encoding functional REP1 to the eye to treat the disease.

As gene therapy of choroideremia is becoming a clinical reality, there is a need for reliable and sensitive assays to determine the activity of exogenously delivered REP1, in particular to test new gene therapy vectors and as a quality control screen for clinical vector stocks.

Existing methods for assaying REP1 use Rab27a as a prenylation substrate (Tolmachova, T. *et al.* (2012) *J. Gene Med.* 14: 158-168; Tolmachova, T. *et al.* (2013) *J. Mol. Med.* 91: 825-837; Vasireddy, V. *et al.* (2013) *PLoS ONE* 8: e61396; and Black, A. *et al.* (2014) *J. Gene Med.* 16: 122-130). This has likely followed from numerous implications of Rab27a in the pathogenesis of choroideremia. For example, it has been shown that Rab27a is present unprenylated in choroideremia cells while other Rabs are properly prenylated (Seabra, M.C.

et al. (1995) J. Biol. Chem. 270: 24420-24427). Furthermore, Rab27a is expressed at high levels in the retinal pigment epithelium and choriocapillaries, the two sites of earliest degeneration in choroideremia.

However, assays relying on the prenylation of Rab27a give rise to very weak signals. As a result, the sensitivity of these assays is low and they may not be suitable for reliable screening of clinical gene therapy vectors. Accordingly, a significant need exists for more reliable and sensitive assays which can be used to determine REP1 activity and test gene therapy vectors.

SUMMARY

The present inventors have surprisingly found that, despite a prevailing understanding that Rab27a provides the most suitable prenylation assay substrate, use of Rab6a as a substrate in a prenylation reaction provides a more sensitive method for determining the activity of Rab escort protein 1 (REP1). Not only does Rab6a provide for increased sensitivity in an assay detecting prenylation, it also provides for beneficial signal-to-noise ratios, better dynamic range of signal and better consistency.

Moreover, the inventors have demonstrated that the increased sensitivity of a Rab6a-based assay may be harnessed to accurately and reliably determine the activity of REP1-encoding vectors, in particular AAV gene therapy vectors, such as those suitable for use in the clinic.

Accordingly, in one aspect, the present disclosure refers to an *in vitro* method for determining the activity of Rab escort protein 1 (REP1)-encoding gene therapy vector for use in the treatment of choroideremia, the method comprising the steps:

- (a) contacting a sample comprising REP1 with Rab6a, Rab geranylgeranyltransferase (Rab GGTase) and a lipid donor substrate, wherein the sample comprising REP1 is from a cell genetically engineered to express REP1 using the REP1-encoding gene therapy vector; and
- (b) detecting the lipidated Rab6a product.

For example, the method of the disclosure may be for testing gene therapy vectors suitable for the delivery of REP1 to a target cell or for quality control analysis of vector stocks (e.g. medicament stocks).

Validation of gene therapy vectors is mandatory for the safe and efficacious implementation of gene therapy in the clinic. For analysis and quality control steps, comparison with control experiments or reference levels may provide a measure of the activity of the gene therapy vector or REP1 relative to a known or accepted standard (e.g. better or worse than a known

or accepted standard). This may be used to validate whether a gene therapy vector stock meets specific targets and regulations.

In one example, comparison is made to a sample of REP1 or REP1-encoding AAV vector that is defined as a primary reference standard. The method of the disclosure may be, for example, carried out in parallel on a test sample and the primary reference standard sample. Potency and/or behaviour of the test sample may be, for example, defined relative to the primary reference standard.

Put another way, the method of the disclosure may, for example, be used for quality control analysis and validation of a gene therapy vector as efficacious (e.g. for the treatment of choroideremia), preferably an AAV vector particle comprising a REP1-encoding nucleotide sequence, preferably wherein an output activity or efficacy of the vector determined by the method of the disclosure above a threshold activity or within a specified target range (e.g. by comparison to a control experiment or reference level) indicates the vector is suitable for gene therapy purposes.

Accordingly, the method of the disclosure is for quality control analysis of a Rab escort protein 1 (REP1)-encoding gene therapy vector (preferably an AAV vector).

Disclosed is a method for quality control analysis of a Rab escort protein 1 (REP1)-encoding gene therapy vector (preferably an AAV vector) comprising the steps:

- (a) transducing a cell with the vector, culturing the cell under conditions suitable for the expression of the REP1 and lysing the cells to provide a sample comprising REP1;
- (b) contacting the sample of step (a) with Rab6a, Rab geranylgeranyltransferase (Rab GGTase) and a lipid donor substrate; and
- (c) detecting the lipidated Rab6a product.

Accordingly, the method may comprise carrying out a plurality of experiments comprising steps (a) to (c) in which parameters relating to the sample comprising REP1 are varied, while other parameters (e.g. parameters relating to the Rab6a, Rab GGTase and lipid donor substrate) are kept constant. Such parameters may include, for example, the amino acid sequence of the relevant protein (e.g. REP1), the REP1-encoding nucleotide sequence comprised in a vector used to express the REP1 in a cell, the type of vector used to deliver a REP1-encoding nucleotide sequence to a cell (e.g. the type of viral vector, such as the type

of adeno-associated viral (AAV) vector), the concentration of REP1 and/or the multiplicity-of-infection (MOI) of a vector used to deliver a REP1-encoding nucleotide sequence to a cell. In a preferred example, the method comprises carrying out a plurality of experiments comprising steps (a) to (c) at different MOIs of a vector used to deliver a REP1-encoding nucleotide sequence to a cell (e.g. to generate a dose-response curve).

In one example, the detection of the lipidated Rab6a product comprises quantifying the amount of the lipidated Rab6a product. In a preferred example, the detection of the lipidated Rab6a product comprises quantifying the amount of the lipidated Rab6a product relative to a control or reference level. The quantification may be, for example, made relative to a sample of REP1 or REP1-encoding AAV vector that is defined as a primary reference standard. The method of the disclosure may be, for example, carried out in parallel on a test sample and the primary reference standard sample. Potency and/or behaviour of the test sample may be, for example, defined relative to the primary reference standard.

In one example, the method comprises a further step of comparing the amount of lipidated Rab6a product (e.g. prenylated, such as geranylgeranylated or biotin-geranylated, Rab6a) with an amount determined from a control experiment, such as an experiment using a known or standard sample of REP1.

In another example, the method comprises a further step of comparing the amount of lipidated Rab6a product (e.g. prenylated, such as geranylgeranylated or biotin-geranylated, Rab6a) with a reference level.

In one example, the sample comprising REP1 is from a cell genetically engineered to express the REP1. Preferably, the sample comprising REP1 is a lysate of a cell genetically engineered to express the REP1. Preferably, a cell is transfected or transduced with a vector comprising a REP1-encoding nucleotide sequence to provide the cell genetically engineered to express the REP1. Preferably, the vector is a viral vector.

In one example, the REP1 is expressed using a viral vector comprising a REP1-encoding nucleotide sequence.

In one example, the viral vector is an adeno-associated viral (AAV) vector. Preferably the viral vector is in the form of a viral vector particle.

The AAV vector may be of any serotype (e.g. comprise any AAV serotype genome and/or capsid protein). Preferably, the vector is capable of infecting or transducing cells of the eye.

In one example, the AAV vector comprises an AAV serotype 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 genome. In another example, the AAV vector comprises an AAV serotype 2, 4, 5 or 8 genome. Preferably, the AAV vector comprises an AAV serotype 2 genome.

In one example, the AAV vector particle comprises an AAV serotype 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 capsid protein. In another example, the AAV vector particle comprises an AAV serotype 2, 4, 5 or 8 capsid protein. The AAV serotype 8 capsid protein may, for example, be a AAV8/Y733F mutant capsid protein. Preferably, the AAV vector particle comprises an AAV serotype 2 capsid protein.

In one example, the AAV vector particle comprises an AAV2 genome and AAV2 capsid proteins (AAV2/2); an AAV2 genome and AAV5 capsid proteins (AAV2/5); or an AAV2 genome and AAV8 capsid proteins (AAV2/8). Preferably, the AAV vector particle comprises an AAV2 genome and AAV2 capsid proteins (AAV2/2).

The AAV vector particle may be a chimeric, shuffled or capsid-modified derivative of one or more naturally occurring AAVs. In particular, the AAV vector particle may comprise capsid protein sequences from different serotypes, clades, clones or isolates of AAV within the same vector (i.e. a pseudotyped vector). Thus, in one example the AAV vector is in the form of a pseudotyped AAV vector particle.

In one example, the REP1 is human REP1.

In one example, the REP1 comprises an amino acid sequence that has at least 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 5, preferably wherein the amino acid sequence substantially retains the natural function of the protein represented by SEQ ID NO: 5.

In one example, the REP1-encoding nucleotide sequence comprises a nucleotide sequence that has at least 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 6 or 7, preferably wherein the protein encoded by the nucleotide sequence substantially retains the natural function of the protein represented by SEQ ID NO: 5.

In one example, the REP1-encoding nucleotide sequence comprises a nucleotide sequence that encodes an amino acid sequence that has at least 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 5, preferably wherein the amino acid sequence substantially retains the natural function of the protein represented by SEQ ID NO: 5.

In one example, the Rab6a and/or Rab GGTase are substantially pure.

In one example, the Rab6a comprises an amino acid sequence that has at least 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 1, preferably wherein the amino acid sequence substantially retains the natural function of the protein represented by SEQ ID NO: 1.

In one example, the Rab GGTase comprises an amino acid sequence that has at least 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 3 or 8, preferably SEQ ID NO: 8, preferably wherein the amino acid sequence substantially retains the natural function of the protein represented by SEQ ID NO: 8; and/or an amino acid sequence that has at least 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 4 or 9, preferably SEQ ID NO: 9, preferably wherein the amino acid sequence substantially retains the natural function of the protein represented by SEQ ID NO: 9.

In another example, the Rab6a:Rab GGTase molar ratio is about 1:0.25-3, 1:0.3-2.9, 1:0.35-2.8, 1:0.4-2.7, 1:0.45-2.6 or 1:0.5-2.5, preferably about 1:0.5-2.5.

In one example, the Rab6a:Rab GGTase molar ratio is about 1:2-3, 1:2.1-2.9, 1:2.2-2.8, 1:2.3-2.7 or 1:2.4-2.6, preferably about 1:2.4-2.6. In one example, the Rab6a:Rab GGTase molar ratio is about 1:2, 1:2.1, 1:2.2, 1:2.3, 1:2.4, 1:2.5, 1:2.6, 1:2.7, 1:2.8, 1:2.9 or 1:3, preferably about 1:2.5.

In another example, the Rab6a:Rab GGTase molar ratio is about 1:0.25-0.75, 1:0.3-0.7, 1:0.35-0.65, 1:0.4-0.6 or 1:0.45-0.55, preferably about 1:0.4-0.6. In one example, the Rab6a:Rab GGTase molar ratio is about 1:0.25, 1:0.3, 1:0.35, 1:0.4, 1:0.45, 1:0.5, 1:0.55, 1:0.6, 1:0.65, 1:0.7 or 1:0.75, preferably about 1:0.5.

In one example, the lipid donor substrate is geranylgeranylpyrophosphate (GGPP) or an analogue thereof. Preferably, the lipid donor substrate is labelled with a detectable marker. For example, the lipid donor substrate may be isotopically labelled (e.g. the lipid donor substrate may comprise ^3H), or may comprise a fluorescent group, epitope or biotin moiety.

In a preferred example, the lipid donor substrate is biotin-geranylpyrophosphate (BGPP).

In one example, the lipidated Rab6a product is detected using an enzyme-linked immunosorbent assay (ELISA), Western blot analysis or autoradiography. In a preferred example, the lipidated Rab6a product is detected using an ELISA. The ELISA may be, for example, a sandwich ELISA.

In a preferred example, a biotin-labelled lipidated Rab6a product is detected using a detection reagent specific for biotin, for example streptavidin. Preferably, the biotin-labelled lipidated Rab6a product is detected using Western blot analysis using a detection reagent specific for biotin, for example streptavidin (e.g. a streptavidin-horseradish peroxidase conjugate). More preferably, the biotin-labelled lipidated Rab6a product is detected using an ELISA using a detection reagent specific for biotin, for example streptavidin.

In one example, the method is for determining the activity of a REP1-encoding gene therapy vector for use in the treatment of choroideremia.

In another aspect, the present disclosure refers to use of Rab6a for determining *in vitro* the activity of Rab escort protein 1 (REP1)-encoding gene therapy vector for use in the treatment of choroideremia, wherein the Rab6a is contacted with Rab geranylgeranyltransferase (Rab GGTase), a lipid donor substrate, and REP1 from a cell genetically engineered to express the REP1 using the REP1-encoding gene therapy vector.

The method of determining the activity of REP1, the Rab6a, Rab GGTase, lipid donor substrate and the REP1 may be as described herein.

Disclosed is a method for determining the efficacy of a vector comprising a Rab escort protein 1 (REP1) encoding nucleotide sequence, wherein the method comprises the steps:

- (a) providing a sample comprising REP1, wherein the REP1 is expressed using the vector comprising a REP1-encoding nucleotide sequence;
- (b) contacting the sample of step (a) with Rab6a, Rab geranylgeranyltransferase (Rab GGTase) and a lipid donor substrate; and
- (c) detecting the lipidated Rab6a product.

Disclosed is the use of Rab6a for determining the efficacy of a vector comprising a Rab escort protein 1 (REP1) encoding nucleotide sequence.

Preferably, the method and use are for determining the efficacy of a vector for use in the treatment of choroideremia.

The vector, REP1, Rab6a, Rab GGTase, lipid donor substrate, lipidated Rab6a product, method of detection and other features of the method and use may be as described herein.

DESCRIPTION OF THE DRAWINGS

Figure 1

In vitro prenylation reaction using Rab27a as a substrate followed by Western blot (WB) analysis of incorporated biotinylated lipid donor using untransduced cells and cells transduced with either AAV-GFP or AAV-REP1 (MOI = 10000 gp/cell).

The experiment involved 3 sets of lysates prepared independently. Prenylation reactions were set up using 10 µg of lysate in a total volume of 12.5 µL. Positive controls were spiked with 2 µM of fish REP1. Detection time was 2 min.

(A) WB analysis of human REP1 levels in cell lysates (1:1000). Untransduced cells (#4, #9 and #12) show endogenous levels of REP1. Cells transduced with AAV-GFP (#5, #10 and #13) show endogenous levels of REP1 similar to untransduced cells. Cells transduced with AAV-REP1 (#6, #11 and #14) show an increase of REP1 levels compared to untransduced and AAV-GFP transduced cells. Positive controls (+ve) show endogenous REP1 levels.

(B) WB analysis of β-actin as loading control (1:15000). The levels of β-actin are similar in all samples analysed.

(C) WB analysis of incorporated biotinylated lipid donor in Rab27a (1:10000). Untransduced cells and AAV-GFP transduced cells show no detectable incorporation of biotin in Rab27a. Cells transduced with AAV-REP1 show some level of biotin incorporated into Rab27a (#6, #11 and #14). Positive controls show the strongest band of all as a result of fish REP1 activity.

(D) Semiquantification of the WB analysis in (C) using Image Studio Lite software.

Figure 2

In vitro prenylation reaction using Rab27a as a substrate followed by Western blot (WB) analysis of incorporated biotinylated lipid donor using untransduced cells and cells transduced with either AAV-GFP or AAV-REP1 (MOI = 10000 gp/cell).

The experiment involved 2 sets of lysates prepared independently. Prenylation reactions were set up using 30 µg of lysate in a total volume of 22 µL. Positive controls were spiked with 1 µM of fish REP1. Detection time was 2 min.

(A) WB analysis of human REP1 levels in cell lysates (1:1000). Untransduced cells (#9 and #12) show endogenous levels of REP1. Cells transduced with AAV-GFP (#10 and #13) show endogenous levels of REP1 similar to untransduced cells. Cells transduced with AAV-REP1 (#11 and #14) show an increase of REP1 levels compared to untransduced and AAV-GFP transduced cells. Positive controls (+ve) show endogenous REP1 levels.

(B) WB analysis of β -actin as loading control (1:15000). The levels of β -actin are similar in all samples analysed.

(C) WB analysis of incorporated biotinylated lipid donor in Rab27a (1:10000). Untransduced cells and AAV-GFP transduced cells show no detectable incorporation of biotin in Rab27a.

5 Cells transduced with AAV-REP1 show some level of biotin incorporated into Rab27a (#11 and #14). Positive controls show the strongest band of all as a result of fish REP1 activity.

(D) Semiquantification of the WB analysis in (C) using Image Studio Lite software.

Figure 3

In vitro prenylation reaction using Rab6a as a substrate followed by Western blot (WB) analysis of incorporated biotinylated lipid donor using untransduced cells and cells transduced with either AAV-GFP or AAV-REP1 (MOI = 10000 gp/cell).

The experiment involved 2 sets of lysates prepared independently. Prenylation reactions were set up using 20 μ g of lysate in a total volume of 20 μ L. Positive controls were spiked with 1 μ M of fish REP1. Detection time was 2 min.

15 (A) WB analysis of human REP1 levels in cell lysates (1:1000). Untransduced cells (#9 and #12) show endogenous levels of REP1. Cells transduced with AAV-GFP (#10 and #13) show endogenous levels of REP1 similar to untransduced cells. Cells transduced with AAV-REP1 (#11 and #14) show an increase of REP1 levels compared to untransduced and AAV-GFP transduced cells. Positive controls (+ve) show endogenous REP1 levels.

20 (B) WB analysis of β -actin as loading control (1:15000). The levels of β -actin are similar in all samples analysed.

(C) WB analysis of incorporated biotinylated lipid donor in Rab6a (1:10000). Untransduced cells and AAV-GFP transduced cells show very low incorporation of biotin in Rab6a. Cells transduced with AAV-REP1 show biotin incorporated into Rab6a (#11 and #14). Positive controls show the strongest band of all as a result of fish REP1 activity.

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(D) Semiquantification of the WB analysis in (C) using Image Studio Lite software.

Figure 4

In vitro prenylation reaction using Rab6a as a substrate followed by Western blot (WB) analysis of incorporated biotinylated lipid donor using untransduced cells and cells transduced with AAV-REP1 (MOI = 250, 1000, 5000, 10000 and 20000 gp/cell).

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Prenylation reactions were set up using 20 µg of lysate in a total volume of 15 µL. The positive control was spiked with 0.5 µM of fish REP1. Detection time was 2 min.

(A) WB analysis of human REP1 levels in cell lysates (1:2500). Untransduced cells show endogenous levels of REP1. Cells transduced with AAV-REP1 show an increase of REP1 levels that directly correlates with the MOI used. The positive control (+ve) shows endogenous REP1 levels.

(B) WB analysis of β-actin as loading control (1:50000). The levels of β-actin are similar in all samples analysed.

(C) WB analysis of incorporated biotinylated lipid donor in Rab6a (1:10000). Untransduced cells show very low of biotin incorporation in Rab6a. Cells transduced with AAV-REP1 show increasingly more biotin incorporated into Rab6a. The positive control shows the strongest band of all as a result of fish REP1 activity.

(D) Semiquantification of the WB analysis in (C) using Image Studio Lite software.

Figure 5

In vitro prenylation reaction using Rab6a as a substrate followed by Western blot (WB) analysis of incorporated biotinylated lipid donor using untransduced cells and cells transduced with AAV-REP1 (MOI = 10000 gp/cell).

Prenylation reactions were set up using 20 µg of lysate in a total volume of 15 µL. The positive control was spiked with 0.5 µM of fish REP1. Detection time was 2 min for REP1/actin and 30 seconds for biotin.

(A) WB analysis of human REP1 levels in cell lysates (1:2500). Untransduced cells show endogenous level of REP1. Cells transduced with AAV-REP1 show an increase of REP1 levels. The positive control (+ve) shows endogenous REP1 levels.

(B) WB analysis of β-actin as loading control (1:50000). The levels of β-actin are similar in all samples analysed.

(C) WB analysis of incorporated biotinylated lipid donor in Rab6a (1:10000). Untransduced cells show very low biotin incorporation in Rab6a. Cells transduced with AAV-REP1 show increased biotin incorporation into Rab6a. The positive control shows the strongest band of all as a result of fish REP1 activity.

(D) Semiquantification of WB analysis in (C) using Image Studio Lite software.

Figure 6

In vitro prenylation reaction, followed by Western blot (WB) analysis of incorporated biotinylated lipid donor in untransduced ARPE-19 cells, and ARPE-19 cells transduced with AAV-REP1.

- 5 Prenylation reactions were set up using 15 µg of lysate in a total volume of 45 µL. Positive control was spiked with 0.1 µM of fish REP1. Detection time for REP1/actin: 2 min; for biotin: 30 seconds.

Experiments were carried out in parallel using the following cells:

- (a) Untransduced cells (#86 and #87); and
 10 (b) Cells + AAV-REP1 MOI 10,000 (#90 and #91) - R&D grade vector.

(A) WB analysis of human REP1 levels in cell lysates (1:2,500). Untransduced cells show endogenous level of REP1. Cells transduced with AAV-REP1 show an increase of REP1 levels. Positive control (+ve) shows endogenous REP1 levels.

- (B) WB analysis of β-actin as loading control (1:50,000). The levels of β-actin are similar in
 15 all samples analysed.

(C) WB analysis of incorporated biotinylated lipid donor in Rab6a (1:10,000). Untransduced cells show very low incorporation of biotin in Rab6a; cells transduced with AAV-REP1 show increased biotin incorporation into Rab6a. Positive control shows the strongest band of all, as a result of fish REP1 activity.

- 20 (D) Semiquantification of WB analysis in (C) using Image Studio Lite software.

Figure 7

In vitro prenylation reaction, followed by Western blot (WB) analysis of incorporated biotinylated lipid donor in untransduced HT1080 cells, and HT1080 cells transduced with AAV-REP1.

- 25 Prenylation reactions were set up using 20 µg of lysate in a total volume of 20 µL. Positive control was spiked with 0.1 µM of fish REP1. Detection time for REP1/actin: 2 min; for biotin: 30 seconds.

Experiments were carried out in parallel using the following cells:

- (a) Untransduced cells (#56 and #57);

(b) Cells + AAV-REP1 MOI 10,000 (#60 and #61) – R&D grade vector; and

(c) Cells + AAV-REP1 MOI 10,000 (#64 and #65) – clinical grade vector.

(A) WB analysis of human REP1 levels in cell lysates (1:2,500). Untransduced cells show endogenous levels of REP1. Cells transduced with AAV-REP1 show an increase of REP1 levels. Positive control (+ve) shows endogenous REP1 levels.

(B) WB analysis of β -actin as loading control (1:50,000). The levels of β -actin are similar in all samples analysed.

(C) WB analysis of incorporated biotinylated lipid donor in Rab6a (1:10,000). Untransduced cells show baseline levels of biotin incorporation in Rab6a; cells transduced with AAV-REP1 show increased biotin incorporation into Rab6a.

(D) Semiquantification of WB analysis in (C) using Image Studio Lite software.

Figure 8

In vitro prenylation reaction, followed by Western blot (WB) analysis of incorporated biotinylated lipid donor in untransduced cells, and cells transduced with AAV-REP1 (MOI = 250, 1000, 5000, 10000 and 20000 gp/cell) comparing Rab6a and Rab27a substrates.

Prenylation reactions were set up using 20 μ g of lysate in a total volume of 15 μ L, and 2 different substrates: Rab27a (left-hand lanes and plots; in red) and Rab6a (right-hand lanes and plots; in blue). Positive controls, one for each substrate, were spiked with 0.1 μ M of fish REP1. Detection time: 2 min.

(A) WB analysis of human REP1 levels in cell lysates (1:2,500). Untransduced cells show endogenous level of REP1. Cells transduced with AAV-REP1 show an increase of REP1 levels that directly correlates with the MOI used. Positive control (+ve) shows endogenous REP1 levels.

(B) WB analysis of β -actin as loading control (1:50,000). The levels of β -actin are similar in all samples analysed.

(C) WB analysis of incorporated biotinylated lipid donor in Rab27a and Rab6a. Untransduced cells show very low incorporation of biotin into the Rab protein, which increases as the MOI increases. Positive controls show strong biotin incorporation, as a result of fish REP1 activity.

(D) Semiquantification of WB analysis in (C) using Image Studio Lite software. Data was plotted using Prism software as shown in the four plots in (E).

(E) Band density values from biotinylated substrates across MOIs used (upper two plots) and ratio between biotinylated substrates and REP1 across MOIs used (lower two plots).

5 Figure 9

In vitro prenylation reaction, followed by Western blot (WB) analysis of incorporated biotinylated lipid donor in untransduced cells comparing different prenylation reaction conditions.

10 Prenylation reactions were set up using 20 µg of lysate in a total volume of 15 µL, and 2 different substrates: Rab27a (in red) and Rab6a (in blue). Positive controls, one for each substrate, were spiked with recombinant human REP1. Detection time: 2 min.

(A) WB analysis of incorporated biotinylated lipid donor in Rab27a and Rab6a. Level of biotin incorporation is directly proportional to the amount of total protein in the reaction. Positive controls show strong biotin incorporation, as a result of fish REP1 activity.

15 (B) WB analysis of β-actin as loading control (1:50,000). The levels of β-actin match the amount of total cell lysates used in the reaction, and are similar between samples.

(C) WB analysis of human REP1 levels in cell lysates (1:2,500). Untransduced cells show endogenous level of REP1. Positive control (+ve) shows higher density of REP1.

20 (D) Semiquantification of WB analysis in (A) using Image Studio Lite software. Data was plotted using Prism software. Values highlighted are for those conditions where a higher difference between substrates was detected.

Figure 10

Comparison between Rab27a and Rab6a as substrates for prenylation in AAV-REP1 transduced cells.

25 DETAILED DESCRIPTION OF THE INVENTION

Various preferred features and embodiments of the present invention will now be described by way of non-limiting examples.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, biochemistry, molecular biology, microbiology and immunology,

which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press; Ausubel, F.M. et al. (1995 and periodic supplements) *Current Protocols in Molecular Biology*, Ch. 9, 13 and 16, John Wiley & Sons; Roe, B., Crabtree, J. and Kahn, A. (1996) *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; Polak, J.M. and McGee, J.O'D. (1990) *In Situ Hybridization: Principles and Practice*, Oxford University Press; Gait, M.J. (1984) *Oligonucleotide Synthesis: A Practical Approach*, IRL Press; and Lilley, D.M. and Dahlberg, J.E. (1992) *Methods in Enzymology: DNA Structures Part A: Synthesis and Physical Analysis of DNA*, Academic Press. Each of these general texts is herein incorporated by reference.

Prenylation

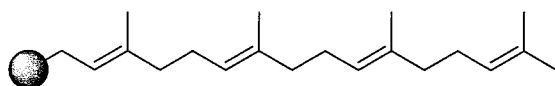
Lipidation of proteins by the addition of isoprenoid moieties is a post-translational modification that affects up to 2% of the mammalian proteome. Such lipidation enables reversible association of the target proteins with cell membranes and can also modulate protein-protein interactions.

Preferably, the lipidation referred to herein is prenylation, such that the lipid donor substrate and lipidated Rab6a product are a prenyl donor substrate and prenylated Rab6a product, respectively.

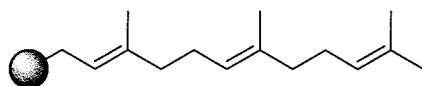
Prenylation is a specific type of post-translational modification in which a geranylgeranyl or farnesyl moiety (or analogue of either) is attached to one or two C-terminal cysteine residues of a protein via a thioether linkage.

Preferably, the prenylation is the addition of a geranylgeranyl moiety or an analogue thereof (e.g. biotin-geranyl moiety) to a target protein (e.g. Rab6a).

A geranylgeranyl moiety attached to a protein (the protein is depicted schematically by the shaded circle) is:

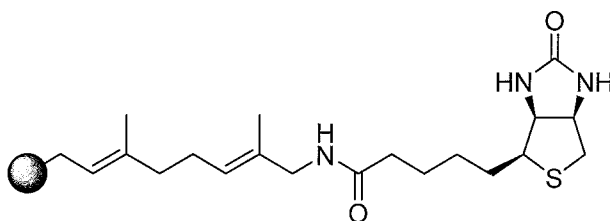


A farnesyl moiety attached to a protein (the protein is depicted schematically by the shaded circle) is:



The term “analogue” is used herein in relation to the lipid (e.g. geranylgeranyl or farnesyl) moiety or lipid donor substrate to refer to a compound which has been modified to comprise a functional group suitable for a particular purpose, such as detection. The analogue is able to be added to a substrate protein by the prenylation machinery (i.e. REP1 and Rab GGTase) in a manner substantially unhindered (for the purposes of the activity assays of the invention) by the modification.

Accordingly, analogues of the above moieties include those which have been artificially created for particular purposes (e.g. labelled moieties which are suitable for detection in an assay). In particular, Nguyen et al. (Nguyen, U.T. et al. (2009) Nat. Chem. Biol. 5: 227-235) developed the following biotin-geranyl moiety that can be detected in in vitro protein prenylation reactions (the biotin-geranyl moiety is shown attached to a protein, which is depicted schematically by the shaded circle):



Rab6a

Rab6a (Ras-related protein Rab-6A) is a member of the mammalian Rab GTPase family, which is itself the largest of the Ras-like super-family of GTPases.

Rab GTPases (also known as Rab proteins) are peripheral membrane proteins and are involved in the regulation of membrane trafficking, including vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion. The main function of Rab6a is understood to be the regulation of protein transport from the Golgi complex to the endoplasmic reticulum.

Rab GTPases are typically anchored to a cell membrane via prenyl groups (in particular, geranylgeranyl groups) which are covalently bound to two C-terminal cysteine residues.

Rab GTPases exhibit two conformations: an inactive, GDP-bound form; and an active, GTP-bound form. Conversion from the GDP- to the GTP-bound forms is catalysed by a GDP/GTP exchange factor (GEF), which thereby activates the Rab GTPase. Conversely, GTP

hydrolysis by Rab GTPases can be enhanced by a GTPase-activating protein (GAP), which thereby leads to Rab inactivation.

In one embodiment, the Rab6a is human Rab6a.

An example amino acid sequence of Rab6a is the sequence deposited under NCBI
5 Accession No. NP_942599.1.

An example amino acid sequence of Rab6a is:

MSTGGDFGNPLRKFKLVFLGEQSVGKTSLITRFMYDSFDNTYQATIGIDFLSKTMYLEDRTVRLQLWDTAGQ
ERFRSLIPSYIRDSTVAVVVYDITNVNSFQOTTKWIDDVTERGSDVIIMLVGNKTDLADKRQVSIIEGERK
AKELNVMFIETSAKAGYNVQQLFRRVAAALPGMESTQDRSREDMIDIKLEKPQEQPVSEGGCSC

(SEQ ID NO: 1)

An example nucleotide sequence encoding Rab6a is the sequence deposited under NCBI
Accession No. NM_198896.1.

An example nucleotide sequence encoding Rab6a is:

ATGTCCACGGGCGGAGACTTCGGGAATCCGCTGAGGAAATTCAGCTGGTGTTCTTGGGGGAGCAAAGCGTT
GGAAAGACATCTTTGATCACCAGATTTCATGTATGACAGTTTTGACAACACCTATCAGGCAACAATTGGCATT
GACTTTTTTATCAAAAACATGTACTTGGAGGATCGAACAGTACGATTGCAATTATGGGACACAGCAGGTCAA
GAGCGGTTTCAGGAGCTTGATTCTAGCTACATTCGTGACTCCACTGTGGCAGTTGTTGTTTATGATATCACA
AATGTTAACTCATTCCAGCAAACACAAAGTGGATTGATGATGTCAGAACAGAAAGAGGAAGTGATGTTATC
ATCATGCTAGTAGGAAATAAAACAGATCTTGCTGACAAGAGGCAAGTGTCAATTGAGGAGGGAGAGAGGAAA
GCCAAAGAGCTGAATGTTATGTTTATTGAAACTAGTGCAAAAGCTGGATACAATGTAAAGCAGCTCTTTCGA
CGTGTAGCAGCAGCTTTGCCGGGAATGGAAAGCACACAGGACAGAAGCAGAGAAGATATGATTGACATAAAA
CTGGAAAAGCCTCAGGAGCAACCAGTCAGTGAAGGAGGCTGTTCTTGCTAA

(SEQ ID NO: 2)

Rab geranylgeranyltransferase (Rab GGTase)

Rab geranylgeranyltransferase (Rab GGTase; also known as geranylgeranyltransferase II) is a protein prenyltransferase which exclusively prenylates the GTPases of the Rab family.

Rab GGTase typically naturally catalyses the transfer of two geranylgeranyl groups to
30 cysteine residues at the C-terminus of Rab GTPases. Each geranylgeranyl group is conjugated to the Rab GTPase via a thioether linkage to a cysteine residue.

Rab GGTase has been shown to be capable of binding a range of derivatised phosphoisoprenoids and can catalyse their addition to Rab GTPase substrates (e.g. Rab6a). For example, Nguyen et al. (Nguyen, U.T. et al. (2009) Nat. Chem. Biol. 5: 227-235)
35 demonstrated the successful addition of a biotin-geranyl moiety to Rab GTPases.

Rab GGTase is a heterodimeric enzyme comprised of alpha and beta subunits.

In one embodiment, the Rab GGTase is human Rab GGTase. In a preferred embodiment, the Rab GGTase is rat Rab GGTase.

Example amino acid sequences of Rab GGTase alpha subunits are the sequences deposited under NCBI Accession Nos. NP_004572.3 and NP_113842.1.

Example amino acid sequences of Rab GGTase alpha subunits are:

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10 MHGRLKVKVTSEEQAEAKRLEREQKLKLYQSATQAVFQKRQAGELDESVLELTSQILGANPDFATLWNCRREV
    LQOLETQKSPEELAALVKAELGFLESLRVNPKSYGTWHHRCWLLGRLPEPNWTRELELCARFLEVDERNFH
    CWDYRRFVATQAAVPPAEELAFDTSLITRNFNSYSSWHYRSCLLPQLHPQPDGSGPQGRLPEDVLLKELELVQ
15 NAFFTDPNQSAWIFYHRWLLGRADPDQALRCLHVSDEACLTVSFSRPLLVGSRMEILLMVDDSPILIVEWR
    TPDGRNRP SHVWLCDLPAASLNDQLPQHTFRVIWTAGDVQKECVLLKGRQEGWCRDSTTDEQLFRCELSVEK
    STVLQSELESCKELEPEPENKWCLLTIIILLMRALDPLLYEKETLQYFQTLKAVDPMRATYLDLRSKFLLE
    NSVLKMEYAEVRVLHLAHKDLTVLCHLEQLLLVTHLDLSHNRLRLTLPALAAALRCLEVLQASDNAIESLDGV
    TNLPRLQELLLCNNRLQQPAVLQPLASCPRLVLLNLQGNPLCQAVGILEQLAELLPSVSSVLT
    (SEQ ID NO: 3)

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and:

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20 MHGRLKVKVTSEEQAEAKRLEREQKLKLYQSATQAVFQKRQAGELDESVLELTSQILGANPDFATLWNCRREV
    LQHLETEKSPPEESAALVKAELGFLESLRVNPKSYGTWHHRCWLLSRLPEPNWARELELCARFLEADERNFH
    CWDYRRFVAAQAAPPAEELAFDTSLITRNFNSYSSWHYRSCLLPQLHPQPDGSGPQGRLPENVLLKELELVQ
25 NAFFTDPNQSAWIFYHRWLLGRAEPHDVLCVHVSREEACLSVCFSRPLTVGSRMGTLMLMDEAPLSVEWR
    TPDGRNRP SHVWLCDLPAASLNDQLPQHTFRVIWTGSDSQKECVLLKDRPECWCWCRDSATDEQLFRCELSVEK
    STVLQSELESCKELEPEPENKWCLLTIIILLMRALDPLLYEKETLQYFSTLKAVIDPMRAAYLDLRSKFLLE
    NSVLKMEYADVRVLHLAHKDLTVLCHLEQLLLVTHLDLSHNRLRALPPALAAALRCLEVLQASDNALENVDGV
    ANLPRLQELLLCNNRLQQAIIQPLVSCPRLVLLNLQGNSLCQEEGIQERLAEMLPSPVSSILT
    (SEQ ID NO: 8)

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Example amino acid sequences of Rab GGTase beta subunits are the sequences deposited under NCBI Accession Nos. NP_004573.2. and NP_619715.1.

Example amino acid sequences of Rab GGTase beta subunits are:

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35 MGTPOKDVIKSDAPDTLLLEKHADYIASYGSKKDDYEYCMSEYLRMSGIYWGLTVMDLMGQLHRMNREEIL
    AFIKSCQHECGGISASIGHDPHLLYTLSAVQILTLYDSINVIDVNKVVVEYVKGQKEDGSFAGDIWGEIDTR
    FSFCAVATLALLGKLDAINVEKAIEFVLSMNFDDGGFGCRPGSESHAGQIYCCTGFLAITSQLHQVNSDLLG
    WWLCERQLPSGGLNGRPEKLPDVCYSWWVLASLKIIGRLHWIDREKLRNFILACQDEETGGFADRPDGMVDP
    FHTLFGIAGLSLLGEEQIKPVNPVFCMPPEEVLQRVNVQPELVS
    (SEQ ID NO: 4)

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and:

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40 MGTQQKDVTIKSDAPDTLLLEKHADYIASYGSKKDDYEYCMSEYLRMSGVYWGLTVMDLMGQLHRMNKEEIL
    VFIKSCQHECGGVSASIGHDPHLLYTLSAVQILTLYDSIHVINVDKVVAYVQSLQKEDGSFAGDIWGEIDTR
    FSFCAVATLALLGKLDAINVEKAIEFVLSMNFDDGGFGCRPGSESHAGQIYCCTGFLAITSQLHQVNSDLLG

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WWLCERQLPSGGLNGRPEKLPDVCYSWWVLASLKIIGRLHWIDREKLRSFILACQDEETGGFADRPGDMVDP
FHTLFGIAGLSLLGEEQIKPVSPVFCMPPEEVLQRVNVQPELVS

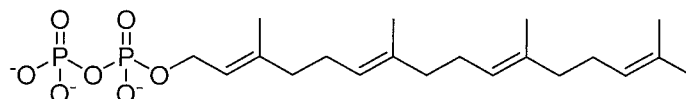
(SEQ ID NO: 9)

5 Lipid donor substrate

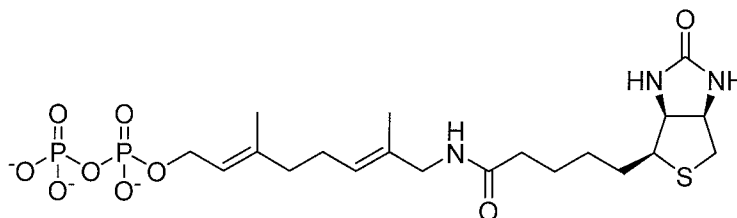
To add a lipid moiety to a Rab GTPase, the Rab GGTase may use the lipid moiety in the form of a lipid (e.g. geranylgeranyl or biotin-geranyl) donor substrate as a substrate. These are typically pyrophosphate derivatives of the lipid moiety.

For example, geranylgeranylpyrophosphate (GGPP) or biotin-geranylpyrophosphate (BGPP) may be used as lipid donor substrates by Rab GGTase to transfer a geranylgeranyl or biotin-geranyl moiety, respectively, to the substrate Rab GTPase.

Geranylgeranylpyrophosphate has the structure:



An example structure of biotin-geranylpyrophosphate is:



Rab escort protein 1 (REP1)

Rab escort proteins (REPs) perform the functions of presenting unprenylated Rab GTPases to Rab GGTases, and carrying prenylated Rab GTPases to their target membranes.

Rab GTPases do not comprise a consensus sequence at the prenylation site that may be recognised by Rab GGTases. However, substrate recognition is effected through REPs, which bind Rab GTPases through a conserved region and then present the Rab GGTase with its substrate for prenylation.

Once prenylated, the lipid anchors render the Rab GTPases insoluble. Accordingly, REPs are required to bind and solubilise the geranylgeranyl groups and aid delivery of the Rab GTPase to the target cell membrane.

REP1 may also be known as Rab protein geranylgeranyltransferase component A. Furthermore, the gene that encodes REP1 may be known as the CHM gene.

In one embodiment, the REP1 is human REP1.

An example amino acid sequence of REP1 is:

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5  MADTLPSSEFDVIVIGTGLPESIIAAACSRSGRRVLHVDSRSYYGGNWASFSSGLLSWLKEYQENS

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(SEQ ID NO: 5)


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An example nucleotide sequence encoding REP1 is:

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(SEQ ID NO: 6)


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A further example nucleotide sequence encoding REP1 is:

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50  GATATCGAATTCCTGCAGCCCGGCGGCACCATGGCGGATACTCTCCCTTCGGAGTTTGATGTGATCGTAATA
    GGGACGGGTTTGCCTGAATCCATCATTCAGCTGCATGTTCAAGAAGTGGCCGGAGAGTTCTGCATGTTGAT
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TCAAGAAGCTACTATGGAGGAACTGGGCCAGTTTTAGCTTTTCAGGACTATTGTCTCTGGCTAAAGGAATAC
 CAGGAAAACAGTGACATTGTAAGTGACAGTCCAGTGTGGCAAGACCAGATCCTTGAAAATGAAGAAGCCATT
 GCTCTTAGCAGGAAGGACAAAACATTTCAACATGTGGAAGTATTTTGTATGCCAGTCAGGATTTGCATGAA
 5 GATGTGCGAAGAAGCTGGTGCCTGCAGAAAAATCATGCTCTTGTGACATCTGCAAACTCCACAGAAGCTGCA
 GATTCTGCCTTCTGCCTACGGAGGATGAGTCATTAAGCACTATGAGCTGTGAAATGCTCACAGAACAACT
 CCAAGCAGCGATCCAGAGAATGCGCTAGAAGTAAATGGTGTGAAAGTGACAGGGGAAAAAGAAAACCATTGT
 GATGATAAACTTGTGTGCCATCAACTTCAGCAGAAGACATGAGTGAAAATGTGCCTATAGCAGAAGATACC
 10 ACAGAGCAACCAAAGAAAAACAGAATTACTTACTCACAAATTATTAAAGAAGGCAGGAGATTTAATATTGAT
 TTAGTATCAAAGCTGCTGTATTCTCGAGGATTACTAATTGATCTTCTAATCAAATCTAATGTTAGTCGATAT
 GCAGAGTTTAAAAATATTACCAGGATTCTTGCATTTTCGAGAAGGACGAGTGGAACAGGTTCCGTGTTCCAGA
 GCAGATGTCTTTAATAGCAAACAACCTTACTATGGTAGAAAAGCGAATGCTAATGAAATTTCTTACATTTTGT
 ATGGAATATGAGAAATATCCTGATGAATATAAAGGATATGAAGAGATCACATTTTATGAATATTTAAAGACT
 CAAAAATTAACCCCCAACCTCCAATATATTGTGCATGCATTCAATTGCAATGACATCAGAGACAGCCAGCAGC
 15 ACCATAGATGGTCTCAAAGCTACCAAAAACCTTTCTTCACTGTCTTGGGCGGTATGGCAACACTCCATTTTGT
 TTTCTTTTATATGGCCAAGGAGAACTCCCCCAGTGTCTTCTGCAGGATGTGTGCTGTGTTGGTGGAATTTAT
 TGTCTTCGCCATTTCAGTACAGTGCCTTGTAGTGGACAAAGAATCCAGAAAATGTAAAGCAATTATAGATCAG
 TTTGGTCAGAGAATAATCTCTGAGCATTTCCTCGTGGAGGACAGTTACTTTCTGAGAACATGTGCTCACGT
 GTGCAATACAGGCAGATCTCCAGGGCAGTCTGATTACAGATAGATCTGTCTTAAAAACAGATTTCAGATCAA
 20 CAGATTTCCATTTTGCAGTGCAGCAGAGGAACCAGGAACCTTTTGTGTTTCGGGTCAATTGAGTTATGTTCT
 TCAACGATGACATGCATGAAAGGCACCTATTTGGTTCAATTTGACTTGCACATCTTCTAAAAACAGCAAGAGAA
 GATTTAGAATCAGTTGTGCAGAAATTGTTTGTTCATATACTGAAATGGAGATAGAAAATGAACAAGTAGAA
 AAGCCAAGAATTCTGTGGGCTCTTTACTTCAATATGAGAGATTCGTCAGACATCAGCAGGAGCTGTTATAAT
 GATTTACCATCCAACGTTTATGTCTGCTCTGGCCCAGATTGTGGTTTAGGAAATGATAATGCAGTCAAACAG
 25 GCTGAAACACTTTTCCAGGAAATCTGCCCCAATGAAGATTTCTGTCCCCCTCCACCAAATCCTGAAGACATT
 ATCCTTGATGGAGACAGTTTACAGCCAGAGGCTTCAGAATCCAGTGCCATACCAGAGGCTAACTCGGAGACT
 TTCAAGGAAAGCACAAACCTTGGAACCTAGAGGAGTCCTCTGAATAA

(SEQ ID NO: 7)

Example variants of REP1 are described further in WO 2012/114090 (incorporated herein by
 30 reference).

Activity determination

In one aspect, the invention provides a method for determining the activity of Rab escort protein 1 (REP1) comprising the steps:

- (a) providing a sample comprising REP1;
- 35 (b) contacting the sample of step (a) with Rab6a, Rab geranylgeranyltransferase (Rab GGTase) and a lipid donor substrate; and
- (c) detecting the lipidated Rab6a product.

In another aspect, the invention provides the use of Rab6a for determining the activity of Rab escort protein 1 (REP1).

40 Assay sensitivity is an important factor to consider, because it enables detection of low levels of a target, which is particularly relevant when small quantities of reagents are present (e.g. as may be the case with gene therapy reagents). However, it is also important to

maximise the dynamic range of an assay's signal, which may, for example, not correlate with reagents that provide low or high sensitivity.

The method and use of the invention are for testing the activity of REP1, rather than testing other agents that are involved in the prenylation of a Rab GTPase, for example, the activity of Rab GGTTases or lipid donor substrates, or the activity of Rab GTPases as prenylation substrates. For example, the method of the invention may be for testing gene therapy vectors suitable for the delivery of REP1 to a target cell or for quality control analysis of vector stocks (e.g. medicament stocks).

In one embodiment, the sample comprising REP1 is from a cell genetically engineered to express the REP1. Preferably, a cell is transfected or transduced with a vector comprising a REP1-encoding nucleotide sequence to provide the cell genetically engineered to express the REP1. Preferably, the vector is a viral vector.

In one embodiment, the REP1 is expressed using a viral vector comprising a REP1-encoding nucleotide sequence.

The cell (which may be as a population of such cells) which is genetically engineered to express the REP1 may be any cell suitable for genetic engineering and expression of REP1, such as a cell from a cell line (e.g. HEK293). The cell may be, for example, a human or mouse cell. Preferably, the cell is a human cell. The cell may, for example, be a retinal cell, such as a retinal pigment epithelial or photoreceptor cell. In one embodiment, the cell is a HEK293 cell. In another embodiment, the cell is an ARPE-19 cell. In another embodiment, the cell is an HT1080 cell.

Preferably, the Rab6a and/or Rab GGTTase are from a standard source such that they provide for minimal or no variation in repeated experiments. Preferably, the Rab6a and/or Rab GGTTase are substantially pure (i.e. comprise substantially no protein contaminants that interfere with the method or use of the invention).

Accordingly, the method or use may comprise carrying out a plurality of experiments (e.g. comprising steps (a) to (c)) in which parameters relating to the sample comprising REP1 are varied, while other parameters (e.g. parameters relating to the Rab6a, Rab GGTTase and lipid donor substrate) are kept constant. Such parameters may include, for example, the amino acid sequence of the relevant protein (e.g. REP1), the REP1-encoding nucleotide sequence comprised in a vector used to express the REP1 in a cell, the type of vector used to deliver a REP1-encoding nucleotide sequence to a cell (e.g. the type of viral vector, such as the type of adeno-associated viral (AAV) vector), the concentration of REP1 and/or the

multiplicity-of-infection (MOI) of a vector used to deliver a REP1-encoding nucleotide sequence to a cell.

The term “activity” is used herein to refer to the ability of REP1 to facilitate the lipidation of a Rab GTPase (e.g. Rab6a). Although the REP1 does not catalyse the lipidation itself, it is required for a Rab GGTase to catalyse the lipidation of its substrate Rab GTPase. Accordingly, the activity of the REP1 may be measured by determining the amount of Rab GTPase (i.e. Rab6a) which is lipidated under certain conditions.

The term “efficacy” is used herein, in relation to efficacy of a vector comprising a REP1-encoding nucleotide sequence, to refer to the ability of the vector to provide REP1 activity to a cell which is transfected or transduced by the vector.

The term “lipidated Rab6a product” as used herein refers to Rab6a to which a lipid moiety has been added. Preferably, the lipidated Rab6a product is a prenylated Rab6a, such as a geranylgeranylated Rab6a or a biotin-geranylated Rab6a.

Preferably, the step of detecting the lipidated Rab6a product provides quantification of the amount of lipidated Rab6a product.

The detection of lipidated Rab6a may be carried out by any suitable method, for example an enzyme-linked immunosorbent assay (ELISA), a Western blot, autoradiography (e.g. utilising an isotopically-labelled, such as tritiated, lipid donor substrate), chromatographic (e.g. HPLC or FPLC) and/or mass spectrometry-based method (e.g. LC/MS).

In one embodiment, the lipidated Rab6a product is detected using a Western blot. In a preferred embodiment, the lipidated Rab6a product is detected using an ELISA.

By way of example, a prenylation reaction may be carried out according to the method of the invention using a biotin-geranylpyrophosphate lipid donor substrate. The product of the reaction may be subjected to Western blot analysis in which the lipidated Rab6a product (i.e. biotin-geranylated Rab6a) may be detected by direct incubation with, for example, streptavidin-horseradish peroxidase conjugate. Quantification of the lipidated Rab6a (i.e. biotin-geranylated Rab6a) may be achieved by densitometric analysis of the resulting Western blot, which may be carried out by any suitable means (e.g. using Image Studio Lite software (LI-COR)).

By way of further example, a prenylation reaction may be carried out according to the method of the invention using a biotin-geranylpyrophosphate lipid donor substrate. The product of the reaction may be subjected to an ELISA analysis in which the Rab6a may be

immobilised on a plate directly or using an antibody that has been attached to the plate (i.e. a sandwich ELISA); and then the lipidated Rab6a product (i.e. biotin-geranylated Rab6a) may be detected by incubation with, for example, streptavidin-horseradish peroxidase conjugate. Quantification of the lipidated Rab6a (i.e. biotin-geranylated Rab6a) may be achieved by any suitable means (e.g. detection using a spectrophotometer, fluorometer or luminometer).

Further detection steps may be incorporated into the method of the invention, as required (e.g. for control purposes), such as the detection of the amount of REP1 present in the reaction or detection of the amount of β -actin (e.g. as a loading control).

In one embodiment, the method comprises a further step of comparing the amount of lipidated Rab6a product (e.g. prenylated, such as geranylgeranylated or biotin-geranylated, Rab6a) with an amount determined from a control experiment, such as an experiment using a known or standard sample of REP1.

In another embodiment, the method comprises a further step of comparing the amount of lipidated Rab6a product (e.g. prenylated, such as geranylgeranylated or biotin-geranylated, Rab6a) with a reference level.

Comparison with such control experiments or reference levels may provide a measure of the activity of the REP1 relative to a known or accepted standard (e.g. better or worse than a known or accepted standard).

The method of the invention may, for example, be used for quality control analysis of a gene therapy vector for the treatment of choroideremia, preferably an AAV vector particle comprising a REP1-encoding nucleotide sequence, wherein an output activity or efficacy of the vector determined by the method of the invention above a threshold activity or within a specified target range (e.g. by comparison to a control experiment or reference level) indicates the vector is suitable for gene therapy purposes.

The conditions of the prenylation reaction (e.g. that occurring in step (b) of the method of the invention) are not particularly limited, providing that they do not substantially interfere with the prenylation of Rab6a.

The sample comprising REP1 may be formulated in any suitable form, for example the sample may be prepared in a prenylation buffer comprising about 50 mM HEPES, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT and protease inhibitor cocktail (Roche) at about pH 7.5.

The sample comprising REP1 may, for example, comprise about 1-100, 1-75, 1-50, 1-40, 1-30, 1-20 or 1-10 μg of total protein. The sample comprising REP1 may, for example, comprise about 10-100, 10-75, 10-50, 10-40, 10-30 or 10-20 μg of total protein. Preferably, the sample comprising REP1 comprises about 10-30 μg of total protein, for example, about 10, 15, 20, 25 or 30 μg of total protein.

The Rab6a may, for example, be at a concentration of about 1-25, 1-20, 1-15, 1-10 or 1-5 μM , preferably about 1-5 μM . The Rab6a may, for example, be at a concentration of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 μM , preferably about 4 μM .

The Rab GGTase may, for example, be at a concentration of about 1-25, 1-20, 1-15, 1-10, 1-5 or 1-2.5 μM , preferably about 1-2.5 μM . The Rab GGTase may, for example, be at a concentration of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 μM , preferably about 2 μM .

The lipid donor substrate (e.g. biotin-geranylpyrophosphate (BGPP)) may, for example, be at a concentration of about 1-25, 1-20, 1-15, 1-10 or 1-5 μM , preferably about 1-5 μM . The lipid donor substrate (e.g. biotin-geranylpyrophosphate (BGPP)) may, for example, be at a concentration of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 μM , preferably about 4 μM .

The prenylation reaction may be carried out in any suitable buffer, for example the reaction may be carried out in a prenylation buffer comprising about 50 mM HEPES, 50 mM NaCl, 2 mM MgCl_2 , 1 mM DTT and protease inhibitor cocktail (Roche) at about pH 7.5.

Prenylation reactions may be carried out for any suitable length of time at any suitable temperature (e.g. about 37°C). For example, prenylation reactions may be carried out for about 1-10, 1-7.5, 1-5, 1-2.5 or 1-2 h, preferably about 1-2 h. Prenylation reactions may, for example, be carried out for about 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 h, preferably about 2 h.

Choroideremia

Choroideremia is a rare X-linked progressive degeneration of the choroid, retinal pigment epithelium and photoreceptors of the eye. The typical natural history in afflicted males is onset of nightblindness during teenage years, and then progressive loss of peripheral vision during the 20's and 30's leading to complete blindness in the 40's. Female carriers have mild symptoms, most notably nightblindness, but may occasionally have a more severe phenotype.

Choroideremia is caused by mutations in the *CHM* gene, which is located on the X chromosome 21q region. Rab escort protein 2 (REP2), which is 75% homologous to REP1, compensates for any REP1 deficiency in most cells of the body. However, for reasons that are not yet clear, REP2 is unable to compensate for REP1 deficiency in the eye. This leads to insufficient Rab escort protein activity to maintain normal prenylation of target Rab GTPases and gives rise to cellular dysfunction and ultimately cell death, primarily affecting the outer retina and choroid.

Choroideremia may be successfully treated by providing functional copies of the REP1 transgene to the affected cells of the eye (MacLaren, R.E. *et al.* (2014) *Lancet* 383: 1129-37).

Vectors

A vector is a tool that allows or facilitates the transfer of an entity from one environment to another. In accordance with the invention, and by way of example, some vectors used in recombinant nucleic acid techniques allow entities, such as a segment of nucleic acid (e.g. a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a target cell. The vector may serve the purpose of maintaining the heterologous nucleic acid (e.g. DNA or RNA) within the cell, facilitating the replication of the vector comprising a segment of nucleic acid or facilitating the expression of the protein encoded by a segment of nucleic acid.

Vectors may be non-viral or viral. Examples of vectors used in recombinant nucleic acid techniques include, but are not limited to, plasmids, chromosomes, artificial chromosomes and viruses. The vector may also be, for example, a naked nucleic acid (e.g. DNA or RNA). In its simplest form, the vector may itself be a nucleotide of interest.

The vectors used in the invention may be, for example, plasmid or viral vectors and may include a promoter for the expression of a polynucleotide and optionally a regulator of the promoter.

Viral Vectors

In a preferred embodiment, the vector of the invention is a viral vector. Preferably, the viral vector is in the form of a viral vector particle.

The viral vector may be, for example, an adeno-associated viral (AAV), retroviral, lentiviral or adenoviral vector. Preferably, the viral vector is an AAV vector.

The term “gene therapy vector” is used herein to refer to a vector which is suitable for use in gene therapy and includes, for example, viral (e.g. AAV) vectors and vector particles.

Variants, derivatives, analogues, homologues and fragments

5 In addition to the specific proteins and nucleotides mentioned herein, the invention also encompasses the use of variants, derivatives, analogues, homologues and fragments thereof.

10 In the context of the invention, a variant of any given sequence is a sequence in which the specific sequence of residues (whether amino acid or nucleic acid residues) has been modified in such a manner that the polypeptide or polynucleotide in question substantially retains its function. A variant sequence can be obtained by addition, deletion, substitution, modification, replacement and/or variation of at least one residue present in the naturally-occurring protein.

15 The term “derivative” as used herein, in relation to proteins or polypeptides of the invention includes any substitution of, variation of, modification of, replacement of, deletion of and/or addition of one (or more) amino acid residues from or to the sequence providing that the resultant protein or polypeptide substantially retains at least one of its endogenous functions.

The term “analogue” as used herein, in relation to polypeptides or polynucleotides includes any mimetic, that is, a chemical compound that possesses at least one of the endogenous functions of the polypeptides or polynucleotides which it mimics.

20 Typically, amino acid substitutions may be made, for example from 1, 2 or 3 to 10 or 20 substitutions provided that the modified sequence substantially retains the required activity or ability. Amino acid substitutions may include the use of non-naturally occurring analogues.

Proteins used in the invention may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent protein.

25 Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues as long as the endogenous function is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar

30 hydrophilicity values include asparagine, glutamine, serine, threonine and tyrosine.

Conservative substitutions may be made, for example according to the table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R H
AROMATIC		F W Y

5 The term “homologue” as used herein means an entity having a certain homology with the wild type amino acid sequence and the wild type nucleotide sequence. The term “homology” can be equated with “identity”.

10 A homologous sequence may include an amino acid sequence which may be at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or 90% identical, preferably at least 95% or 97% or 99% identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the invention it is preferred to express homology in terms of sequence identity.

15 A homologous sequence may include a nucleotide sequence which may be at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or 90% identical, preferably at least 95% or 97% or 99% identical to the subject sequence. Although homology can also be considered in terms of similarity, in the context of the invention it is preferred to express homology in terms of sequence identity.

20 Preferably, reference to a sequence which has a percent identity to any one of the SEQ ID NOs detailed herein refers to a sequence which has the stated percent identity over the entire length of the SEQ ID NO referred to.

Homology comparisons can be conducted by eye or, more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate percent homology or identity between two or more sequences.

25 Percent homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is

called an “ungapped” alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion in the nucleotide sequence may cause the following codons to be put out of alignment, thus potentially resulting in a large reduction in percent homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting “gaps” in the sequence alignment to try to maximise local homology.

However, these more complex methods assign “gap penalties” to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible, reflecting higher relatedness between the two compared sequences, will achieve a higher score than one with many gaps. “Affine gap costs” are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum percent homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux et al. (1984) *Nucleic Acids Res.* 12: 387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al. (1999) *ibid* – Ch. 18), FASTA (Atschul et al. (1990) *J. Mol. Biol.* 403-410) and the GENWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel et al. (1999) *ibid*, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program. Another tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequences (see FEMS Microbiol. Lett. (1999) 174: 247-50; and FEMS Microbiol. Lett. (1999) 177: 187-8).

Although the final percent homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix – the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see the user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate percent homology, preferably percent sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

“Fragments” of full length polypeptides or polynucleotides of the invention are also variants and the term typically refers to a selected region of the polypeptide or polynucleotide that is of interest either functionally or, for example, in an assay. “Fragment” thus refers to an amino acid or nucleic acid sequence that is a portion of a full-length polypeptide or polynucleotide.

Such variants may be prepared using standard recombinant DNA techniques such as site-directed mutagenesis. Where insertions are to be made, synthetic DNA encoding the insertion together with 5' and 3' flanking regions corresponding to the naturally-occurring sequence either side of the insertion site may be made. The flanking regions will contain convenient restriction sites corresponding to sites in the naturally-occurring sequence so that the sequence may be cut with the appropriate enzyme(s) and the synthetic DNA ligated into the cut. The DNA is then expressed in accordance with the invention to make the encoded protein. These methods are only illustrative of the numerous standard techniques known in the art for manipulation of DNA sequences and other known techniques may also be used.

Codon optimisation

The polynucleotides used in the present invention may be codon-optimised. Codon optimisation has previously been described in WO 1999/41397 and WO 2001/79518. Different cells differ in their usage of particular codons. This codon bias corresponds to a bias in the relative abundance of particular tRNAs in the cell type. By altering the codons in the sequence so that they are tailored to match with the relative abundance of corresponding tRNAs, it is possible to increase expression. By the same token, it is possible to decrease expression by deliberately choosing codons for which the corresponding tRNAs are known

to be rare in the particular cell type. Thus, an additional degree of translational control is available.

EXAMPLES

Materials and methods (Examples 1-2)

5 *Cell transduction and harvesting*

Cultured HEK293 cells were treated with rAAV2/2-REP1 at a range of multiplicities of infection (MOI, genome particles/cell). rAAV2/2-GFP was used in parallel as a control vector, and fluorescence was monitored for onset of transgene expression.

10 Experiments on untransduced, and +AAV-GFP transduced and +AAV-REP1 transduced cells were conducted in parallel.

Cell lysates were prepared at day 5 post-transduction using the following protocol: cells were washed with PBS and incubated for 5 min with prenylation buffer, pH 7.5 (50 mM HEPES, 50 mM NaCl, 2 mM MgCl₂, 1 mM DTT and protease inhibitor cocktail (Roche)) on ice; cells were then scraped using a cell scraper into a 1.5 mL tube and incubated on ice for 15 min; subsequently, cells were disrupted by pushing them 20 times through a 26-G syringe needle attached to a 1 mL syringe.

Lysed cells were centrifuged for 5 min at 1500×g at 4°C. The supernatant was then transferred to cellulose propionate tubes and centrifuged for 1 h at 100000×g at 4°C. The supernatant from the second centrifugation step was used for the in vitro prenylation reactions (described below).

Total protein quantification

Total cell protein concentration was quantified using the Bradford method according to the manufacturer's instructions (Quick Start™ Bradford 1x Dye Reagent, BioRad, #500-0205). Sample values were extrapolated from a standard curve.

25 *In vitro prenylation reaction*

Prenylation reactions were set up using frozen cell lysate (10-30 µg), 2 µM Rab GGTase, 4 µM Rab protein (Rab27a or Rab6a) and 5 µM biotin-geranylpyrophosphate (BGPP) as the lipid donor, in prenylation buffer. All reactions were supplemented with fresh GDP (guanosine diphosphate, 20 µM) and DTT (1 mM).

For positive control samples, fish REP1 (see individual experiments for the amount) was added to the prenylation reaction containing lysate from untransduced cells.

Reactions were incubated for 2 h at 37°C and then stopped by addition of sample buffer (Laemmli buffer, 2× concentrate, Sigma #S3401). This buffer contains 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH approx. 6.8.

Western blots (WB) were performed to detect human REP-1, β -actin (as a loading control) and biotinylated Rab protein (Rab27a or Rab6a).

For detection of human REP1, a mouse monoclonal antibody from Millipore was used (clone 2F1, #MABN52). For detection of β -actin, a mouse monoclonal antibody from Thermo Fisher Scientific was used (clone AC-15, #AM4302). Both detections were followed by a secondary antibody-labelling step (donkey anti-mouse HRP, Abcam, #ab98799).

The incorporation of biotinylated lipid donor into the appropriate Rab substrate was detected by direct incubation with streptavidin-HRP (Thermo Fisher Scientific, #43-4323).

All membranes were detected using ECL substrate and Odyssey FC detection system (LI-COR). The intensities of the bands were quantitatively analysed using Image Studio Lite software (LI-COR).

Example 1 – Rab27a as a prenylation substrate

To test the sensitivity of a prenylation assay using Rab27a as a substrate, experiments were carried out in parallel using the following cells:

- (a) untransduced cells;
- (b) cells transduced with AAV-GFP at a MOI of 10000; and
- (c) cells transduced with AAV-REP1 at a MOI of 10000.

Prenylation reactions were set up using 10 μ g of lysate in a total volume of 12.5 μ L. Positive controls were spiked with 2 μ M of fish REP1.

The results indicate that Rab27a is a substrate for the prenylation assay to assess REP1 function following transduction of cells with AAV-REP1 (Figure 1). However, the signal from the WB semiquantification is not very strong.

This study was repeated with an increased amount of total cell protein to increase the WB band intensity.

Prenylation reactions were set up using 30 µg of lysate in a total volume of 22 µL. Positive controls were spiked with 1 µM of fish REP1.

The results confirm that Rab27a works as a substrate for the prenylation assay to assess REP1 function following transduction of cells with AAV-REP1 (Figure 2). Furthermore, the strength of the WB signal has increased compared to the data obtained using 10 µg of lysate. However, the signal is still not very strong. Ideally, a larger increase of prenylated Rab protein when cells are transduced with AAV-REP1 would be observed.

Example 2 – Rab6a as a prenylation substrate

To test the sensitivity of a prenylation assay using Rab6a as a substrate, experiments were carried out in parallel using the following cells (same cell lysates used in Example 1):

- (a) untransduced cells;
- (b) cells transduced with AAV-GFP at a MOI of 10000; and
- (c) cells transduced with AAV-REP1 at a MOI of 10000.

Prenylation reactions were set up using 20 µg of lysate in a total volume of 20 µL. Positive controls were spiked with 1 µM of fish REP1.

The results indicate that Rab6a is an effective substrate for the prenylation assay to assess REP1 function following transduction of cells with AAV-REP1 (Figure 3).

The strength of the WB signal has increased approximately 10-fold for AAV-REP1 transduced cells, compared to the data shown in Figure 2, even though less total protein was used. Furthermore, the band intensity for the positive controls is approximately 100-fold greater compared to the data shown in Figure 2, confirming the increased sensitivity of the Rab6a-based assays.

The data also demonstrate the increased sensitivity enables the detection of differences at endogenous levels too, which makes the assay more accurate.

Following the successful demonstration of increased assay sensitivity provided by the use of Rab6a as the prenylation substrate, we repeated the assay using different MOIs of AAV-REP1 study whether Rab6a prenylation correlates with the amount of AAV-REP1.

Experiments were carried out in parallel using the following cells:

- (a) untransduced cells;
- (b) cells transduced with AAV-REP1 at a MOI of 250;

- (c) cells transduced with AAV-REP1 at a MOI of 1000;
- (d) cells transduced with AAV-REP1 at a MOI of 5000;
- (e) cells transduced with AAV-REP1 at a MOI of 10000; and
- (f) cells transduced with AAV-REP1 at a MOI of 20000.

5 Prenylation reactions were set up using 20 µg of lysate in a total volume of 15 µL. The positive control was spiked with 0.5 µM of fish REP1.

The results confirm that Rab6a is an effective substrate for the prenylation assay to assess REP1 function following transduction of cells with AAV-REP1 (Figure 4) and furthermore demonstrate that the incorporation of biotinylated lipid donor in Rab6a correlates with the
10 amount of AAV-REP1 used for cell transduction.

Following the validation of Rab6a as an effective assay substrate, we then tested the AAV-REP1 vector currently in use in our Phase 1 clinical trial (MacLaren, R.E. et al. (2014) Lancet 383: 1129-37).

Experiments were carried out in parallel using the following cells:

- 15 (a) untransduced cells (#29, #30 and #31);
- (b) cells transduced with AAV-REP1 at a MOI of 10000 (#32, #33 and #34); and
- (c) cells transduced with GMP grade AAV-REP1 at a MOI of 10000 (#35, #36 and #37).

Prenylation reactions were set up using 20 µg of lysate in a total volume of 15 µL. The positive control was spiked with 0.5 µM of fish REP1.

20 The results are in keeping with the previous experiments and confirm that the incorporation of biotinylated lipid donor in Rab6a correlates with the amount of AAV-REP1 used for cell transduction (Figure 5).

Example 3 – Rab6a as a substrate in the prenylation reaction using ARPE-19 cells

Cell transduction and harvesting

25 Cultured ARPE-19 cells were treated with rAAV2/2-REP1 at an MOI of 10,000 genome particles/cell. Cell lysates were prepared at day 13 post-transduction: cells were washed with PBS and incubated with prenylation buffer, pH 7.5 (50 mM HEPES, 50 mM NaCl, 2 mM MgCl₂, 1 mM DTT and protease inhibitor cocktail (Roche)) on ice. Cells were scraped using a cell scraper into a 1.5 mL tube, and incubated on ice for 15 min. Cells were disrupted by
30 pushing them 20 times through a 26-G syringe needle attached to a 1 mL syringe. Cells were spun for 5 min at 1,500 g, 4°C. The supernatant was then transferred to cellulose

propionate tubes and centrifuged at 100,000 g for 1 h at 4°C. The supernatant was used for the in vitro prenylation reaction.

Total protein quantification

5 Total cell protein was quantified using the Bradford method according to the manufacturer's instructions (Quick Start™ Bradford 1x Dye Reagent, BioRad, #500-0205).. Sample values were extrapolated from a standard curve.

In vitro prenylation reaction

10 The prenylation reactions were set up using frozen cell lysate (15 µg), 2 µM Rab GGTase, 4 µM of Rab protein (Rab6a) and 5 µM of biotin-geranylpyrophosphate as lipid donor, in prenylation buffer. All reactions were supplemented with fresh GDP (20 µM) and DTT (1 mM). In positive control samples, fish REP1 (see experiments for amount) was added to the prenylation reaction containing untransduced cell lysate.

The reactions were incubated for 2 h at 37°C and then stopped by addition of SDS-PAGE sample buffer.

15 Western blotting (WB) was performed to detect human REP-1, β-actin (loading control) and biotinylated Rab protein (Rab27a or Rab6a). For detection of human REP1, a mouse monoclonal antibody from Millipore was used (clone 2F1, #MABN52). For detection of β-actin, a mouse monoclonal antibody from Thermo Fisher Scientific was used (clone AC-15, #AM4302). Both detections were followed by a secondary antibody-labelling step (donkey
20 anti-mouse HRP, Abcam, #ab98799). The incorporation of biotinylated lipid donor into the appropriate Rab substrate was detected by direct incubation with streptavidin-HRP (Thermo Fisher Scientific, #43-4323). All membranes were detected using ECL substrate and Odyssey FC detection system (LI-COR). The intensities of the bands were quantitatively analysed using Image Studi Lite software (LI-COR).

25 *Results and discussion*

To test the prenylation assay using Rab6a as a substrate in ARPE-19 cells (human retinal pigment epithelium cells), experiments were carried out in parallel using the following cells:

- (a) Untransduced cells (#86 and #87); and
- (b) Cells + AAV-REP1 MOI 10,000 (#90 and #91) - R&D grade vector.

30 Prenylation reactions were set up using 15 µg of lysate in a total volume of 45 µL. Positive control was spiked with 0.1 µM of fish REP1.

The results indicate that Rab6a works as a substrate for the prenylation assay to assess REP1 function following transduction of ARPE-19 cells with AAV-REP1 (Figure 6).

Example 4 – Rab6a as a substrate in the prenylation reaction using HT1080 cells

Cell transduction and harvesting

- 5 Cultured HT1080 cells were treated with rAAV2/2-REP1 at an MOI of 10,000 genome particles/cell. Cell lysates were prepared at day 5 post-transduction: cells were washed with PBS and incubated with prenylation buffer, pH 7.5 (50 mM HEPES, 50 mM NaCl, 2 mM MgCl₂, 1 mM DTT and protease inhibitor cocktail (Roche)) on ice. Cells were scraped using a cell scraper into a 1.5 mL tube, and incubated on ice for 15 min. Cells were disrupted by
10 pushing them 20 times through a 26-G syringe needle attached to a 1 mL syringe. Cells were spun for 5 min at 1,500 g, 4°C. The supernatant was then transferred to cellulose propionate tubes and centrifuged at 100,000 g for 1 h at 4°C. The supernatant was used for the in vitro prenylation reaction.

Total protein quantification

- 15 Total cell protein was quantified using the Bradford method according to the manufacturer's instructions (Quick Start™ Bradford 1x Dye Reagent, BioRad, #500-0205). Sample values were extrapolated from a standard curve.

In vitro prenylation reaction

- The prenylation reactions were set up using frozen cell lysate (20 µg), 2 µM Rab GGTase, 4
20 µM of Rab protein (Rab6a) and 5 µM of biotin-geranylpyrophosphate as lipid donor, in prenylation buffer. All reactions were supplemented with fresh GDP (20 µM) and DTT (1 mM). In positive control samples, fish REP1 (see experiments for amount) was added to the prenylation reaction containing untransduced cell lysate.

- The reactions were incubated for 2 h at 37°C and then stopped by addition of SDS-PAGE
25 sample buffer.

- Western blotting (WB) was performed to detect human REP-1, β-actin (loading control) and biotinylated Rab protein (Rab27a or Rab6a). For detection of human REP1, a mouse monoclonal antibody from Millipore was used (clone 2F1, #MABN52). For detection of β-actin, a mouse monoclonal antibody from Thermo Fisher Scientific was used (clone AC-15, #
30 AM4302). Both detections were followed by a secondary antibody-labelling step (donkey anti-mouse HRP, Abcam, #ab98799). The incorporation of biotinylated lipid donor into the

appropriate Rab substrate was detected by direct incubation with streptavidin-HRP (Thermo Fisher Scientific, #43-4323). All membranes were detected using ECL substrate and Odyssey FC detection system (LI-COR). The intensities of the bands were quantitatively analysed using Image Studi Lite software (LI-COR).

5 *Results and discussion*

To test the prenylation assay using Rab6a as a substrate in HT1080 cells, experiments were carried out in parallel using the following cells:

- (a) Untransduced cells (#56 and #57);
- (b) Cells + AAV-REP1 MOI 10,000 (#60 and #61) – R&D grade vector; and
- 10 (c) Cells + AAV-REP1 MOI 10,000 (#64 and #65) – clinical grade vector.

Prenylation reactions were set up using 20 µg of lysate in a total volume of 20 µL. Positive control was spiked with 0.1 µM of fish REP1.

The results indicate that Rab6a works as a substrate for the prenylation assay to assess REP1 function following transduction of HT1080 cells with AAV-REP1 (Figure 7).

15 **Example 5 – Comparison of Rab27a and Rab6a as substrates in prenylation reactions**

The same cell lysates were used as in the experiment shown in Figure 4:

- (a) Untransduced cells;
- (b) Cells + AAV-REP1 MOI 250;
- (c) Cells + AAV-REP1 MOI 1,000;
- 20 (d) Cells + AAV-REP1 MOI 5,000;
- (e) Cells + AAV-REP1 MOI 10,000; and
- (f) Cells + AAV-REP1 MOI 20,000.

Prenylation reactions were set up using 20 µg of lysate in a total volume of 15 µL, and 2 different substrates: Rab27a and Rab6a. Positive controls, one for each substrate, were
25 spiked with 0.1 µM of fish REP1. Samples were run in parallel on SDS-PAGE and detected simultaneously.

Results and discussion

Both Rab27a and Rab6a work as a substrate for the prenylation assay to assess REP1 function following transduction of cells with AAV-REP1.

The incorporation of biotinylated lipid donor correlates with the amount of AAV-REP1 used for cell transduction for each of the substrates used (Figure 8).

The band density from biotinylated Rab6a is higher than for Rab27a, which indicates Rab6a is a more suitable substrate for a parallel line analysis for determination of relative potency.

5 **Example 6 – Comparison of Rab27a and Rab6a performance as substrates in prenylation reactions using different conditions**

Untransduced lysate of 293 cells was prepared for use in this experiment using Rab27a and Rab6a. The conditions tested are shown in the tables in Figure 9. Samples were run in parallel on SDS-PAGE and detected simultaneously.

10 *Results and discussion*

Both Rab27a and Rab6a work as a substrate for the prenylation assay to assess endogenous REP1 function.

The incorporation of biotinylated lipid donor correlates with the amount of total protein in the reaction for each of the substrates used.

15 Comparing the conditions, the concentration of Rab substrate in the reaction seems to affect the signal the most.

There is a 2.5-fold increase in the biotinylated substrate when Rab6a is used, compared to Rab27a.

20 **Example 7 – Comparison of Rab27a and Rab6a as substrates in prenylation reactions in lysates transduced with AAV2-REP1**

New lysates (in triplicate) were prepared using increasing MOIs of AAV2-REP1 (R&D material):

- (a) Untransduced cells;
- (b) Cells + AAV-REP1 MOI 100;
- 25 (c) Cells + AAV-REP1 MOI 500;
- (d) Cells + AAV-REP1 MOI 1,000;
- (e) Cells + AAV-REP1 MOI 5,000;
- (f) Cells + AAV-REP1 MOI 10,000;
- (g) Cells + AAV-REP1 MOI 20,000; and
- 30 (h) Cells + AAV-REP1 MOI 50,000.

Prenylation reactions were prepared using 20 µg of total protein, 2 µM of Rab substrate (Rab27a or Rab6a) and 2 µM of Rab GGTase, in a total volume of 10 µL. Positive controls, one for each substrate, were spiked with 0.1 µM of fish REP1.

Samples from each replicate were run in parallel on SDS-PAGE and detected for biotinylated substrate (1:10,000), β-actin (1:50,000) as loading control and human REP1 (1:2,500) using Image Studio Lite software. Data from semiquantification of band density for biotinylated substrate was plotted using Prism software (Figure 10).

Results

The levels of β-actin were similar in all samples analysed. Untransduced cells (and positive control samples) showed endogenous level of REP1. Cells transduced with AAV-REP1 showed an increase of REP1 levels that directly correlates with the MOI used. Positive controls show stronger biotin incorporation, as a result of fish REP1 activity.

A two-way ANOVA analysis of all three replicates with substrate and MOI as factors found that both were highly significant ($p < 0.0001$). Bonferroni's multiple comparisons test for the effect of the substrate at a given MOI showed a significant pairwise difference at MOI of 5,000 ($p = 0.0023$) and all above ($p < 0.0001$).

Discussion

Both Rab27a and Rab6a work as a substrate for the prenylation assay to assess REP1 function following transduction of cells with AAV-REP1.

Semiquantification of band density for biotinylated substrate only shows the values for Rab6a to be significantly higher than those obtained for Rab27a.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and uses of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention, which are obvious to those skilled in biochemistry and biotechnology or related fields, are intended to be within the scope of the following claims.

CLAIMS

1. An *in vitro* method for determining the activity of Rab escort protein 1 (REP1)-encoding gene therapy vector for use in the treatment of choroideremia, the method comprising the steps:
 - 5 (a) contacting a sample comprising REP1 with Rab6a, Rab geranylgeranyltransferase (Rab GGTase) and a lipid donor substrate, wherein the sample comprising REP1 is from a cell genetically engineered to express REP1 using the REP1-encoding gene therapy vector; and
 - (b) detecting the lipidated Rab6a product.
- 10 2. The method of claim 1, wherein the REP1 encoding gene therapy vector is a viral vector comprising a REP1-encoding nucleotide sequence.
3. The method of claim 2, wherein the viral vector is an adeno-associated viral (AAV) vector, preferably, wherein the adeno-associated viral vector is AAV serotype 2 (AAV2).
- 15 4. The method of any preceding claim, wherein the Rab6a and/or Rab GGTase are pure.
5. The method of any preceding claim, wherein the Rab6a:Rab GGTase molar ratio is 1:2-3, preferably 1:2.5.
- 20 6. The method of any preceding claim, wherein the lipid donor substrate is geranylgeranylpyrophosphate (GGPP) or an analogue thereof, preferably biotin-geranylpyrophosphate (BGPP).
7. The method of any preceding claim, wherein the lipidated Rab6a product is detected using an enzyme-linked immunosorbent assay (ELISA), Western blot analysis or autoradiography.
- 25 8. The method of any preceding claim, wherein the method is for quality control analysis of a REP1-encoding gene therapy vector for use in the treatment of choroideremia.
9. The method of any preceding claim, wherein the detection of the lipidated Rab6a product comprises quantifying the amount of the lipidated Rab6a product, preferably quantifying the amount relative to a control or reference level.

10. Use of Rab6a for determining *in vitro* the activity of Rab escort protein 1 (REP1)-encoding gene therapy vector for use in the treatment of choroideremia, wherein the Rab6a is contacted with Rab geranylgeranyltransferase (Rab GGTase), a lipid donor substrate, and REP1 from a cell genetically engineered to express the REP1 using the REP1-encoding gene therapy vector.
11. The use of claim 10, wherein the REP1 is in a lysate from the cell genetically engineered to express the REP1.
12. The use of claim 10 or 11, wherein the REP1 is expressed using a viral vector comprising a REP1-encoding nucleotide sequence.
13. The use of claim 12, wherein the viral vector is an adeno-associated viral (AAV) vector.
14. The use of any one of claims 10-13, wherein the Rab6a is pure.
15. The method of any one of claims 1-9 or the use of any one of claims 10-14, wherein the REP-1 encoding nucleotide sequence of the REP1-encoding gene therapy vector comprises a nucleotide sequence that encodes an amino acid sequence that has at least 80% identity to SEQ ID NO: 5, wherein the amino acid sequence substantially retains the natural function of the protein represented by SEQ ID NO: 5.