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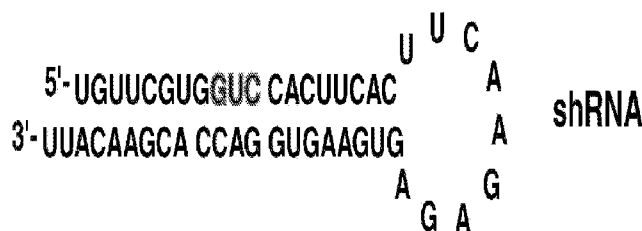
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(54) Title: COMPOSITIONS AND METHODS FOR THERAPY OF MACULAR DEGENERATION

Figure 9

Human *RHO* 725 shRNA

(SEQ ID NO:21)

(57) Abstract: Provided are compositions and methods for therapy of macular degeneration including dry age-related macular degeneration (dAMD), juvenile macular degenerations (JMDs) where toxic retinoids are known to accumulate as part of the pathogenesis, such as Stargardt disease, and Best disease, and neovascular wet age-related macular degeneration. The method entails administering to an individual in need of therapy for macular degeneration a first polynucleotide that can facilitate a reduction in the amount of rod opsin (RHO) mRNA in the individual; or a second polynucleotide that can facilitate a reduction in the amount of RPE65 mRNA in the individual; or a combination thereof. The polynucleotides of the invention are hammerhead ribozymes or shRNAs. The polynucleotides target a sequence in RHO mRNA or RPE65 mRNA and facilitate reduction in the target mRNA via ribozymatic cleavage of the target, or by hybridization to the target, which leads to RNAi mediated degradation of the target mRNA.



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COMPOSITIONS AND METHODS FOR THERAPY OF MACULAR DEGENERATION

This application claims priority to U.S. application serial no. 61/174,435, filed on
5 April 30, 2009, the entire disclosure of which is incorporated herein by reference.

This invention was made with government support under R01 EY13433 awarded by the National Eye Institute. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

10 Macular degeneration is a clinical term that is used to describe a family of diseases that are all characterized by a progressive loss of central vision associated with abnormalities of Bruch's membrane, the choroid, the neural retina and/or the retinal pigment epithelium. These disorders include very common conditions that affect older subjects (age-related macular degeneration or AMD) as well as rarer, earlier-onset
15 dystrophies that in some cases can be detected in the first decade of life. Other maculopathies include and dry age-related macular degeneration (dAMD), juvenile macular degenerations (JMDs) where toxic retinoids are known to accumulate as part of the pathogenesis (e.g., Stargardt disease, and Best disease), neovascular and wet age-related macular degeneration.

20 Age-related macular degeneration (AMD), the most prevalent macular degeneration, is associated with progressive diminution of visual acuity in the central portion of the visual field, changes in color vision, and abnormal dark adaptation and sensitivity. Two principal clinical manifestations of AMD have been described as the dry, or atrophic, form, and the wet, or exudative, form. Dry AMD is the most common form
25 of macular degeneration and affects millions of people in the US alone. There is an ongoing and unmet need for therapies of macular degeneration.

SUMMARY OF THE INVENTION

30 The present invention provides compositions and methods for therapy of macular degeneration. The method comprises administering to an individual in need of therapy for macular degeneration a composition comprising i) a first polynucleotide that can facilitate a reduction in the amount of rod opsin (RHO) mRNA in the individual; or ii) a second polynucleotide that can facilitate a reduction in the amount of RPE65 mRNA in
35 the individual; or iii) a combination of i) and ii). Performance of the method of the invention results in macular degeneration in the individual being inhibited.

The polynucleotides of the invention are hammerhead ribozymes or shRNAs. The polynucleotides target a sequence in RHO mRNA or RPE65 mRNA and facilitate reduction in the target mRNA via ribozymatic cleavage of the target, or by hybridization to the target, which leads to RNAi mediated degradation of the target mRNA.

The forms of macular degeneration that the individual who is treated using the method of the invention include but are not necessarily limited to dry age-related macular degeneration (dAMD), juvenile macular degenerations (JMDs) where toxic retinoids are known to accumulate as part of the pathogenesis (e.g., Stargardt disease, and Best disease), and neovascular wet age-related macular degeneration.

DESCRIPTION OF THE FIGURES

Fig. 1 provides a graphical representation of rank order of candidate opsin ribozyme NUH sites by SEAP assay. Of 34 hhRzs tested, 25 significantly reduced SEAP protein expression ($p < 0.01$, $n=8$). These sites were rank ordered based on amount of SEAP activity knockdown. The target site at 725 showed a 28.97% knockdown of SEAP reporter protein activity in the stable Rho-IRES-SEAP cell line, and resides in a region we found to be highly accessible by *in silico* and experimental assays of accessibility.

Fig. 2a and 2b provides data evidencing effective shRNA Knockdown of RHO expression. a) shRNA construct targeted to 725 site (Rhoi725) was compared to a known successful shRNA construct (Rhoi2 from Cashman *et. al.* 2005) using qRT-PCR. Full-length Rho constructs with single and double mutation(s) in the target site showed significant resistance to Rhoi725 indicating specificity of cleavage site. b) Knock-down observations were confirmed at the protein level (western blot).

Fig. 3. provides data obtained from evaluation of RPE65 knockdown using hhRz. Data were obtained from western blots of protein encoded by RPE65 mRNA and quantitation of the protein.

Fig. 4 provides a description of mouse *RPE65* Hammerhead Ribozyme Sequences and the sequences they target.

Figure 5 provides a description of an shRNA that targets human RPE65 at site 221 (human *RPE65* 221 shRNA).

Figure 6 provides a description of an shRNA that targets human RPE65 at site 520 (Human *RPE65* 520 shRNA).

Figure 7 provides a description of an shRNA that targets human RPE65 at site 933 (Human RPE65 933 shRNA).

Figure 8 provides a description of an shRNA that targets human RPE65 at site 2108 (RPE65 2108 shRNA).

5 Figure 9 provides shRNA that targets human RHO mRNA. This sequence forms an shRNA hairpin to attack Human RHO 725 site.

DETAILED DESCRIPTION OF THE INVENTION

10 The present invention provides compositions and methods for therapy of macular degeneration. The method comprises administering to an individual in need of therapy for macular degeneration a composition comprising:

- i) a first polynucleotide that can facilitate a reduction in the amount of rod rhodopsin (*RHO*) mRNA in the individual; or
- 15 ii) a second polynucleotide that can facilitate a reduction in the amount of RPE65 mRNA in the individual; or
- iii) a combination of i) and ii);

 wherein macular degeneration in the individual is inhibited subsequent to the administration. Those skilled in the art can readily determine whether macular
20 degeneration has been inhibited using any of a variety of well known clinical and/or molecular biological techniques.

 The invention is based in part on the disease etiology of several forms of macular degeneration that are caused by and/or positively correlated with accumulation of toxic retinoids. The forms of macular degeneration for which the present invention is expected
25 to provide a therapeutic benefit include but are not necessarily limited to dry age-related macular degeneration (dAMD), juvenile macular degenerations (JMDs) where toxic retinoids are known to accumulate as part of the pathogenesis (e.g., Stargardt disease, and Best disease), and neovascular wet age-related macular degeneration. Thus, in various embodiments, the method comprises administering a composition comprising a first
30 polynucleotide that can facilitate a reduction in the amount *RHO* mRNA in the individual, or a second polynucleotide that can facilitate a reduction in the amount of RPE65 mRNA in the individual, or a combination of such polynucleotides, to an individual in need of therapy for a macular degeneration, wherein the macular degeneration is selected from dAMD, JMDs where toxic retinoids are known to
35 accumulate as part of the pathogenesis, and wet neovascular age-related macular

degeneration. In one embodiment, the individual does not have and/or has not previously been treated for an eye disorder that is not a macular degenerative disorder, such as retinitis pigmentosa.

Polynucleotides that can “facilitate a reduction in mRNA” encompass RNA
5 polynucleotides or and/or modified RNA polynucleotides that can participate in a biological process that results in degradation of RHO mRNA and/or RPE65 mRNA. It will be recognized that the net result of mRNA degradation is a reduction in the intracellular amount of protein encoded by the mRNAs. Thus, the invention provides a method for post-transcription gene silencing (PTGS) of the RHO and RPE65 genes.

10 In various embodiments, the polynucleotide that can facilitate a reduction in the amount of mRNA is a ribozyme, such as a hammerhead ribozyme (“hhRz”) or a hairpin ribozyme, or an shRNA. It will thus be apparent that the polynucleotide that can facilitate a reduction in the amount of mRNA may act directly on the mRNA to catalyze its degradation (e.g., a ribozyme), or it may facilitate reduction in the amount of mRNA
15 by hybridization to the mRNA to direct the mRNA to an RNAi-mediated degradation pathway. For instance, in one embodiment, the method comprises administering to an individual a first shRNA polynucleotide targeted to RHO mRNA, and/or a second shRNA polynucleotide targeted to RPE65 mRNA. As is known in the art, shRNAs adopt a typical secondary structure that contains a paired sense and antisense portion, and a
20 short loop sequence between the paired sense and antisense portions. shRNA is delivered to the cytoplasm where it is processed by dicer into siRNAs. The siRNAs are recognized by RNA-induced silencing complex (RISC), and once incorporated into RISC, siRNAs facilitate cleavage and degradation of targeted mRNA. Each of the shRNAs used in the present invention can consist of between 45-100 nucleotides, inclusive, and including all
25 integers between 45 and 100.

In the case of a ribozyme that can cleave a target mRNA, the ribozyme binds to an accessible region of the target mRNA, cleaves it, and then dissociates from the target and performs the same series of reactions (enzymatic turnover) with other substrate mRNA molecules. Ribozymes generally bind to target RNA with sufficient strength to
30 insure a hybrid lifetime that allows chemical cleavage of target RNA, but not so strongly that the product dissociation is slow and inhibits turnover (product inhibition). Hammerhead ribozymes are known to cleave at a consensus sequence triplet with a central U which can be described as NUH↓, where N can be any nucleotide and H any nucleotide but G, and where the arrow identifies the cleavage site.

The invention provides multiple novel shRNAs and ribozymes that target RHO mRNA or RPE65 mRNA. Further, shRNAs that are directed to each of these mRNAs are commercially available. For example, THERMO SCIENTIFIC Open Biosystems offers a variety of shRNAs against human and mouse RPE65 mRNA (i.e., GenBank entries
5 NM_000329 and NM_029987, respectively). The same entity offers a variety of shRNAs against human and mouse RHO mRNA (i.e., GenBank entries NM_000539 and NM_145383, respectively).

Additionally, the method comprises ribozymes and shRNAs that can cleave target mRNA at a specific site, or cause a target mRNA to be degraded via RNAi-mediated
10 RNA degradation. For instance, in one embodiment, the method employs a ribozyme that cleaves an mRNA comprising the sequence of SEQ ID NO:1, wherein the cleavage occurs between nucleotides 725 and 726 of SEQ ID NO:1.

The method also comprises administering one or more viral vectors encoding a polynucleotide that can facilitate a reduction in the amount of *RHO* mRNA in the
15 individual and/or encoding a polynucleotide that can facilitate a reduction in the amount of RPE65 mRNA in the individual. In this regard, any viral vector or nanoparticle vector capable of expressing the coding sequences for the polynucleotides can be used. Examples of suitable vectors include but are not limited to viral based vectors, such as adenovirus (AV) vectors, adeno-associated virus (AAV) vectors, retroviral vectors [e.g.,
20 lentiviruses (LV) or murine leukemia virus], rhabdoviruses [rabies virus or vesicular stomatitis virus (VSV)]. In one embodiment, a chicken adenovirus (CELO virus) may be used. In another embodiment, recombinant adeno-associated virus (rAAV) type with high specificity for photoreceptor transduction (rAAV2-7 or rAAV2-8) is used. As used herein, “administering” an shRNA or a ribozyme is intended to encompass administering
25 a vector encoding the shRNA or the ribozyme that is transcribed from the vector. The first and second polynucleotides can be encoded by the same or distinct vectors.

The method of the invention can be performed by administering the polynucleotides as naked polynucleotides, or in combination with any suitable pharmaceutically acceptable carriers, excipients and/or stabilizers. Some suitable
30 examples of pharmaceutically acceptable carriers, excipients and stabilizer can be found in *Remington: The Science and Practice of Pharmacy* (2005) 21st Edition, Philadelphia, PA. Lippincott Williams & Wilkins.

The method can also be performed by administering the polynucleotides with a delivery agent. Suitable delivery agents include but are not limited to the Mirus Transit

TKO lipophilic reagent; lipofectin; lipofectamine; cellfectin; or polycations (e.g., polylysine), or liposomes.

In one embodiment, the polynucleotides are administered via intraocular injection (e.g., sub-retinal injection), but the administration may be performed by any other
5 suitable route, including but not limited to an eye-drop solution, an implantable composition, a time-release composition, or any other suitable method and/or composition. The polynucleotides can be delivered as a single dose or as multiple doses over days, weeks, months, or longer. The administrations can occur simultaneously or sequentially in any order. In one embodiment, there is an opportunity for multiple PTGS
10 agents that target independent accessible regions of a single target to enhance the a reduction in target mRNA, and such enhancement may be greater than additive (i.e., it may result in a synergistic effect).

Polynucleotides for the compositions and methods of the invention can be made using any acceptable technique, including conventional and commercially available
15 chemical synthesis techniques, in vitro transcription, etc., such as by expression from expression vectors, and isolated and purified as necessary or desired. Further, various types of polynucleotide modifications are contemplated so as to improve the capability of the polynucleotides to resist endonuclease degradation and/or improve facilitation of reduction of their mRNA targets. For example, in addition to RNA, the polynucleotides
20 can comprise RNA:DNA hybrids. Other modifications that can be comprised by the polynucleotides include but are not limited to modified ribonucleotides or modified deoxyribonucleotides. Such modifications can include without limitation substitutions of the 2' position of the ribose moiety with an --O-- lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an --O-aryl group having 2-6 carbon
25 atoms, wherein such alkyl or aryl group may be unsubstituted or may be substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl, or amino groups; or with a hydroxy, an amino or a halo group. In addition to phosphodiester linkages, the nucleotides can be connected by a synthetic linkage, i.e., inter-nucleoside linkages other than phosphodiester linkages. Examples of inter-
30 nucleoside linkages that can be used in the invention include but are not limited to phosphodiester, alkylphosphonate, phosphorothioate, phosphorodithioate, phosphate ester, alkylphosphonothioate, phosphoramidate, carbamate, carbonate, morpholino, phosphate trister, acetamidate, carboxymethyl ester, or combinations thereof.

The invention also provides compositions comprising polynucleotides that can facilitate a reduction in the amount of *RHO* mRNA in the individual and/or that can facilitate a reduction in the amount of RPE65 mRNA via ribozymatic activity or via RNAi mediated mRNA degradation. The polynucleotides can be any of those discussed
5 *supra* as suitable for being used in the method of the invention, and accordingly include shRNAs, ribozymes, and vectors that encode such polynucleotides. Representative polynucleotide sequences that the compositions can comprises are described more fully below. The compositions can comprise, consist essentially of, or consist of shRNAs, ribozymes, or viral vectors encoding the same.

10 The pathogenesis and symptomology of the forms of macular degeneration for which the present invention provides therapeutic compositions and methods are well characterized. In general, in orphan JMDs (e.g. Stargardt, Best) and common dAMD there is accumulation of cellular and biochemical debris within and beneath the RPE. Each day the distal tips (10%) of rod and cone PR outer segment are shed according to a
15 circadian cycle. Expression and trafficking of new phototransduction and cellular materials maintains the outer segment length and structure to support scotopic and photopic realms of human vision. In the rod rich parafoveal area of the human retina, where both dAMD and JMDs begin (1-3 mm from fovea, 3.5-10° eccentricity from fixation), there are approximately 30-40 PRs per single RPE cells and most of these are
20 rods. The RPE cell phagocytizes the distal PR tips for lysosomal digestion. This intracellular digestion process is incomplete and results in accumulation of outer segment materials in RPE phagolysosomes including retinoids. This material is called lipofuscin (LF). LF is composed of numerous protein, lipid and carbohydrate components. LF accumulates in the RPE over time as the individual ages. Materials in LF contribute to
25 sub-RPE deposits such as flecks and drusen in juvenile macular degenerations and dry AMD. LF has a brilliant autofluorescence under blue light excitation due to the presence of a *bis*-retinoid pyridinium salt, N-retinylidene-N'-retinyl-ethanolamine (A2E), and retinaldehyde dimers (RetDi). The process of A2E and RetDi formation occurs spontaneously in the PR outer segments, and is derived from the chemical covalent
30 ligation of two molecules of all-*trans*-retinal (ATR) with a single molecule of the membrane aminolipid, PE.

ATR forms because light bleaches rod and cone visual pigments. Because of finite enzymatic limits within the retinoid visual cycle, some unknown fraction of the

total ATR that forms has sufficient time to react with PE, and/or another ATR molecule, to form A2E and RetDi. Precursors to A2E/RetDi form in outer segments and are a component of shed rod and cone outer segment tips and the completion of formation of A2E/RetDi occur in the RPE phagolysosome. Due to their unique structures these chemicals cannot be metabolized and accumulate in RPE cells over time. Accumulation reflects the normal daily production of ATR from the visual cycle, which varies with light exposure, and normal RPE phagocytosis of PR outer segment tips, integrated over many years. In the case of juvenile MD or dAMD the rate of accumulation of A2E/RetDi is greatly accelerated, and thus can be used to define an individual in need of the presently provided compositions and methods. Additionally, RPE cells in these diseases become physically swollen with numerous engorged phagolysosomes such that their surface area expands. While RPE cells may secrete some of this material onto underlying Bruch's membrane and contribute to formation of the drusen of dAMD or the flecks or yolk-like accumulations in JMDs, the prevailing model is that death of focal RPE cells deposits undigested bulk material onto Bruch's membrane that forms clinically visible lesions of these syndromes. The accumulation of A2E, retinal dimer, and other toxic retinoids in the RPE cells also has a direct toxic influence on these cells and can directly promote cell death. As RPE cells are post-mitotic, surrounding cells do not divide after local neighbor loss, but rather expand and spread out to fill the void in the RPE matrix and reconstitute the outer blood-retinal barrier. In doing so, single RPE cells cover larger areas and more PRs, which compounds the cumulative daily stress to fewer cells that must provide for nutrient exchange from the choroid, phagocytosis of outer segment tips, and retinoid metabolism. With progressive local death in the RPE matrix, there are insufficient numbers to sustain PR coverage and a stable hole in the matrix is formed. Overlying PRs without the critical interaction with the underlying RPE cells die secondarily. This results in focal areas of visual sensitivity loss in the JMDs, or the emergence of geographic atrophy of end stage dAMD. These areas expand with disease progression and A2E accumulates in RPE cells at the edge of the wake of geographic atrophy prior to RPE and PR cell death. Thus, the present provides a therapy for forms of macular degeneration that are caused by, or are positively correlated with, the accumulation of toxic retinoids, and the invention embraces a metabolic engineering approach that can reduce the accumulation of the toxic retinoids.

It is notable that within the rod-rich human parafovea, where A2E/RetDi accumulates and JMDs and dAMD begin, a single human RPE cell overlies 30-40 rod

PRs and only a few cone PRs. This reflects the absolute numbers of rods (120×10^6) and cones ($6-7 \times 10^6$) in the human retina, and their nonuniform distribution. JMDs and dAMD originate in the parafoveal region of the retina where the rod density is high and the foveal cone density has already fallen strongly. With 10% of the volume of outer segments of rod or cone PRs being shed each day, a single RPE cell phagocytizes the equivalent of 2.5 full rod PR outer segments and 0.1 cone PR outer segments per day. The RPE is one of the most metabolically active cells in the human body. The parafoveal RPE is presented with 25-fold greater rod than cone mass material on a daily basis. Light bleached visual pigment is the source of essentially all ATR molecules that might form A2E/RetDi. Thus, the invention takes advantage of the novel discovery that decreased stress on the parafoveal RPE and RPE in general should result in decreases in the bulk mass of rod outer segment material that is presented to the RPE by its local associated population of rod PRs. Therefore, by reducing the overall metabolic load on the RPE, stress levels will be lower, and the probability of both RPE and PR cell death will decrease accordingly. The invention also takes advantage of the discovery that decreased amounts of A2E/RetDi should be formed if there were both decreased levels of visual pigment to form ATR during bleaching, and decreased turnover (bleaching followed by recovery) of the available levels of rod visual pigment integrated over many cycles into the life of the individual. Accordingly, and without intending to be bound by theory, it is expected that by decreasing the steady-state level of total retinoid molecules in the rod-RPE retinoid visual cycle that are available to form ATR, the level of A2E/RetDi that otherwise forms from two serial 2nd-order biochemical reactions should decrease significantly, and this would reduce the toxic impact on RPE structure and metabolism.

In view of the foregoing, it will be apparent to those skilled in the art that post-transcriptional gene silencing (or reducing the level of mRNA) for *RHO* and *RPE65* should confer a therapeutic benefit for individuals who suffer the aforementioned biochemical/ocular irregularities that lead to macular degeneration associated therewith. In more detail, and again without intending to be bound by any particular theory, it is believed that A2E-mediated or toxic retinoid-mediated RPE toxicity in dAMD or JMDs can be slowed by programming kinetic bottlenecks in the generation and metabolic processing of ATR. In particular, suppressing a fraction of *RHO* expression specifically in rod photoreceptors would clamp a major source of ATR formation that leads to A2E

and other toxic retinoids. Likewise, suppressing a fraction of *RPE65* in RPE would clamp turnover of retinoid backbones in visual pigment regeneration.

PTGS in particular cell types is a highly specific means of reducing levels of target mRNAs and proteins to modulate distributed cellular system functionality, but RNA biocomplexity limits development of potent and safe PTGS agents as mRNA targets which are densely folded and large, stable, single-stranded PTGS annealing platforms are rare. We developed and utilize here a proven platform of high throughput screening technologies to: 1) identify rare strongly accessible regions in target mRNAs, 2) screen sets of PTGS agents in cultured cell expression systems to identify lead candidates, and 3) optimize polynucleotides that can facilitate a reduction in *RHO* and *RPE65* mRNA. The screening procedure is described in US patent publication no. 20080227103, the entire disclosure of which is incorporated herein by reference.

In the present invention, we achieved highly potent ribozyme and shRNA PTGS agents against *RHO* mRNA (90% suppression). For *RPE65*, substantial and significant target suppression was achieved.

Human rod opsin mRNA (transcribed from the *RHO* gene) has the following sequence:

AGAGUCAUCCAGCUGGAGCCCUGAGUGGCUGAGCUCAGGCCUUCGCAGCAUUCUUGGGUG
GGAGCAGCCACGGGUCAGCCACAAGGGCCACAGCCAUGAAUGGCACAGAAGGCCCUAACU
UCUACGUGCCCUUCUCAAUGCGACGGGUGUGGUACGCAGCCCCUUCGAGUACCCACAGU
ACUACCUGGCUGAGCCAUGGCAGUUCUCCAUGCUGGCCGCCUACAUGUUUCUGCUGAUCG
UGCUGGGCUUCCCCAUACAUCUCCUACGCUCUACGUCACCGUCCAGCACAAGAAGCUGC
GCACGCCUCUCAACUACAUCUCCUACAACCUAGCCGUGGCUGACCUCUUAUGGUCCUAG
GUGGCUUACCCAGCACCCUCUACACCUCUCUGCAUGGAUACUUCGUCUUCGGGCCACAG
GAUGCAAUUUGGAGGGCUUCUUUGCCACCCUGGGCGGUGAAAUGCCCUGUGGUCCUUGG
UGGUCCUGGCCAUCGAGCGGUACGUGGUGGUGUGUAAGCCCAUGAGCAACUCCGCUUCG
GGGAGAACCAUGCCAUCAUGGGCGUUGCCUUCACCUUGGGUCAUGGCGCUGGCCUGCGCCG
CACCCCCACUCGCCGGCUGGUCCAGGUACAUCCCCCGAGGGCCUGCAGUGCUCGUGUGGAA
UCGACUACUACAGCUACAAGCCGAGGUACAACAGAGUCUUUUGUCAUCUACAUGUUCG
UGGUCCACUUCACCAUCCCCAUGAUUAUCAUCUUUUCUGCUAUGGGCAGCUCGUCUUA
CCGUCAAGGAGGCCGUGCCAGCAGCAGGAGUCAGCCACCACACAGAAGGCAGAGAAGG
AGGUCACCCGCAUGGUCAUCAUCAUGGUCAUCGCUUCCUGAUCUGCUGGGUGCCCUACG
CCAGCGUGGCAUUCUACAUCUACCCACCAGGGCUCCAACUUCGGUCCCAUCUUAUGA
CCAUCCCAGCGUUCUUUGCCAAGAGCGCCGCCAUCUACAACCCUGUCAUCUAUAUCAUGA
UGAACAAAGCAGUUCGGAACUGCAUGCUCACCACCAUCUGCUGCGGCAAGAACCACUGG
GUGACGAUGAGGCCUCUGCUACCGUGUCCAAGACGGAGACGAGCCAGGUGGCCCCGGCCU
AAGACCUGCCUAGGACUCUGUGGCCGACUAUAGGCGUCUCCCAUCCCCUACACCUUCCCC
CAGCCACAGCCAUCCCACCAGGAGCAGCGCCUGUGCAGAAUGAACGAAGUCACAUAGGCU
CCUUAUUUUUUUUUUUUUUUAAGAAUAUAUUAUGAGGCUCUCACUACCUGGGACA
GCCUGAGAAGGGACAUCACCAAGACCUACUGAUCUGGAGUCCACGUUCCCCAAGGCCA
GCGGGAUGUGUGCCCCUCCUCCUCCAAUCUAUCUUUCAGGAACACGAGGAUUCUUGCUU
UCUGGAAAAGUGUCCAGCUUAGGGAUAAGUGUCUAGCACAGAAUGGGGCACACAGUAGG
UGC UAAUAAAUGCUGGAUGGAUGCAGGAAGG (SEQ ID NO:1).

The sequence of mouse rod opsin mRNA is as follows:

1 CGUCAGUGGC UGAGCUCGCC AAGCAGCCUU GGUCUCUGUC UACGAAGAGC CCGUGGGGCA
61 GCCUCGAGAG CCGCAGCCAU GAACGGCACA GAGGGCCCCA AUUUUUUUGU GCCCUUCUC
121 AACGUCACAG GCGUGGUGCG GAGCCCCUUC GAGCAGCCGC AGUACUACCU GGCGGAACCA

181 UGGCAGUUCU CCAUGCUGGC AGCGUACAUG UUCCUGCUCA UCGUGCUGGG CUUCCCCAUC
 241 AACUUCUCA CGCUCUACGU CACCGUACAG CACAAGAAGC UGCGCACACC CCUCAACUAC
 301 AUCCUGCUCA ACUUGGCCGU GGCUGACCUC UUCAUGGUCU UCGGAGGAUU CACCACCACC
 361 CUCUACACA CACUCCAUGG CUACUUCGUC UUUGGGCCCC CAGGCUGUAA UCUCGAGGGC
 5 421 UUCUUUGCCA CACUUUGAGG UGAAAUCGUC CUGUGGUCCC UGGUGGUCCU GGCCAUUGAG
 481 CGCUACGUGG UGGUCUGCAA GCCGAUGAGC AACUUCGCU UCGGGGAGAA UACGCUAUC
 541 AUGGGUGUGG UCUUCACCUG GAUCAUGGCG UUGGCCUGUG CUGCUCUCCC ACUCGUUGGC
 601 UGGUCCAGGU ACAUCCUGA GGGCAUGCAA UGUUCAUGCG GGAUUGACUA CUACACACUC
 661 AAGCCUGAGG UCAACAACGA AUCCUUUGUC AUCUACAUGU UCGUGGUCCA CUUCACCAUU
 10 721 CCUAUGAUCG UCAUCUUCUU CUGCUAUGGG CAGCUGGUCU UCACAGUCAA GGAGGCGGCU
 781 GCCCAGCAGC AGGAGUCAGC CACCACUCAG AAGGCAGAGA AGGAAGUCAC CCGCAUGGUU
 841 AUCAUCAUGG UCAUCUUCUU CCUGAUCUGC UGGCUUCCCU ACGCCAGUGU GGCCUUCUAC
 901 AUCUUCACCC ACCAGGGCUC CAACUUCGUC CCCAUUCCA UGACUCUGCC AGCUUUCUUU
 961 GCUAAGAGCU CUUCCAUCUA UAACCCGUC AUCUACAUCU UGUUGAACAA GCAGUUCGCG
 15 1021 AACUGUAUGC UCACCACGCU GUGCUGCGGC AAGAAUCCAC UGGGAGAUGA CGACGCCUCU
 1081 GCCACGCUU CCAAGACGGA GACCAGCCAG GUGGCUCAG CCUAAGCCUG GCCAGAGACU
 1141 GUGGCUGAAA GUAGGAGUCU CCUGUCCCCA CACACCCCAG CCACAGCCCC CACCAGGAGC
 1201 AGCACCCGUU GGAUGAGGU CAUGCAGGCU CCCUCAGUGU UCUUUUCUUU GUUUUUAUUG
 1261 AAUUCAGAA AGCAAAUUGA GGCUCUCCAC UCAAUGGGGA CAGCUUGACA AAGGGCAUCC
 20 1321 AUCCACCAAG ACCAUCCUCA ACCUGAGGUC CCCAAUUCU GGGGGGCCAG CGGUAUCUGU
 1381 ACCCUUCCCU CAGCUUGUCU AUCAGGAACA UGACAAGUGU CCCGGCUUAG GGCUAAAUGU
 1441 CUAGGACAGA AUGGAACACA UAGUAGCUGA UUAUAAAUG CUAGCUGGAU GAAGGGAGGA
 1501 AUGAGUGACU GACUGAGUGG AUUAUAGAGU GAAGGGAUUA AUGGAAGGGA ACAUGGAUGU
 1561 CCUCAGGUGC CCAACCUGGC AGAUCCAGUC AUGUCUGGCU GGAAUCUAUA AGCAGUUUUA
 25 1621 CAUACCUGCC CUGGUUUUCU CUGCCCCAC CCCCACCCA GUUGGAUCUC CCAAUCCAG
 1681 GGCCUGAUA GAAUUGGCU GCUUCAAGA CAGAGAGAUG AGGGGAGGGA GGGGGGAGGG
 1741 AGAGAGGAG GGAGGGAGAC ACAGAGAGG AAUAUGUGUG AUGCGUGUGU AUGUGUGUUA
 1801 GUGUGUGUGU AAACACUUUG UAUUAAAAGA GUACAGCUGG UAGUUAUGUU ACAAGUAACA
 1861 CCGACUAAUA UAAUUAUUA ACCAUCCUAA UGGUCUCUGC UUGUUAGUGA CUGCUUGGGA
 30 1921 AUUAGGCAGG GCCCAAGCAC UCAGAUAAAG UAUUUCUCC AGCCUCAGUA GGCUUUUGCA
 1981 AAUGACCCAG GCUUCAGGC CUGUGCAGGG CUAGAGCUGG AUUACAGAGA UAAAUGACAG
 2041 UGACAGCAAC GUGAGCUGCA GCUUUAAGGA CUGAGAAAGC AUCGAGACCA GGGGUCUCCG
 2101 GCAAGGCCUA GUUCCUCCU UCAGUAUGGA AACCUUGCCU CAUGUCUCUC AGCCUCCUUG
 2161 GCCUGGGAG AUCCAGCCU UCCUCUUGG UUCUGGAUAC AUUUGCUCU CUACACCAGC
 35 2221 AACCAAGUGG CAACAGUUC AGGCCAGUAU GGAGUUUUG AAGCCAUGCC AAUAUGCCCA
 2281 CCUUCAGGGA GCAGCUGAGU CCUUGAUGCC ACCCUUGUUC UGAAGAGUUC AGAAACACAG
 2341 UGCAAGACAU GACCAGGCCU CAUCCUAGG AUGCUCAUGG AUCCAGUUCU UAGCUCUCCU
 2401 GUUGGAUAUG CUGUUUUCU UGGCCUUUG UCUUUUCUU AUCCAGAGG GUUUUGGCUU
 2461 UAAGGCCAAC AGGAACUAUG GGUUACCAGA AUUGAGCAGC CUCAGUCUGC AUCCUCCUC
 40 2521 UAUAGAACCA CAGCUGGGCC CUCAGCAGG CCAACUCUGC AUGGGGACAG AGGCAUUAUA
 2581 AGCUCAGCUC CUACACUUGG UGCAGUGGU GGUCUGUUGC UCUAAGCUC UUUCAAAUG
 2641 GAUGGAAACU GGGACGCUUC CCUGACCCU GGUUAUGAAA GACUAGACUG UGUGGGGACA
 2701 AACAGUCCAG AGUCCCGGGG AAUGUGAUAG AGCAGCUCCA UCAUUUUUAG AAACCCAAU
 2761 UGAGGCAGUA UAGAGAGAUG GUGACCUCUA UAAGCCUCUG UAUCUGCAAA GAGGAGCUUA
 45 2821 GACCUGCCCU UGAGGGGAU AUUAUGAGAU UAAGGGACU AUGUGGCCAG CCUACUCCU
 2881 GGCAUGCUGA AGACAUUGG ACACUCUGGU AUUCUAGACC UUGGCUCAGA GCUGCCUUA
 2941 CUAGGAUACU GUCACUAGC AAAAGAAUGG GAUGGAGCCU CAGAUGUGGA GUGACACCAU
 3001 CUCCAAGAA GGAAAGGGUG CCAGGGUCUG GGAUGAAAGC CCUUGGUGC UAUGUUGGGC
 3061 AAGGGCGAGU GCCAGCAAGG GGUUAUUUGC UUGCUCUCUC CAUCAGUGAU GAGGUUCCAU
 50 3121 UUGGUCACAA GAAAUUACC CCAAUUGCUG AAACAGAGGC UGACUAUUG CUUAUAGGCA
 3181 UGAAACCCCA CUCCCUCCA CUUCAGGCUG GCUAGAUUA AAGCUCAGAC CUGUGAAAAA
 3241 AAAAAAAAA (SEQ ID NO:2)

Polynucleotides suitable for use in the present invention also include the following:

55

Stabilized 6bp hhRz sequences

Rho 725

5'-UGAAGUG CUGAUGAGCGGUCUUCGGACCGCGAA ACCUCGU-3'(SEQ ID NO:3)

60

Rho 731

5'-GGAUGGU CUGAUGAGCGGUCUUCGGACCGCGAA AAGUGGA-3' (SEQ ID NO:4)

Rho 266

5 5'-AGAGCGU CUGAUGAGCGGUCUUCGGACCGCGAA AGGAAGU-3' (SEQ ID NO:5)

Classical 4bp HH16 hhRz sequences

Rho 725-HH16

10 5'-UGAAGUG CUGAUGAGGCCGAAAGGCCGAA ACCUCGU-3' (SEQ ID NO:6)

Rho 731-HH16

5'-GGAUGGU CUGAUGAGGCCGAAAGGCCGAA AAGUGGA-3' (SEQ ID NO:7)

Rho 266-HH16

5'-AGAGCGU CUGAUGAGGCCGAAAGGCCGAA AGGAAGU-3' (SEQ ID NO:8)
15

Mini 2bp hhRz sequences

Rho 725-mini

5'-UGAAGUG CUGAUGAGCUUUUGCGAA ACCUCGU-3' (SEQ ID NO:9)

Rho 731-mini

20 5'-GGAUGGU CUGAUGAGCUUUUGCGAA AAGUGGA-3' (SEQ ID NO:10)

Rho 266-mini

5'-AGAGCGU CUGAUGAGCUUUUGCGAA AGGAAGU-3' (SEQ ID NO:11)

Tertiary modified hhRz sequences / SM1 (Schistosoma Mansoni tertiary elements)

25 **Rho 725-SM1** 5'-UGGU GUAC GAAGUG CUGAUGAGUCCCCAAAUAGGACGAA ACCUC-3 (SEQ ID NO:12)

Rho 731-SM1 5'-UGGG GUAC GAUGGU CUGAUGAGUCCCCAAAUAGGACGAA AAGUG-3 (SEQ ID NO:13)

Rho 266-SM1

30 5'-CGUA GUAC GAGCGU CUGAUGAGUCCCCAAAUAGGACGAA AGGAA-3' (SEQ ID NO:14)

RzB (Peach latent mosaic viroid (PLMVD) tertiary elements)

Rho 725-RzB 5'-UGGUG UAA AAGUG CUGAUGAGUCGCGUGGGAUGCGACGAA ACCUCGU-3' (SEQ ID NO:15)

Rho 731-RzB 5'-UGGGG UAA AUGGU CUGAUGAGUCGCGUGGGAUGCGACGAA AAGUGGA-3' (SEQ ID NO:16)

5 Rho 266-RzB

5'-CGUAG UAA AGCGU CUGAUGAGUCGCGUGGGAUGCGACGAA AGGAAGU-3' (SEQ ID NO:17)

The following sequences comprise hammerhead ribozyme and RNAi target sites for human *RHO* mRNA

10 A. Hammerhead Ribozyme Human *RHO* 725 GUC↓ Target Site and hhRz Sequence

1) GUC↓ 725 Target Sequence in Human *RHO*: 5'-UCGUGGUC↓CACUUCA-3' (SEQ ID NO:18)

The "C" of GUC↓ is position 725 in the human *RHO* mRNA sequence.

2) The 725 GUC↓ hhRz sequence is as follows:

15 3'-AGCACCAAAGCCGGAAAGCCGGAGUAGUCGUGAAGU-5' (SEQ ID NO:19***

USE THIS IN SEQ ID for 5'-6' direction

UGAAGUGCUGAUGAGGCCGAAAGGCCGAAACCACGA (SEQ ID NO:19)

20 The underlined antisense (in 2) flank sequences anneal to the respective underlined regions of the target sequence listed above (in 1). The center region of the hhRz sequence that is between the underlined antisense sequences is the core catalytic enzyme and Stem II (HH16). The hhRz RNA is written from the 3' to the 5' position in the way that it would anneal to the target sequence in *RHO*.

B. shRNA (RNAi) 725 Human *RHO* Target Site and shRNA Sequence:

25 1. Rhoi725 Target sequence in Human *RHO* is as follows:

5'-UGUUCGUGGUCCACUUCAC-3' (SEQ ID NO:20)

This sequence forms an shRNA hairpin (Figure 9) to attack Human *RHO* 725 site.

This shRNA was expressed from an H1 promoter in pSUPER vector (OligoEngine).

5 The sequence shown in Figure 9 is SEQ ID NO:21. CHANGED FROM 20

The sequence of human RPE65 mRNA is as follows. Sequences shown in bold and italics represent RPE65 shRNA targets identified according to the invention. Novel shRNAs that attack these target sequences are presented in Figures 5-8.

10

1UCCUUCUUCAUUCUGCAGUUGGUGCCAGAACUCUGGAUCCUGAACUGGAAGAAAAUGUCU
 61AUCCAGGUUGAGCAUCCUGCUGGUGGUACAAGAAACUGUUUGAAACUGUGGAGGAACUG
 121UCCUCGCCGCUCACAGCUCAUGUAACAGGCAGGAUCCCCCUCUGGCUCACCGGCAGUCUC
 181CUUCGAUGUGGGCCAGGACUCUUUGAAGUUGGAUCUGAGC**CAUUUUACCACCGUUUGAU**
 15 241GGGCAAGCCCUCUGCACAAGUUUGACUUUAAAGAAGGACAUGUCACAUACCACAGAAGG
 301UUCAUCCGCACUGAUGCUUACGUACGGGCAAUGACUGAGAAAAAGGAUCGUCAUAACAGAA
 361UUUGGCACCUGUGCUUUCCAGAUCCUGCAAGAAUUAUUUUUCCAGGUUUUUUUCUUC
 421UUUCGAGGAGUAGAGGUUACUGACAAUGCCCUUGUUAUUGUCUACCCAGUGGGGGAAGAU
 481UACUACGCUUGCAGAGACCAACUUUAUUACAAAGAUU**AAUCCAGAGACCUUGGAGACA**
 20 541AUUAAGCAGGUUGAUCUUUGCAACUAUGUCUCUGUCAAUGGGGCCACUGCUCACCCCCAC
 601AUUGAAAAUGAUGGAACCGUUUACAUAUUGGUAAUUGCUUUGGAAAAAAUUUUUCAAUU
 661GCCUACAACAUUGUAAAGAUCACCACUGCAAGCAGACAAGGAAGAUCCAAUAAGCAAG
 721UCAGAGAUUGUACAAUUCUCCUGCAGUGACCGAUUCAAGCCAUCUUACGUUCAUAGU
 781UUUGGUCUGACUCCCAACUAUAUCGUUUUUGUGGAGACACCAGUCAAAAUAACCUGUUC
 25 841AAGUCCUUUUUUGAUGGAGUCUUUUGGGGAGCCAACUACAUGGAUUGUUUGUACUCCAAU
 901GAAACCAUGGGGUUUGGCUUACAUAUUGCUGA**CAAAAAAGGAAAAAGUACC**CUCAAUAU
 961AAAUACAGAACUUCUCCUUUCAACCUCUCCAUCACAUAACACCUAUGAAGACAAUGGG
 1021UUUCUGAUUGUGGAUCUCUGCUGCUGGAAAGGAUUUGAGUUUGUUUAUAAUUAUUUAU
 1081UUAGCCAAUUUACGUGAGAACUGGGAAGAGGUGAAAAAAAUGCCAGAAAGGCUCUCCCAA
 30 1141CCUGAAGUUAGGAGAUUGUACUCCUUUGAAUAUUGACAAGGCUGACACAGGCAAGAAU
 1201UUAGUCACGCUCUCCCAAUACAACUGCCACUGCAAUUCUGUGCAGUGACGAGACUAUCUGG
 1261CUGGAGCCUGAAGUUCUCUUUUCAGGGCCUCUGCAAGCAUUUGAGUUUCCUCAAUCAAU
 1321UACCAAGAUUGUGGGAACCUUACACAUAUGCGUAUGGACUUGGCUUGAAUCACUUU
 1381GUUCAGAUAGGCUCUGUAAGCUGAAUGUCAAAACUAAAGAAACUUGGGUUUGGCAAGAG
 35 1441CCUGAUUCAUACCCAUCAGAACCAUCUUUGUUUCUACCCAGAUUGCCUUGGAAGAAGAU
 1501GAUGGUGUAGUUCUGAGUGUGGUGGUGAGCCCAGGAGCAGGACAAAAGCCUGCUUAUCUC
 1561CUGAUUCUGAAUGCCAAGGACUUAAGUGAAGUUGCCCGGGCUGAAGUGGAGAUUAAACUC
 1621CCUGUCACCUUUAUGGACUGUUCAAAAAUCUUGAGCAUACUCCAGCAAGAUUAGUUUU
 1681UGGUAGCAAAACUGAGAAAAUCAGCUUCAGGUCUGCAAUCAAUUCUGUUCAAUUUUAGC
 40 1741CUGCUAUAUGUCAUGGUUUUAACUUGCAGAUUGCACAAUUAUUGCAAUGUUUUACAGAAA
 1801GCACUGAGUUGAGCAAGCAAUUCCUUUAUUUAAAAAAGUACGUUUUUAGAUAAUUA
 1861UACUUCUUCUGUGAGACAGGCCAUAAACUGAAAAACUCUUAUUUAUUUAGCAAUCAAUAG
 1921GAAAUGAUGUGGACUUACUAAAUGGCUUUUAAUCCUUAUUUAAGAGCAUAAUUUAGGU
 1981ACCUAUCUGCUCCAAUUAUUAUUUUUAACAUUUAAAAACCAAAGUCCUCUACACUUGAUUU
 45 2041UAUUUAUUGUGGCUUUGCUGAGUCAAGGAAGUAUCAUGCAAUAAGGCUUAAUUACUAAA
 2101UGUCAAA**CCAAACUUUUUCUCAAACC**AGGGACUAUCAUCUAAGAUUAAUUACAGUAAUUA
 2161UUUUGCGUAUACGUAAACUGCUCAAAGAUUAUGAAUCUUAUGAAUGUUAACCUUCCGUUU
 2221AUUACAAGCAAGUACUAUUUUUCUGAUUUUAUAAUAAAGAAAAUCUGUGUUUUAUCAACU
 2281GAGGCCUCUCAACCAAAUAAACAUUCACAGAGAUUAAAGUUUAUUAUUAAAAAGCUUAUGUAA
 50 2341AUAAAAGCAAGUACAUAUAGUAGUGACUAUAUUUAAAAAAACAGCAUAAAAUGCUUAAAA
 2401AUGUAAUUAUUUACUAAAAUCAGAUUAUGGGAUAAUGUUGCAGGAUUAUACUUUAUUGCAU
 2461CUUUUUUGUUUAAUUGUAUUUAAGCAUUGUGCAAUCACUUGGGAAAAAUUUAAUUUAUU
 2521AACAUUGAGGUAAUUAUACAUUUUAAGCCUUUUUGUUUUUAAUUUUCUUUUCUCCAGAGA
 2581UUGUUUAAAAUAAUUAUUGACAAAAAU (SEQ ID NO:22)

55

Mouse RPE65 mRNA has the following sequence:

```

1 UCCUCAUCCU ACAGCUGGUA CCAGAACUCU CUCUAAUCUU CACUGGAAGA AAAUGUCUAAU
61 CCAAAUUGAA CACCCUGCUG GUGGCUACAA GAAACUAAUU GAAACUGUGG AGGAACUGUC
5 121 CUCACCACUA ACAGCUCAUG UCACAGGCAG GAUUCCCCUC UGGCUCACUG GCAGUCUCCU
181 CCGAUGUGGG CCAGGGCUCU UUGAAGUUGG AUCUGAGCCU UUCUAUCACC UGUUUGAUGG
241 ACAAGCCCUCU UUGCACAAGU UUGACUUCAA GGAGGGCCAU GUCACAUACC ACAGAAGAUU
301 CAUCCGCACU GAUGCUGAUG UUCGAGCAAU GACUGAGAAG AGGAUUGUCA UACAGAAAUU
10 361 UGGCACCUGU GCUUCCCCAG ACCCCUGCAA GAAUAUAAUU UCCAGGUUUU UUUCUUACUU
421 UAAAGGAGUA GAGGUUACUG ACAAUUGCCC UGUAAAUAUC UACCCAGUGG GAGAAGAUUA
481 CUAUGCAUGC ACAGAGACCA ACUUUAUCAC AAAGAUUAAC CCAGAGACCU UGGAGACAAU
541 UAAGCAGGUU GAUCUUUGCA ACUAUAUUUC UGUCAAUGGU GCCACUGCUC AUCCACAUAU
601 UGAAAGUGAU GGAACAGUUU ACAACAUUGG GAAUUGCUUU GGAAAAAAUU UUACAGUUGC
661 CUACAACAUU AUUAAGAUGC CUCCACUGAA AGCAGACAAG GAAGAUCCAA UAAACAAGUC
15 721 AGAAGUUGUU GUGCAGUUC CCUGCAGUGA UCGUUUCAAG CCAUCUUAUG UACACAGUUU
781 UGGUCUGACU CCCAACUAUA UCGUUUUUGU GGAGACUCCA GUCAAAAUUA ACCUUUUCAA
841 GUUUCUUGU UCGUGGAGUC UUUGGGGAGC CAACUACAUG GACUGUUUCG AGUCCAAUGA
901 AAGCAUGGGG GUUUGGCUUC AUGUUGCUGA UAAAAAAGA AGAAAAUACU UCAAUAACAA
961 AUACAGGACU UCCCCUUAU AUCUCUCCA UCAUAUCAAU ACUUAUGAAG ACAAUUGGAU
20 1021 UCUGAUUGUG GAUCUCUGU GCUGGAAAGG GUUUGAAUUU GUUUAUAAUU ACUUAUAAUU
1081 AGCCAAUUUA CGUGAGAAUU GGGAAGAAGU UAAAAAGAAU GCUAUGAAGG CUCCUCAGCC
1141 UGAAGUCAGG AGAUUGUAC UUCUUUGAC AAUUGACAAG GUCGACACAG GCAGAAAUUU
1201 AGUCACACUG CCCCAUACAA CUGCCACAGC CACUCUGCGC AGUGAUGAGA CCAUAUGGCU
1261 GUAACUGAG GUUCUCUUU CAGGGCCUCG UCAAGCCUUU GAAUUUCCU AAUCAAUUA
25 1321 CCAGAAAUUU GGAGGGAAAC CUUAUACUUA UGCAUACGGA CUUGGGUUGA AUCACUUUGU
1381 UCCUGACAAG CUCUGUAAGA UGAACGUCAA AACUAAAGAA AUCUGGAUGU GGCAAGAGCC
1441 AGAUUCUUAC CCAUCUGAAC CCAUCUUUGU UUCUCAACCA GAUGCUCUGG AAGAAGAUGA
1501 UGGUGUGGUU CUGAGUGUGG UGGUGAGCCC UGGGGCAGGG CAAAAGCCUG CAUAUCUCCU
1561 GGUUCUGAAU GCCAAAGACU UGAGUGAAAU UGCCAGGGCU GAAGUGGAGA CUAUAUCCU
30 1621 UGUGACCUUC CAUGGACUGU UCAAAAGAUC CUGAACAUU UCCAGAGAUG GCUCAGCAGU
1681 ACAACACUGA CUGCCUUCU ACAGAUCGUG UGUUCAAUUC CCAAAGAUCA CCUGGUGACU
1741 CACUCCAUC UGAGACGGAU UCCAUGCCC UCUUAUGCUG UUUCUGAAGA CAGCAAAAGU
1801 GUACUCAUUA AUACAUAUGA UAAUAAAUC UUUAAAAAAA ACAAAAAAAA AAAAAAAAAA
1861 AA (SEQ ID NO:23)
35

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Figure 4 provides mouse *RPE65* Hammerhead Ribozyme Sequences identified in performance of the present invention.

Figures 5-8 provide shRNAs that target human RPE65.

In general, the foregoing HhRz and shRNA polynucleotides capable of

40 facilitating a reduction in *RHO* or *RPE65* target mRNA were developed against accessible regions of *RHO* and *RPE65* mRNA regions we identified using a variety of techniques. In particular, full length mRNAs for human *RHO* mRNA, and human and mouse *RPE65* mRNAs were archived from GenBank. RNA secondary structure folding was conducted with MFold (vers. 3.2), SFold and OligoWalk algorithm (RNAstructure,

45 vers 4.5, Mathews, 1999, 2006). Independent sampling outputs were convolved into a final statistical predictor of accessibility which we call multiparameter prediction of RNA accessibility (mppRNA). Local regional accessibility was compared statistically between the human and mouse *RHO* targets for the different algorithms by parametric t-tests at standard criterion significance ($p < 0.05$). Transfection experiments evaluating

50 knockdown by shRNA and ribozyme vectors and controls were subject to one-way

ANOVA to evaluate the null hypothesis of equivalent means. *Post-hoc* t-tests were used to evaluate differences between samples and controls and between samples.

hhRz were expressed within an engineered adenoviral VAI chimera RNA. HhRzs against accessible and inaccessible regions were tested in a 96-well screening assay where the target was the dicistronic mRNA, *RHO*-IRES-SEAP, that expressed both *RHO* and *SEAP* proteins (HEK293S-*RHO*-IRES-SEAP cells). SEAP is secreted from live cells and measured in a fluorescence enzyme assay. shRNAs were cloned into pSUPER (Oligoengine). Maximum knockdown in this assay is expected at 50%. A range of knockdown efficacies were identified. The most efficient hhRz was a polynucleotide targeting the 725 GUC site. The most potent PTGS agent is an shRNA which suppresses 92% of *RHO* mRNA and 83% of *RHO* protein (see Figures 1 and 2a and 2b). There are substantial differences between the mean KD (knock-down) levels by hhRzs targeting common accessible regions (e.g. the 250 region embracing 250, 257, 263, 266, 272, 274; 725 region embracing 725, 730, 731, 737). HhRzs targeting inaccessible sites (control) (e.g. hhRz GUC↓ 785) exerted no KD. Three hhRzs promoted the greatest amount of SEAP protein KD from the *RHO*-IRES-SEAP dicistronic expressing cell line (266, 725, 731).

In separate studies, accessible regions in mouse *RPE65* mRNA were predicted, and hhRz were constructed and tested using cotransfection of a VAI-hhRz expression plasmid and *RPE65* expression construct in HEK293S cells (Fig. 3).

For cell culture and transfection, suspension adapted Human Embryonic Kidney cells (HEK-293S) were maintained in Dulbecco's Modified Eagle's Medium/F-12 nutrient mix (DMEM/F12) with 10% (v/v) heat inactivated calf serum and antibiotics. In transient transfections cells in 24 well plates were co-transfected (Lipofectamine 2000, InVitrogen) with 150 ng of CMV expression plasmids for human *RHO* or mouse *RHO*, and 5 µg of shRNA or hammerhead ribozyme expression plasmids per well. Control ribozyme vectors had a nonspecific adapter in place of the ribozyme sequence and control shRNA vectors had an irrelevant scrambled sequence. After 48 hrs cells were harvested, total RNA was purified (RNAeasy, Qiagen), and first strand cDNA was synthesized, or cells were extracted for western analysis.

For SEAP Assays: 50µL of conditioned cell culture media was transferred to separate wells in black-walled 96 well plates and incubated at 65°C for 30 minutes. After cooling samples to room temp., 45µL of Diethanolamine Assay Buffer (pH 9.8,

2mM MgCl₂, 1mM L-Homoarginine) was added per well. 5μL of 4-methyl umbelliferyl phosphate (4-MUP) fluorescent substrate was added to each well for a final concentration of 0.04mM per well, and fluorescence (480nm excitation/530nm emission) assayed on a Fluoroskan FL plate reader after incubating for 55 minutes at room temperature in the dark.

For Real-Time Quantitative RT-PCR, total RNA was purified from transfected cells with RNeasy kit (Qiagen) and treated with TURBO DNase (Ambion). cDNA synthesis was performed using 500ng of total RNA with the AffinityScript Reverse Transcriptase system (Stratagene) using the supplied oligo(dT) primers. Quantitative PCR for human Rho mRNA was performed in a Smart Cycler II (Cepheid) thermocycler. Primers that spanned adjacent exons and a probe primer containing FITC at the 5' end and a quenching dye at the 3' end were designed using primer quest software (IDT).

For quantitative analysis, transfection experiments evaluating knockdown by shRNA and ribozyme vectors and controls were subject to one-way ANOVA to evaluate the null hypothesis of equivalent means. *Post-hoc* t-tests were used to evaluate differences between samples and controls and between samples.

While the invention has been illustrated by specific working embodiments, those skilled in the art will recognize that minor modifications can be made without deviating from the spirit of the invention.

We claim:

1. A method for therapy of macular degeneration comprising administering to an individual in need of therapy for the macular degeneration a composition comprising:
 - i) a first polynucleotide that can facilitate a reduction in the amount of rod opsin (*RHO*) mRNA in the individual; or
 - 5 ii) a second polynucleotide that can facilitate a reduction in the amount of RPE65 mRNA in the individual; or
 - iii) a combination of i) and ii);wherein the macular degeneration in the individual is inhibited subsequent to the
10 administration.
2. The method of claim 1, wherein the first polynucleotide or the second polynucleotide is a hammerhead ribozyme.
- 15 3. The method of claim 1, wherein the first polynucleotide targets SEQ ID NO:19 or SEQ ID NO:21 in human *RHO* mRNA.
4. The method of claim 1, wherein the second polynucleotide comprises the sequence of SEQ ID NO:30 or SEQ ID NO:31.
- 20 5. The method of claim 1, wherein the first polynucleotide comprises the sequence of SEQ ID NO:20.
6. The method of claim 1, wherein the first polynucleotide is a ribozyme that cleaves
25 an mRNA comprising the sequence of SEQ ID NO:1, wherein the cleavage occurs between nucleotides 725 and 726 of SEQ ID NO:1.
7. The method of claim 1, wherein the macular degeneration is selected from the group consisting of dry age-related macular degeneration (dAMD), Stargardt disease,
30 Best disease, and neovascular wet age-related macular degeneration.
8. The method of claim 6, wherein the macular degeneration is dAMD.

9. The method of claim 1, wherein the first polynucleotide and the second polynucleotide are administered simultaneously.
10. The method of claim 1, wherein the first polynucleotide and the second
5 polynucleotide are administered sequentially.
11. The method of claim 1, wherein only the first polynucleotide or the second polynucleotide is administered to the individual.
- 10 12. A composition comprising a polynucleotide, wherein the polynucleotide comprises the sequence of SEQ ID NO:21, SEQ ID NO:30, or SEQ ID NO:31.
13. The composition of claim 12, wherein the the polynucleotide comprises the sequence of SEQ ID NO:21.
- 15 14. The composition of claim 12, wherein the polynucleotide comprises the sequence of SEQ ID NO:30.
15. The composition of claim 12, wherein the polynucleotide comprises the sequence
20 of SEQ ID NO:31.

Figure 1

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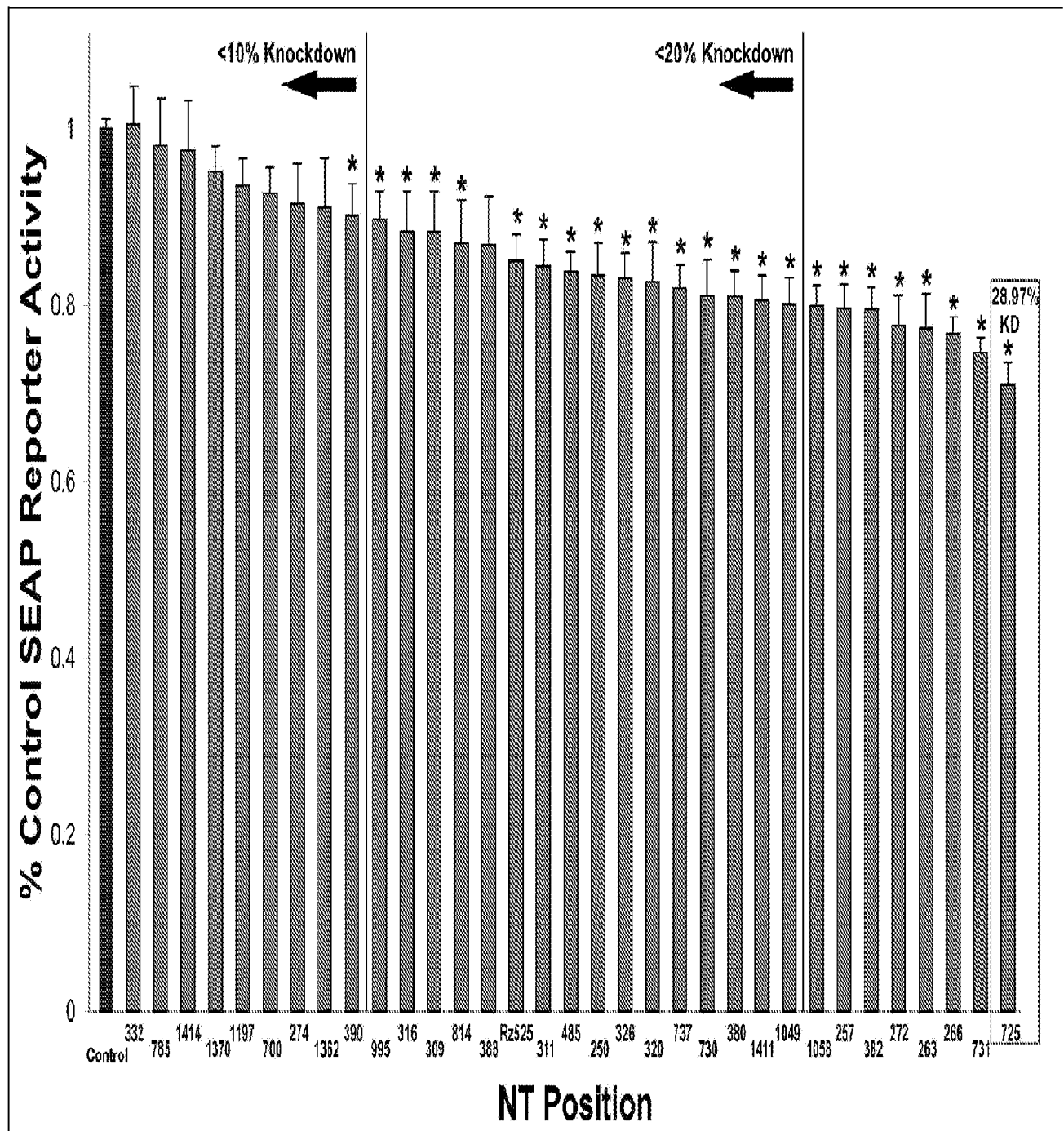


Figure 2a

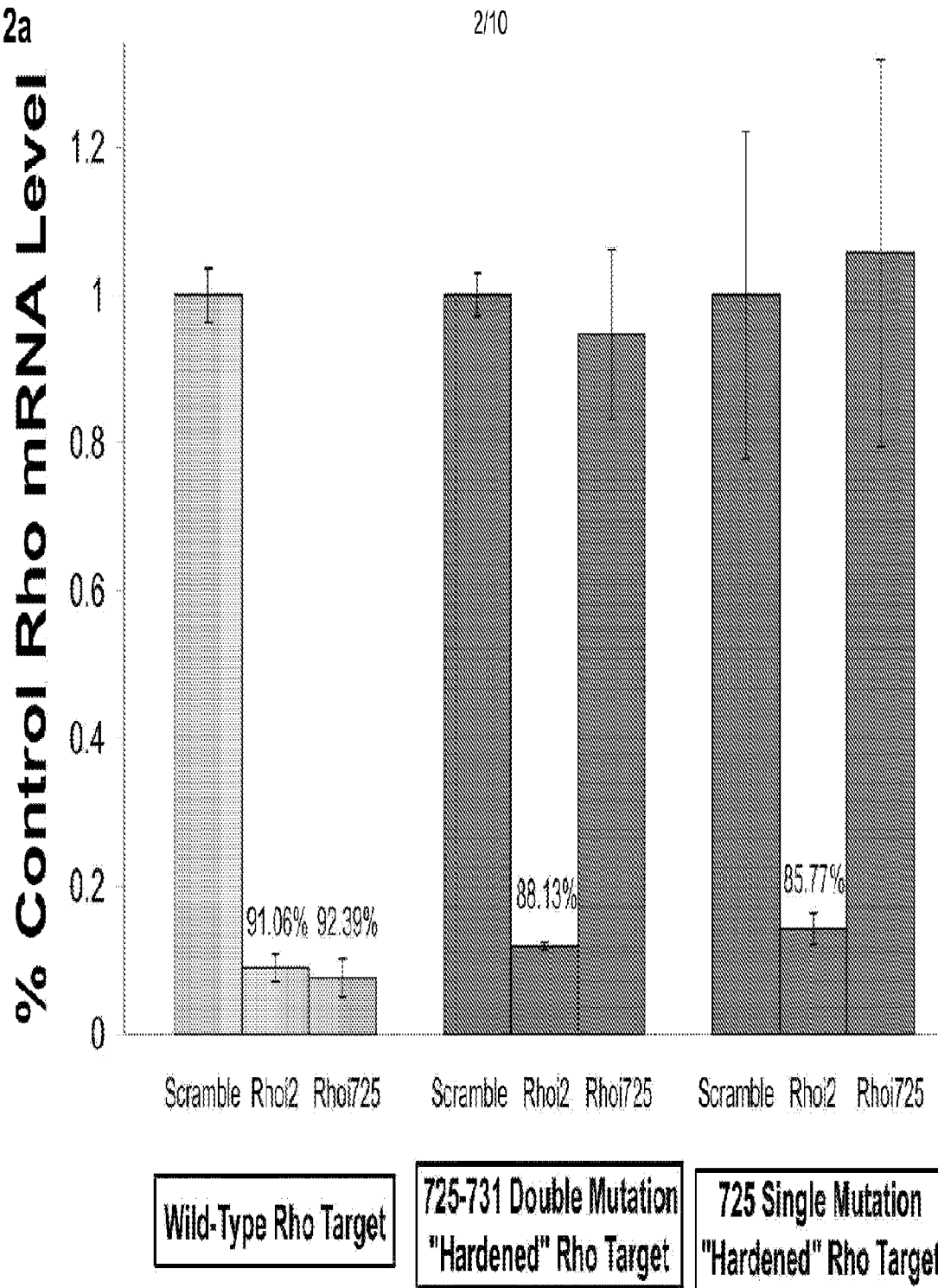


Figure 2b

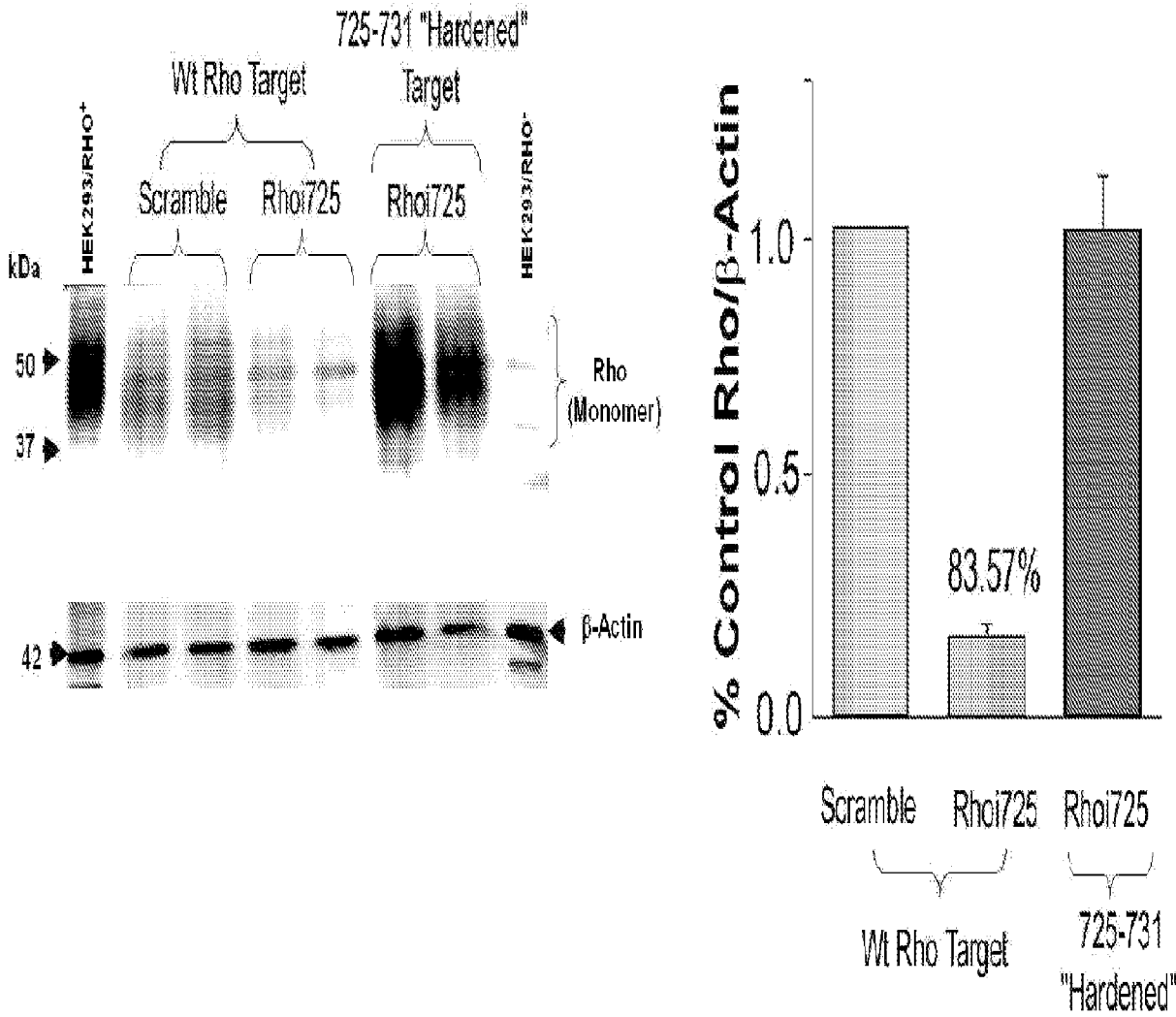


Figure 3

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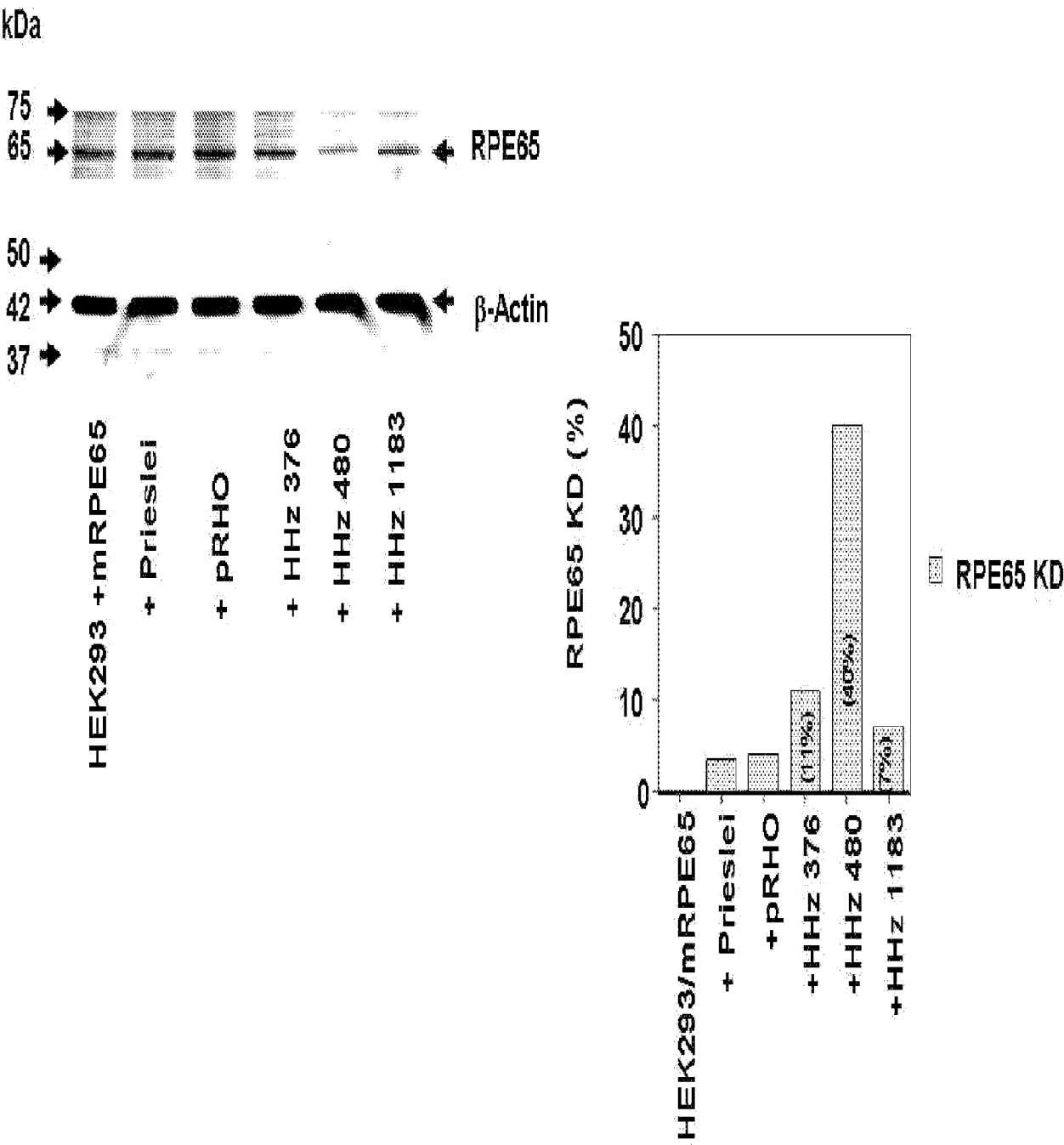


Figure 4.

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Mouse RPE65 Hammerhead Ribozyme Sequences**1. Mouse RPE65 UUC↓ 376 Hammerhead Ribozyme****5' TCGACGGTCTGGCTGATGAGGCCGAAAGGCCGAAAAAGCACCTGCA 3' (SEQ ID NO:24)****Targeted sequence (in bold below):****5' GUGCUUUC↓CCAGACC 3' (SEQ ID NO:25)****2. Mouse RPE65 UUA↓ 480 Hammerhead Ribozyme****5' TCGACTGCATAGCTGATGAGGCCGAAAGGCCGAAATCTTCCTGCA 3' (SEQ ID NO:26)****Targeted sequence (in bold below):****5' GAAGAUUA↓CUAUGCA 3' (SEQ ID NO:27)****3. Mouse RPE65 GUC↓ 1183 Hammerhead Ribozyme****5' TCGACCTGTGTCCTGATGAGGCCGAAAGGCCGAAACCTTGTCTGCA 3' (SEQ ID NO:28)****Targeted sequence (in bold below):****5' ACAAGGUC↓GACACAG 3' (SEQ ID NO:29)**

The first four nucleotides are designed to ligate into a XhoI site and the last four nucleotides are designed to ligate into a Pst I site in the vector. The underlined sequences are the core of the hammerhead ribozyme. The core sequences of the hammerhead ribozyme are flanked by antisense sequences (not underlined) that anneal to the targeting sequence in the *RPE65* mRNA.

Figure 5

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Human *RPE65* 221 shRNA:

5'- CAUUUUUACCACCUGUUUGAUUCAAGAGAUCAAACAGGUGGUAAAAUGUU-3'

(SEQ ID NO:30)

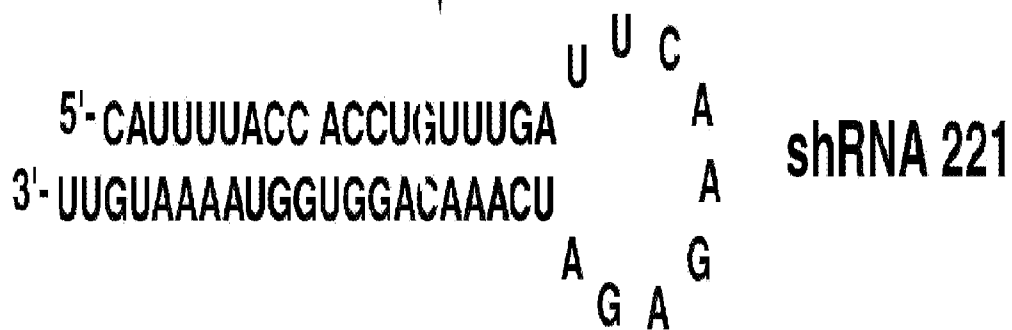


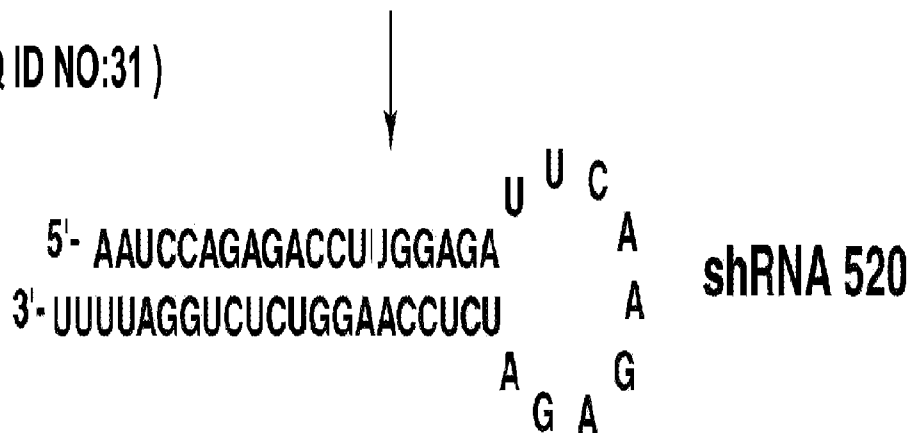
Figure 6

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Human RPE65 520 shRNA:

5'- AAUCCAGAGACCUUGGAGAUUCAAGAGAUCCUCCAAGGUCUCUGGAUUUU-3'

(SEQ ID NO:31)



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Figure 7

Human RPE65 933 shRNA:

5'-CAAAAAAAGGAAAAAGUACUUCAGAGAGUACUUUUUCCUUUUUUUGUU-3'

(SEQ ID NO:32)

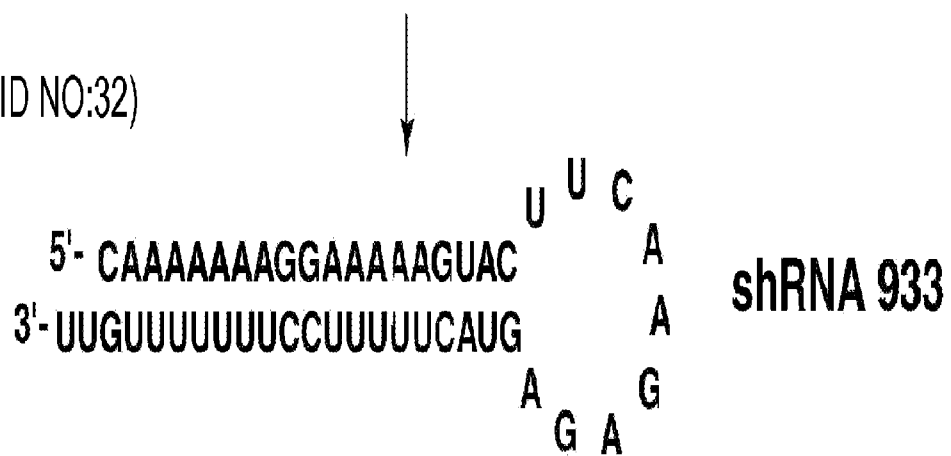


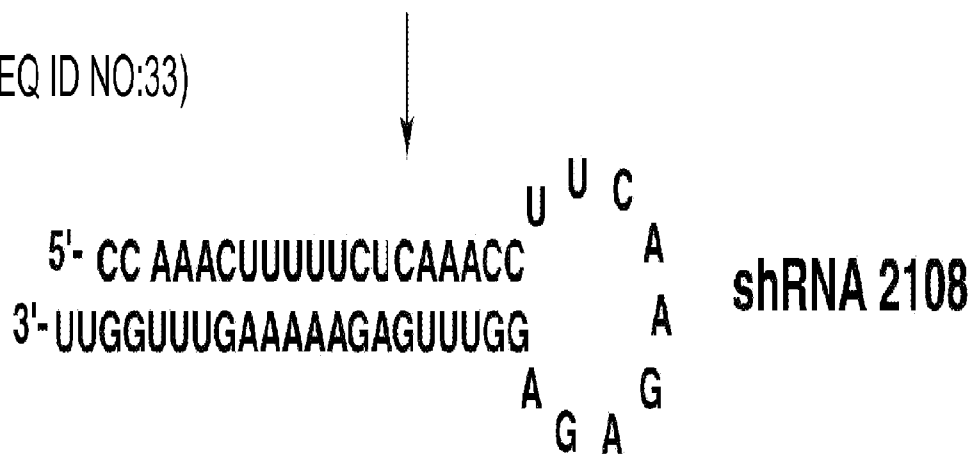
Figure 8

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Human RPE65 2108 shRNA:

5'-CCAAACUUUUUCUCAAAACCUUCAAGAGAGGUUUGAGAAAAAGUUUGGUU-3'

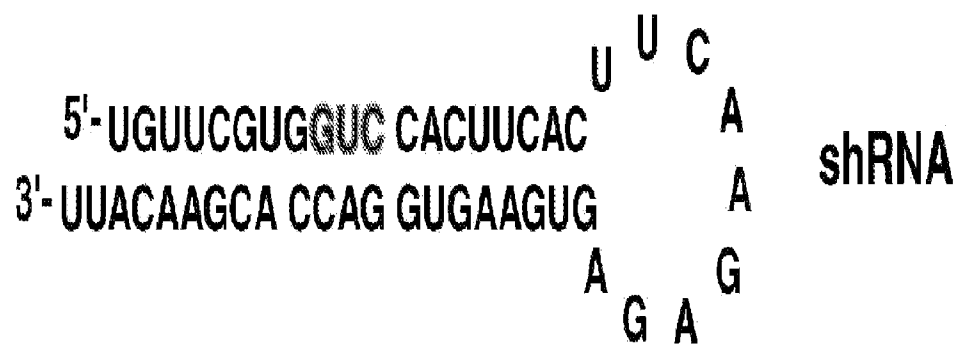
(SEQ ID NO:33)



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Figure 9

Human *RHO* 725 shRNA



(SEQ ID NO:21)