# Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Salus University

2. **Reporting Period (start and end date of grant award period):** 01/01/2013-04/30/2014

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Alexander M. Dizhoor, PhD

4. **Grant Contact Person's Telephone Number:** 215-780-1468

5. **Grant SAP Number:** 4100062219

6. **Project Number and Title of Research Project:** 01 – Interaction of Guanylyl Cyclase GUCY2D with its Regulatory Proteins: Effects of Mutations Causing Inherited Blindness

7. **Start and End Date of Research Project:** January 1, 2013 – April 30, 2014

8. **Name of Principal Investigator for the Research Project:** Alexander M. Dizhoor, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $40,082.56

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

| Last Name, First Name | Position Title | % of Effort on Project | Cost |
|---|---|---|---|
| None | | | |

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

| Last Name, First Name | Position Title | % of Effort on Project |
|---|---|---|
| Dizhoor, Alexander | Principal Investigator | 20% |

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

| Type of Scientific Equipment | Value Derived | Cost |
|---|---|---|
| Low-temperature freezer | Improves long-term storage of biological samples, cell lines and reagents in Research department. | $10,000 |

**10. Co-funding of Research Project during Health Research Grant Award Period.** Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No__X___

If yes, please indicate the source and amount of other funds:

**11. Leveraging of Additional Funds**

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No__X

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

| A. Title of research project on grant application | B. Funding agency (check those that apply) | C. Month and Year Submitted | D. Amount of funds requested: | E. Amount of funds to be awarded: |
|---|---|---|---|---|
| None | ☐NIH<br>☐ Other federal (specify:_____)<br>☐ Nonfederal source (specify:_) | | $ | $ |

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes_X___ No_____

If yes, please describe your plans:

CURE formula grants from PA Department of Health have been used by the PI for short-term small-scale pilot projects designed to identify the directions for the subsequent in-depth studies. In the recent past, we were able to repeatedly leverage funds from federal sources based in part on the results from the CURE-supported preliminary results (the most recent NIH grant was received in 2013). For this CURE-supported project, we plan to continue studies initiated under the Specific Aim 1 designed to identify the mechanisms of multiple mutations causing human blindness. We expect that further development in this direction of the project will provide a competitive basis for applying for larger scale grants from federal sources.

**12. Future of Research Project.** What are the future plans for this research project?

We find that Specific Aim 1 was successful in developing tools that allow us to distinguish between possible mechanisms through which mutations in human GUCY2D gene cause congenital blindness. In particular, they show how the part of regulation caused by disruption of normal protein-protein interactions can be identified and visualized in a cell-based test. Further development of this project will be two-fold. First, we plan to continue identification of the molecular basis for photoreceptor malfunction in connection with various mutations linked to Leber congenital amaurosis. Second, we will use additional point mutations in various parts of GUCY2D surrounding the LCA-related mutations in order to further dissect fine molecular mechanisms of GUCY2D interactions with its regulatory proteins and thus provide better knowledge of the fundamental molecular process underlying visual signaling.

13. **New Investigator Training and Development**. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes_____ No__X__

If yes, how many students? Please specify in the tables below:

| | Undergraduate | Masters | Pre-doc | Post-doc |
|---|---|---|---|---|
| Male | | | | |
| Female | | | | |
| Unknown | | | | |
| **Total** | | | | |

| | Undergraduate | Masters | Pre-doc | Post-doc |
|---|---|---|---|---|
| Hispanic | | | | |
| Non-Hispanic | | | | |
| Unknown | | | | |
| **Total** | | | | |

| | Undergraduate | Masters | Pre-doc | Post-doc |
|---|---|---|---|---|
| White | | | | |
| Black | | | | |
| Asian | | | | |
| Other | | | | |
| Unknown | | | | |
| **Total** | | | | |

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No__X___

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes__X___ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

As a result of this short-term pilot research, we expanded the collection of DNA and cell clones as tools in long-range studies of molecular mechanisms of vision and congenital vision impairment continuing at Salus University. The funds from the CURE formula grant were used to improve conditions for storage of samples requiring a low-temperature freezer.

**16. Collaboration, business and community involvement.**

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes___X__ No_____

If yes, please describe the collaborations:

Part of the project resulting in the use of modified vectors for RetGC and GCAP expression in HEK293 cells was involved in a collaborative study with Dr. Ames Lab, Chemistry department, UC Davis.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No__X _____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No__X _____

If yes, please describe involvement with community groups that resulted from the research project:

**17. Progress in Achieving Research Goals, Objectives and Aims.**

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations

at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

**There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.**

During the entire grant period the main objectives were met and the progress toward both original Specific Aims was achieved as follows.

Specific Aim 1 - To establish how mutations in GUCY2D gene linked to autosomal recessive LCA1 affect the ability of the retinal guanylyl cyclase to bind GCAP1 and Rd3. We hypothesize that mutations in GUCY2D can produce the loss-of-function phenotype through different mechanisms, either the loss of regulatory response from other proteins, or through the loss of catalytic activity (even if the ability to contact regulatory proteins remains). The objective of the study is to test this possibility using recombinant proteins produced in cultured cells.

Leber congenital amaurosis (LCA1) can be caused by mutations in many different genes, and one of the most frequently affected genes linked to LCA1 is GUCY2D coding for retinal membrane guanylyl cyclase 1 (RetGC1). RetGC1 is one of the key enzymes in photoreceptor signaling, because it produces a second messenger of phototransduction-cyclic GMP (cGMP). The activity of RetGC1 in photoreceptors responds to light versus dark conditions, because illumination of photoreceptors alters Ca2+ flux that regulates RetGC1 via calcium-sensor proteins GCAPsp; in the dark GCAPs bind Ca2+ and inhibit RetGC activity, while in the light they release Ca2+ and stimulate RetGC to produce cGMP at a higher rate. RetGC1 also binds with high affinity a protein inhibitor RD3, whose functional role remains unclear at the moment, but is required to maintain normal content of RetGC in photoreceptors.

Mutations in GUCY2D linked to LCA1 are believed to affect its functional activity, but such loss of activity may result from different mechanisms and involve both catalytic capacity of RetGC1 as the enzyme or hamper its interaction with the regulatory proteins. Therefore, we made a preliminary evaluation of how different mutations in GUCY2D associated with LCA1 could propagate the adverse effects on the cyclase regulation at the molecular levels.

Constructs mimicking human mutations in GUCY2D linked to LCA1, R1091x, W708R and I734T, as well as short variants of RetGC1 lacking segments in the N-terminal and C-terminal portions of the molecule were expressed in HEK293 cells from modified pRCCMV vector using co-transfection with RD3 and GCAP1 tagged with green fluorescent protein (GFP) and expressed from pQBI vector. The advantage of this technique is that under optimized conditions (Peshenko et al., 2008; 2014) it makes possible to reliably distinguish between the diffuse pattern of unbound RD3-GFP or GCAP1-GFP, spread throughout the cytoplasm and the nucleus, and its complexes with RetGC1, when RD3-GFP or GCAP1-GFP fluorescence acquires a well-defined pattern of association with the endoplasmic reticulum (ER) and plasma membrane, anchored to the membranes by RetGC1 (Fig. 1)

We found that R1091x RetGC1, a low-activity mutant lacking a short C-terminal peptide (Jacobson et al., 2013), in our experiments effectively binds both GCAP1 and RD3 (Fig. 1). Therefore, the low activity of the RetGC1 mutants encoded by the LCA1 GUCY2D allele found in previous studies by Jacobson et al., 2013, in this case is due to reduced catalytic efficiency of the enzyme rather than its interaction with other proteins.

We also found that removal of the so-called extracellular domain (ECD), which in photoreceptors is actually located inside photoreceptor disks, does not affect the ability of RetGC1 to bind either RD3 or GCAP1 (Fig. 2 and 3). This portion of the molecule can even be replaced with a red fluorescent protein (mOrange) without inactivation of its RD3- and GCAP1-binding capacity (Fig. 3). This strongly indicates that LCA-linked mutations located in the extracellular portion of RetGC1 such as E970Q, E103K, W150R, E255K, T312P, T312M or R365W (Stone 2007) exert their adverse effect through a mechanism unrelated to disruption of RetGC1 interactions with GCAP1 and RD3. Among the remaining possibilities there are incorrect intracellular trafficking of GUCY2D product and/or its intracellular stability, which could only be addressed in vivo using relevant transgenic models.

The removal of the C-terminal portion of the cyclase downstream from Arg941 reduces binding of the inhibitory protein, RD3, yet has little effect of GCAP1 binding (Fig. 2). Therefore, we can effectively rule out that such LCA-linked mutations as C984Y, P999S, S1007L, and M1009L located in the C-terminal portion of GUCY2D (Stone, 2007) act through disruption of GCAP1 binding with the cyclase. Most likely, these mutations directly affect catalytic activity of the cyclase, similarly to the R1091x or H980L mutations (Jacobson et al., 213).

Similar to R768W (Fig. 1) or D639Y mutation (Peshenko et al., 2010), two newly tested LCA-linked mutations, W708R and I734T, in the kinase-homology domain of GUCY2D fail

to bind GCAP1-GFP (Fig. 3). This opens a possibility that these residues are directly involved in forming the interface structure for GCAP1 on the cyclase.

These mutations were also unable to bind RD3 (Fig.4), in a sharp contrast to a cyclase in which the intracellular segment was that of the wild type and the extracellular domain was substituted with mOrange. Hence, both newly characterized LCA-linked substitutions, W708R and I734T, of GUCY2D that are located in kinase-homology domain of the cyclase completely block its protein interactions with both GCAP1 and RD3.

2) Toward Specific Aim 2 - To test how the mutation in GCAP2 causing retinitis pigmentosa associates with the GUCY2D guanylyl cyclase.

A number of mutations associated with GCAP1 are known to cause dominant cone and cone-rod dystrophies in humans (Jiang et al., 2011) by shifting Ca2+-sensititvity of cGMP synthesis by RetGC1 in vitro and in vivo (Dizhoor et al., 1998; Olshevskaya et al., 2004; 2012; Woodruff et al., 2007). In 2005, Sato and colleagues reported the occurrence of a mutation in GUCA1B gene coding for the second RetGC-activating protein, GCAP2, in a family affected by retinitis pigmentosa. GCAP2 regulates cGMP synthesis in rods (Makino et al., 2008; Xu et al., 2013), the type of photoreceptors primarily affected by retinitis pigmentosa. We therefore hypothesized that the disease-causing mutation, G157R, found in human patients alters GCAP2 interaction with the GUCY2D. GCAPs are calcium/magnesium - binding proteins that exchange Mg2+ for Ca2+ in three EF-hand domains as ambient conditions change between light and dark and by doing so either stimulate or inhibit the cGMP production by the guanylyl cyclase. The mutation found in patients produces a Gly→Arg substitution in position 4 (non-coordinating side chain) of the 12-amino acid calcium-binding loop of EF-hand 4.

The use of recombinant bovine GCAPs and native rod outer segment (ROS) membranes was previously proven instrumental in identifying biochemical consequences of multiple disease-causing mutations in GCAP1 (Dizhoor et al., 1998; Olshevskaya et al., 2004; Woordfuff et al., 2007). We therefore introduced the corresponding Gly→Arg substitution in $4^{th}$ position of the 12-amino acid calcium-binding loop of EF-hand 4 in bovine GCAP2 homolog resulting from the G157R mutation, and tested its biochemical properties in regulation of the native guanylyl cyclase in isolated rod outer segment membranes that we purified from dark-adapted bovine retinas.

Mutation in GCAP2 cDNA inserted in pET11 expression vector was confirmed by DNA sequencing and the recombinant protein was produced in E. coli harboring N-myristoyl transferase–expressing plasmid in growth medium supplemented with myristic acid to generate a proper posttranslational modification (N-myristoylation). The presence of N-myristoylation was further verified by mass-spectrometry (Fig. 5) and it’s mass was found to be consistent with the expected molecular mass of properly fatty acylated protein. We then compared both dose-dependence of the cyclase activation by wild type and mutant GCAP2 and its Ca2+-sensitivity in native rod outer segment membranes. To our surprise, we found no indication that such substitution has any effect on either of the two major parameters of

cGMP synthesis regulation (Fig. 6). While we would not expect to see a gain-of-function effect based on the recessive nature of the GCAP2-linked retinitis pigmentosa reported by Sato et al., we nevertheless expected that the mutation would instead cause a loss-of-function effect – either a lack of cyclase activation or abnormally high sensitivity to inhibition by Ca2+. However, neither the activity of GCAP2 nor its Ca2+ sensitivity changed as a result of this mutation. This result leaves two possibilities - either the intracellular expression or stability of the mutant GCAP2 in vivo is affected by the mutation, such that the protein becomes destroyed in living photoreceptors (this question cannot be addressed using in vitro biochemistry and can only be resolved by using animal models) or the reliability of the reported direct genetic link between GCAP2 and retinal degeneration needs to be revisited.

Figures

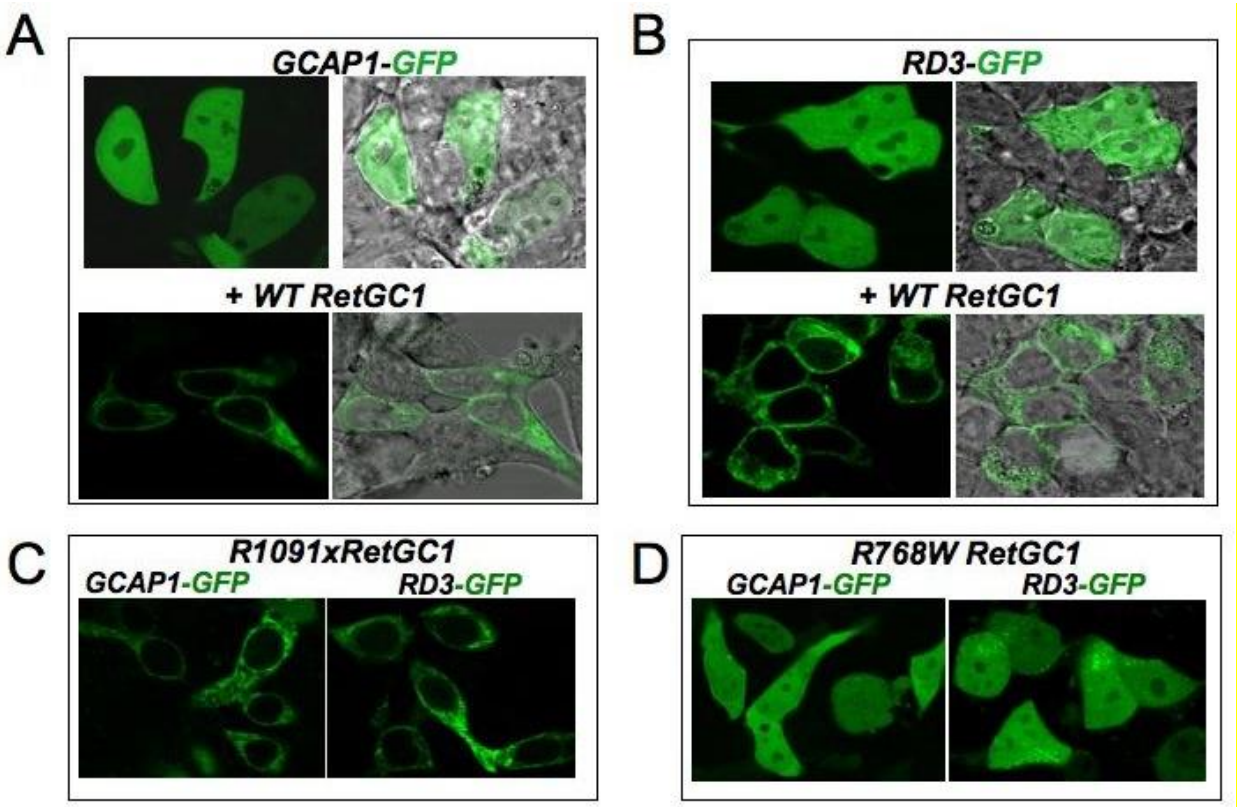


Figure 1. In the absence of RetGC1, GCAP1-GFP (A) and RD3-GFP (B) are diffusely distributed through the cytoplasm and karyoplasms of HEK293 cells, but acquire membrane localization pattern when co-expressed with wild type RetGC1. The same membrane localization pattern for both GCAP and RD3 is observed in the presence of the R1091x RetGC1 mutant (C) in contrast to the R768W RetGC1, which binds neither GCAP1-GFP nor RD3-GFP (D).

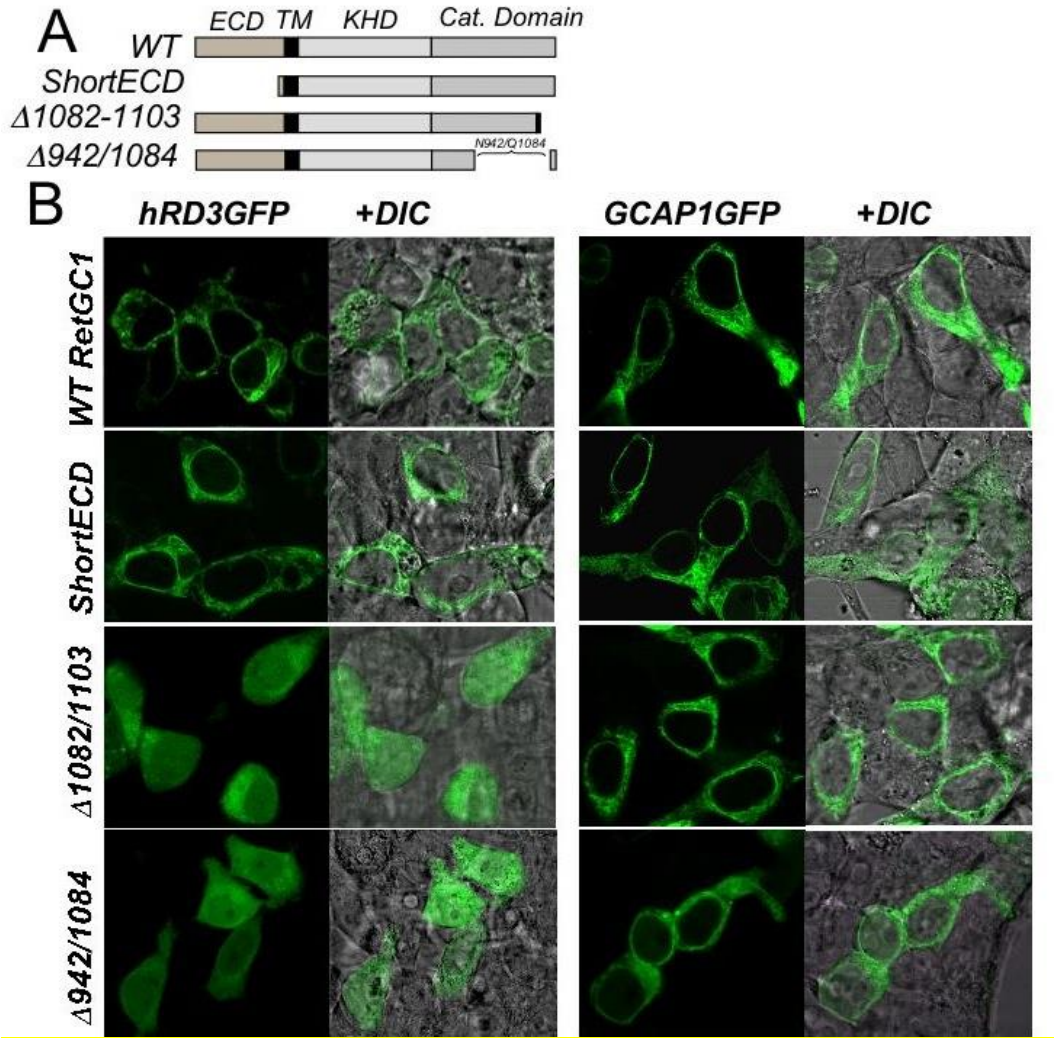


Figure 2. A. Schematic representation of wild type RetGC1 coded by GUCY2D gene and its variants. *Top to bottom*: wild type, 'Short ECD" lacking extracellular segment; carboxy-terminus truncations after resides 1081 or 942. B human RD3-GFP (left column) or GCAP1-GFP (right column) pattern in HEK293 cells expressing wild type (first row from top), Short ECD RetGC1 (second row), truncation after residues 1081 (third row) and 941 (bottom row). Each fluorescence image is also shown superimposed on differential contrast image (DIC) in the right part of each column. Note membrane pattern of GCAP1GFP in all cases and diffused pattern of RD3 after carboxy-terminal deletions.

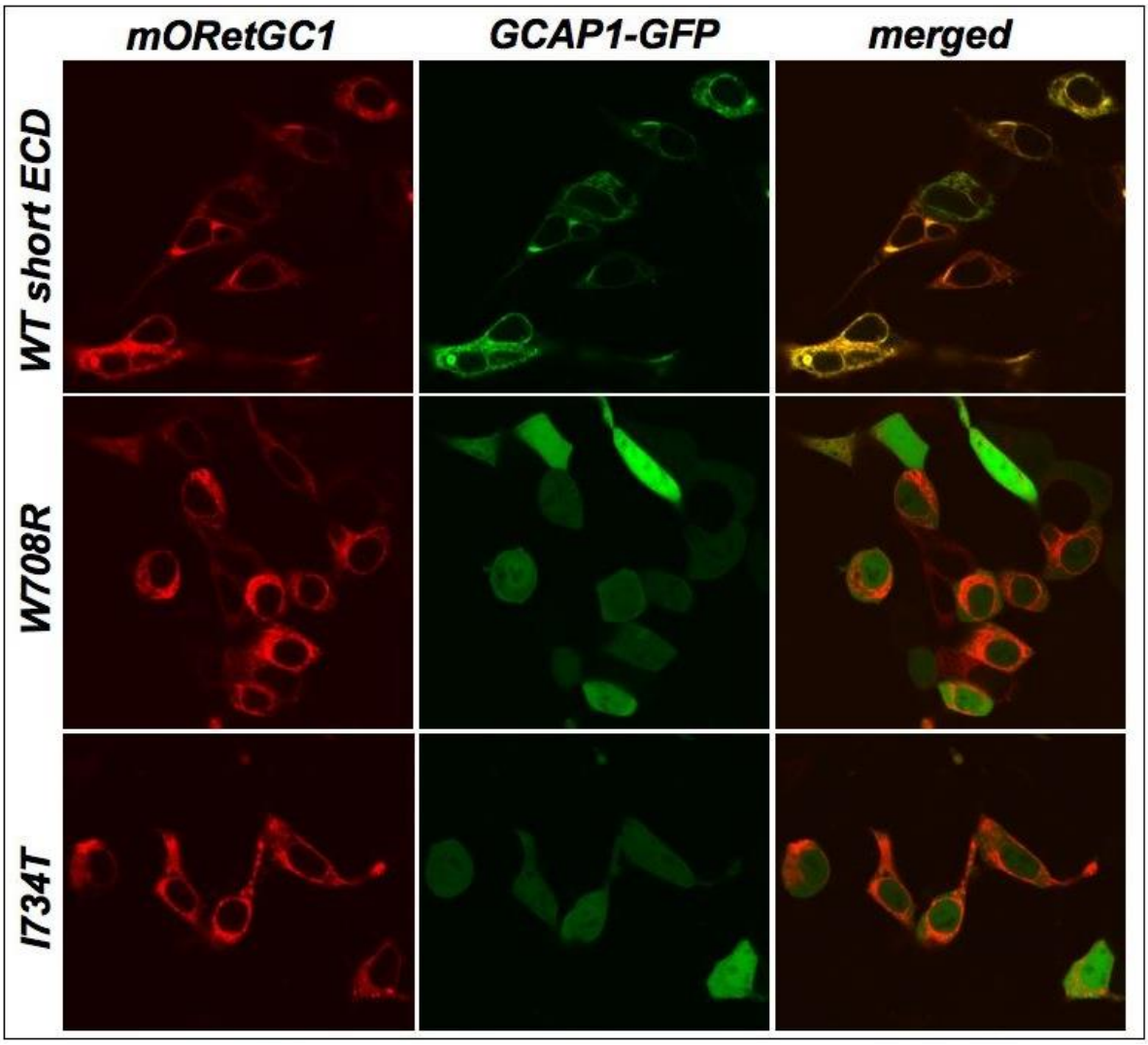


Figure 3. Co-expression of fluorescently labeled RetGC1 (coded by *GUCY2D* cDNA) and GCAP1. The extracellular segment of human GUCY2D was substituted with the mOrange red fluorescent protein tag positioned between the leader peptide and the transmembrane domain. Green fluorescent protein (GFP) tag was placed at the C-terminus of GCAP1. The mixture of mOrangeGUCY2D and GCAP1GFP – coding plasmids was transfected into HEK293 cells using a Promega FuGene HD reagent. The confocal images were taken using Olympus FV1000 Spectral system. *Left to right*: mOrange-RetGC1, GCAP1-GFP, both images merged. *Top to bottom*: mOrange-RetGC1 containing wild type intracellular segment; mOrange-RetGC1 containing W708R substitution in kinase homology domain; mOrange-RetGC1 containing I734T substitution in kinase homology domain. While GCAP1 co-localizes with the wild type mOrangeGucy2D in membranes, the LCA-linked mutations, W708R and I734T, do not bind GCAP1-GFP, which makes GCAP1 uniformly spread through the cytoplasm and the nucleus of the cell.

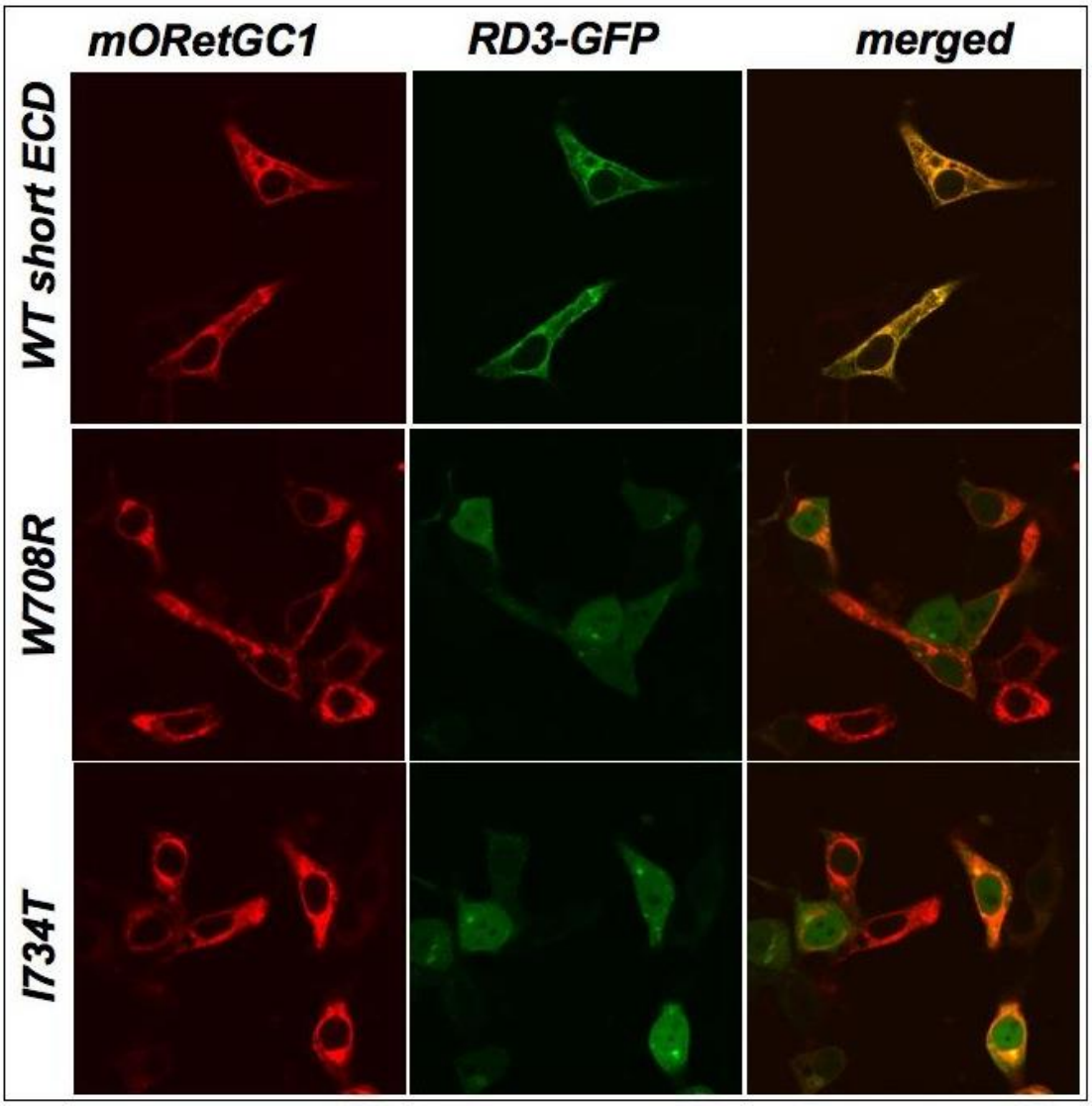


Figure 4. Co-expression of fluorescently labeled RetGC1 (coded by *GUCY2D* cDNA) and RD3. The extracellular segment of human GUCY2D was substituted with the mOrange red fluorescent protein tag. Green fluorescent protein (GFP) tag was placed at the C-terminus of RD3. The mixture of mOrangeRetGC1 and RD3-GFP – coding plasmids was transfected into HEK293 cells using a Promega FuGene HD reagent. *Left to right*: mOrange-RetGC1, RD3-GFP and both images merged. *Top to bottom*: mOrangeRetGC1 containing wild type intracellular segment; mOrange-RetGC1 containing W708R substitution; mOrange-RetGC1 containing I734T substitution. While RD3 co-localizes with the wild type cyclase in membranes, the LCA-linked mutations, W708R and I734T, are unable to bind RD3-GFP, which makes RD3 spread through the cytoplasm and the nucleus.

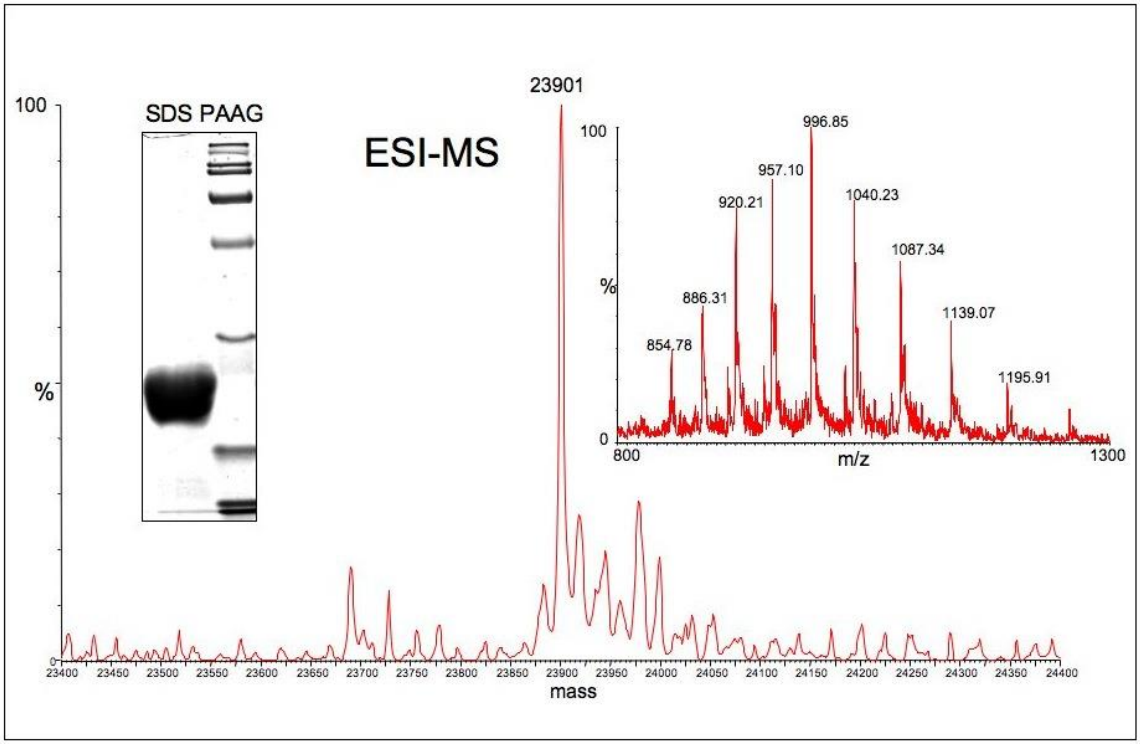


Figure 5. Expression and mass-spectrometry analysis of the mutant GCAP2. Mutated myristoylated GCAP2 purified from E. coli harboring N-myristoyl transferase is shown on Coomassie-stained SDS-PAAG next to Bio-Rad molecular weight protein markers. Electrospray ionization mass-spectrometry (ESI-MS) presented as mass/charge (m/z) distribution (right inset) and deconvoluted spectrum of molecular masses were within the accuracy of 5 mass units consistent with the C14:0 (myristoyl)-acylated GCAP2 harboring the Gly→Arg substitution as the predominant component.

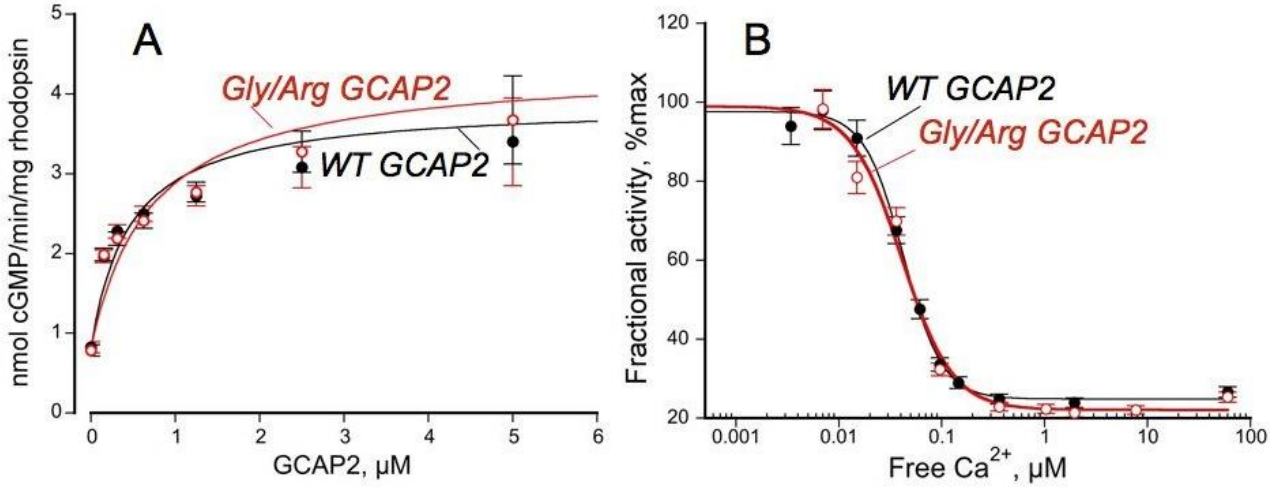


Figure 6. Regulatory properties of GCAP2 mimicking human G157R mutation. A: Dose dependence of guanylyl cyclase activation in native bovine rod outer segment (ROS). ROS membranes were isolated using Ficoll density gradient centrifugation and hypotonically extracted to deplete the endogenous GCAPs. The washed membranes were reconstituted with wild type GCAP2 (black closed symbols) or GCAP2 harboring substitution Gly→Arg in the 4$^{th}$ position of EF-hand 4 calcium binding loop (red open symbols). The guanylyl cyclase activity was assayed under infrared illumination using [$^{32}$P-α]GTP as a substrate in the presence of 2 mM EGTA and saturating 10 mM $MgCl_2$. B: Fractional activity of guanylyl cyclase in washed ROS membranes reconstituted with 10 μM wild type (black) or mutant (red) GCAP2 in the presence of 2 mM EGTA/$Ca^{2+}$/$Mg^{2+}$ buffers varying free $Ca^{2+}$ concentrations at a constant 1 mM free $Mg^{2+}$. The activity at each tested $Ca^{2+}$ concentration was normalized by the maximal activity in each set and the data were fitted using Kaleidagraph software assuming sigmoidal Hill function.

**18. Extent of Clinical Activities Initiated and Completed**. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

**If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed.** (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

**Note**: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

**19. Human Embryonic Stem Cell Research.** Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

**20. Articles Submitted to Peer-Reviewed Publications**.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

**Note:** The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

| Title of Journal Article: | Authors: | Name of Peer-reviewed Publication: | Month and Year Submitted: | Publication Status (check appropriate box below): |
|---|---|---|---|---|
| 1. Identification of Target Binding Site in Photoreceptor Guanylyl Cyclase-activating Protein 1 (GCAP1) | Igor V. Peshenko, Elena V Olshevskaya, Sunghyuk Lim, James B. Ames, and Alexander M. Dizhoor | The Journal Of Biological Chemistry Vol. 289, No. 14, pp. 10140–10154. | December 2013 | ☐Submitted<br>☐Accepted<br>☑Published |

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes__X___ No_____

If yes, please describe your plans:

We plan additional publication of the findings down the road. The short-term pilot study under this project laid a solid foundation for further in-depth analysis of molecular mechanisms altered by mutations in GUCY2D causing LCA blindness. We will continue the analysis of additional mutations and characterization of their biochemical and cellular effects. In our past experience, it typically takes a year or so from the completion of a pilot study to accumulate publication-quality data suitable for high-visibility journals. This is what we expect for continuation of the experiments under Specific Aim 1. After that, we plan to submit the results to a peer review journal of wide readership and acknowledge the support from Pennsylvania Department of Health in a resulting publication.

**21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project.** Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

This project establishes a larger diversity of molecular mechanisms related to Leber congenital amaurosis caused by mutations in GUCY2D gene. This diversity relates to different modes of interaction between a calcium-sensor protein GCAP1 and its target, guanylyl cyclase RetGC1. The diversity of these mechanisms should be taken into consideration in future plans for gene therapy of the corresponding GUCY2D-related cases of LCA.

**22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment**. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

The major finding which resulted from this study was wide evidence for diversity of molecular mechanisms underlying Leber congenital amaurosis caused by mutations in *GUCY2D* gene. We find that mutations in the N-terminal and C-terminal portions of the RetGC1 coded by the *GUCY2D* gene do not affect binding of its protein activator, GCAP1, while point mutations in a kinase homology domain of the cyclase completely block interaction with GCAP1. We have also found that there is no apparent biochemical evidence for the link between mutation in *GUCA1B* gene reported in connection with retinitis pigmentosa and regulatory properties of GCAP2 encoded by this gene as a calcium sensor in guanylyl cyclase regulation.

**23. Inventions, Patents and Commercial Development Opportunities.**

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:

Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No___

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes__ No___

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No__X___

If yes, please describe your plans:

**24. Key Investigator Qualifications.** Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

**Alexander M. Dizhoor, PhD,** Professor, Salus University

EDUCATION:

| | | | |
|---|---|---|---|
| Moscow University, Moscow, USSR | BS | 1977 | Biochemistry, Virology |
| Moscow University | PhD | 1985 | Molecular Biology |

POSTDOCTORAL TRAINING:

Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow, USSR
1985-1989 Enzymology, Phototransduction,
Howard Hughes Medical Institutes and Department of Biochemistry, University of Washington, 1989-1993 Molecular Biology

PERSONAL STATEMENT.

My group investigates $Ca^{2+}$ binding proteins and cGMP synthesis in the retina. With my colleagues, I identified and characterized several new proteins in phototransduction. My laboratory developed multiple mouse genetic models to delineate the *in vivo* regulatory pathways leading to human retinal diseases caused by abnormalities in cGMP regulation. Independently or through multiple collaborations, my group identified biochemical and cellular mechanisms underlying retinal degeneration caused by mutations in GCAP1 and RetGC1. We identified the targeted RetGC isozymes for individual GCAPs *in vivo* and the role of GCAP isoforms in shaping rods photoresponse at different phases of the recovery.

POSITIONS AND EMPLOYMENT

1977-1979 Junior Researcher, Biological Faculty, Moscow State University
1979-1985 Graduate student and pre-doctoral Junior Investigator, Moscow State University
1985-1990 Investigator and Senior Investigator, Moscow State University
1989-1990 Visiting scientist at Dept. of Biochemistry, University of Washington
1990-1992 Leading Investigator, Institute of Physico-Chemical Biology, Moscow, USSR
1991-1996 Associate, Howard Hughes Medical Institute, Senior Fellow, Research Assistant Professor, Dept. of Biochemistry, University of Washington, Seattle, WA.
1997-2002 Assistant Professor, then Associate Professor of Ophthalmology, Pharmacology, Anatomy and Cell Biology, and Adjunct Professor of Anatomy and Cell Biology, Wayne State University, Detroit, Michigan
2002-pres. Hafter Chair and Professor of Pharmacology, Pennsylvania College of Optometry (since 2008 – part of Salus University), Elkins Park, PA

OTHER PROFESSIONAL EXPERIENCE AND MEMBERSHIPS

Study Sections (NIH CSR): BDPE (2005-2010); CSR/ZRG1, MIST (2010-2012).

SELECTED RECENT PEER-REVIEWED PUBLICATIONS (OUT OF 65)

1. Peshenko IV, Olshevskaya EV, Lim S, Ames JB, Dizhoor AM (2014) Identification of Target Binding Site in Photoreceptor Guanylyl Cyclase Activating Protein 1 (GCAP1). J Biol Chem. 289, 10140–10154
2. Xu J, Morris L, Thapa A, Ma H, Michalakis S, Biel M, Baehr W, Peshenko IV, Dizhoor AM, Ding X-Q (2013) cGMP Accumulation Causes Photoreceptor Degeneration in CNG Channel Deficiency: Evidence of cGMP Cytotoxicity Independently of Enhanced CNG Channel Function. Journal of Neuroscience 33 33(37): 14939-48.

3. Boye SL, Peshenko IV, Huang WC, Min SH, McDoom I, Liu X, Dyka FM, Foster TC, Umino Y, Karan S, Jacobson SG, Baehr W, Dizhoor AM, Hauswirth WW, Boye SE (2013) AAV-mediated gene therapy in the guanylate cyclase (RetGC1/RetGC2) double knockout mouse model of Leber congenital amaurosis. Human Gene Therapy 24(2):189-202.
4. Makino CL, Wen X-H, Olshevskaya EV, Peshenko IV, Savchenko AB, Dizhoor AM (2013) Enzymatic relay mechanism stimulates cyclic GMP synthesis in rod photoresponse: biochemical and physiological study in Guanylyl Cyclase Activating Protein 1 knockout mice. PLOS ONE 7 (10), e47637, 1-12.
5. Jacobson SG, Cideciyan AV, Peshenko IV, Sumaroka A, Olshevskaya EV, Schwartz SB, Roman AJ, Olivares MB, Sadigh S, Heon E, Stone EM, Dizhoor AM (2013) Determining consequences of retinal membrane guanylyl cyclase (RetGC1) deficiency in human Leber congenital amaurosis en route to therapy: residual cone photoreceptor vision correlates with biochemical properties of the mutants. Human Molecular Genetics 22:168-183.
6. Olshevskaya EV, Peshenko IV, Savchenko AB, Dizhoor AM (2012) Retinal guanylyl cyclase isozyme 1 is the preferential in vivo target for constitutively active GCAP1 mutants causing congenital degeneration of photoreceptors. Journal of Neuroscience 32: 7208-7217.