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Functional study of two biochemically unusual mutations in *GUCY2D* Leber congenital amaurosis expressed via adenoassociated virus vector in mouse retinas

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Purpose: To test, in living photoreceptors, two mutations, S248W and R1091x, in the *GUCY2D* gene linked to Leber congenital amaurosis 1 (LCA1) that fail to inactivate the catalytic activity of a heterologously expressed retinal membrane guanylyl cyclase 1 (RetGC1).
Methods: *GUC2YD* cDNA constructs coding for wild-type human (hWT), R1091x, and S248W GUCY2D under the control of the human rhodopsin kinase promoter were expressed in *Gucy2e*$^{-/-}$*Gucy2f*$^{-/-}$ knockout (GCdKO) mouse retinas, which lack endogenous RetGC activity. The constructs were delivered via subretinally injected adenoassociated virus (AAV) vector in one eye, leaving the opposite eye as the non-injected negative control. After testing with electroretinography (ERG), the retinas extracted from the AAV-treated and control eyes were used in guanylyl cyclase activity assays, immunoblotting, and anti-RetGC1 immunofluorescence staining.
Results: Cyclase activity in retinas treated with either hWT or R1091x GUCY2D transgenes was similar but was undetectable in the S248W GUCY2D-treated retinas, which starkly contrasts their relative activities when heterologously expressed in human embryonic kidney (HEK293) cells. Rod and cone ERGs, absent in GCdKO, appeared in the hWT and R1091x GUCY2D-injected eyes, while the S248W mutant failed to restore scotopic ERG response and enabled only rudimentary photopic ERG response. The hWT and R1091x GUCY2D immunofluorescence was robust in the rod and cone outer segments, whereas the S248W was detectable only in the sparse cone outer segments and sporadic photoreceptor cell bodies. Robust RetGC1 expression was detected with immunoblotting in the hWT and R1091x-treated retinas but was marginal at best in the S248W GUCY2D retinas, despite the confirmed presence of the S248W GUCY2D transcripts.
Conclusions: The phenotype of S248W GUCY2D in living retinas did not correlate with the previously described normal biochemical activity of this mutant when heterologously expressed in non-photoreceptor cell culture. This result suggests that the S248W mutation contributes to LCA1 by hampering the expression, processing, and/or cellular transport of GUCY2D, rather than its enzymatic properties. In contrast, the effective restoration of rod and cone function by R1091x GUCY2D is paradoxical and does not explain the severe loss of vision typical for LCA1 associated with that mutant allele.

The *GUCY2D* gene (Gene ID: 3000; OMIM 600179) codes for the human retinal membrane guanylyl cyclase 1 (RetGC1) isozyme and is one of the genes most frequently associated with the early onset, inherited recessive blindness, Leber congenital amaurosis type 1 (LCA1) [1-3]. The acute loss of vision in patients with *GUCY2D* LCA1 results not from the severe degeneration of rods and cones [3] but from the inefficient production in the diseased photoreceptors of the second messenger in phototransduction, cyclic GMP (cGMP) [4]. Of the two existing retinal membrane guanylyl cyclase isozymes, RetGC1 (*GUCY2D* in humans) and RetGC2 (*GUCY2F*) [5,6], the former catalyzes most of the cGMP production in mammalian photoreceptors [7,8]. Note that the RetGC1 ortholog in mice is coded by the *Gucy2e gene* [6]; therefore, only the human RetGC1 will be further referred to as GUCY2D (wherever the difference in gene-based nomenclature between species can cause confusion, we use the biochemical names, RetGC1 and RetGC2). cGMP synthesis becomes decelerated in the dark and accelerated in the light by guanylyl cyclase activating proteins (GCAPs) [9]. These proteins are Ca^{2+}-sensor proteins that switch between their Ca^{2+}- and the Mg^{2+}-bound states following the respective increase or decrease of Ca^{2+} influx through the cGMP gated channels between dark and light [10]. Another photoreceptor protein, retinal degeneration 3 (RD3) [11,12], reduces RetGC1 activity by competing with GCAPs [13] and is essential for the normal production of RetGC1 and its transport to the

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outer segment [14-16]. We previously showed, in vitro, that different mutations linked to *GUCY2D* LCA1 inactivate the catalytic activity of RetGC1 and/or suppress its ability to bind GCAPs and RD3 [15,17].

Rod and cone function can be sustainably restored in mice that lack endogenous orthologs of RetGC1 (*Gucy2e*$^{-/-}$) and RetGC2 (*Gucy2f*$^{-/-}$; GCdKO) [18] by delivering functional GUCY2D cDNA with adenoassociated viral (AAV) vectors [19,20]. Because the majority of patients with *GUCY2D* LCA1 exhibit preservation of the retinal structure, an opportunity exists to consider a human gene therapy to rescue vision in this disease [3,21]. Among the reported recessive alleles linked to *GUCY2D* LCA1 [1-3], the properties of two mutations in *GUCY2D* in vitro would not explain the virtually complete lack of photoresponses in patients with LCA1 [3]. These mutations include a missense mutation, Ser248→Trp, in the N-terminal extracellular domain of the enzyme (Figure 1) [3], and a nonsense mutation, R1091x [1,3], that truncates a short C-terminal region, downstream from the catalytic domain (Figure 1). Unlike other LCA1-linked *GUCY2D* mutants shown in vitro [3,17,22] to completely inactivate the enzymatic activity and regulation of the RetGC1 isozyme by Ca^{2+} and GCAPs, the biochemical properties remained substantially preserved in R1091x and remained completely normal in S248W.

By using subretinal delivery via AAV in the present study, we expressed in mouse retinas that lacked endogenous cyclase activity variants of human GUCY2D cDNA that harbor the two mutations linked to LCA1 and tested their physiologic effects in living photoreceptors. We found that S248W GUCY2D, despite its maintenance of full catalytic capacity as a recombinant protein in vitro, was completely unable to sustain rod function and failed to accumulate in rod outer segments in GCdKO mice. S248W GUCY2D appeared in only a small number of sparse GCdKO cone outer segments, thus mediating barely detectable cone responses. In contrast, AAV-mediated R1091x GUCY2D accumulated in GCdKO photoreceptor outer segments and enabled well-detectable rod and cone photoresponses.

METHODS

Animals: The mouse *Gucy2e*$^{-/-}$ *gene* knockout line [23] that originated from the D. Garbers laboratory (University of Texas) and the *Gucy2f*$^{-/-}$ *gene* knockout line [18] that originated from the W. Baehr laboratory (University of Utah) were bred into a homozygous (*Gucy2e*$^{-/-}$*Gucy2f*$^{-/-}$) state. All experiments were approved by the University of Florida and Salus University's Institutional Animal Care and Use Committee and were conducted in accordance with the Association for Research in Vision and Ophthalmology's (ARVO's) Statement for the Use of Animals in Ophthalmic and Vision Research and with National Institutes of Health regulations. Animals were maintained at the University of Florida Health Science Center Animal Care Services Facility under a 12 h:12 h light-dark cycle. Food and water were available ad libitum. Animals were shipped to Salus University for terminal assays.

AAV vector construction and virus packaging: The original vector backbone has been previously described in detail [24]. The recombinant AAV vector plasmids contained flanking AAV2 inverted terminal repeats, human rhodopsin kinase promoter, an SV40 splice donor-acceptor site, human *GUCY2D* cDNA coding for wild-type or LCA1 mutants, and a bovine growth hormone polyadenylation signal. Wild-type *GUCY2D* cDNA (hWT) was synthesized de novo and contained silent mutations that remove several restriction sites to facilitate molecular cloning. The S248W *GUCY2D*-containing vector plasmid was constructed by replacing the Eco47III-flanked fragment coding for the S248W mutation. The R1091x *GUCY2D* vector was constructed by replacement of the NarI/SalI fragment containing the TGA codon at position 1091. All constructs were verified with DNA sequencing and packaged using a modified AAV8 capsid protein containing a single Tyr733Phe substitution. The

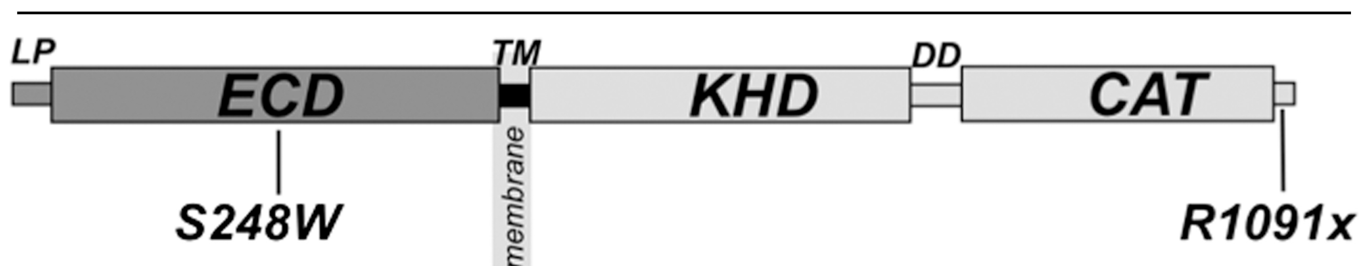

Figure 1. Schematic representation of the GUCY2D (RetGC1) primary structure [5,6]. The S248W missense mutation is located in the extracellular domain, and the R1091x truncation is located near the C-terminus, downstream of the catalytic domain. LP = leader peptide; ECD = extracellular domain; TM = transmembrane domain; KHD = kinase homology domain; DD = dimerization domain; CAT = catalytic domain.

AAV particles were manufactured, purified, and titered as described elsewhere [25,26]. The titers of the viruses harboring the hWT, S248W, and R1091x mutants were 4.06 × 10^{13}, 5.16 × 10^{13}, and 7.64 × 10^{13} vg/ml, respectively. All were contained in a balanced salt solution (Alcon, Ft. Worth, TX) supplemented with 0.014% Tween-20.

Subretinal Injections: Subretinal injections were performed as previously described [27]. Briefly, eyes of postnatal day 30 (P30) GCdKO mice were dilated 1 h before injection with 1% atropine, followed by 2.5% phenylephrine (Akorn, Inc., Lake Forest, IL). The mice were then anesthetized with ketamine (72 mg/kg)/xylazine (4 mg/kg; Phoenix Pharmaceutical, St. Joseph, MO). Trans-corneal subretinal injections of un-diluted virus (1 µl) were performed using a 33-gauge blunt needle on a Hamilton syringe.

QRT PCR: Treated and untreated eyes from GCdKO mice were enucleated 4 weeks post-injection, and each retina was immediately dissected from the eyecup and submerged in RNA Protect (Qiagen, Inc., Valencia, CA) and then was homogenized in RLT Buffer (RNeasy Protect Mini Kit; Qiagen, Inc.) containing 2-mercaptoethanol for 45 s. RNA was extracted and reverse transcribed as reported previously [5]. Real-time PCR was conducted using Bio-Rad iQ SYBR Green Supermix interfaced with C1000 Touch thermal cycler (Bio-Rad Laboratories, Hercules, CA) to measure the content of hWT and S248W using a forward, 5′-ATC CCA CCC GAG CGG CGA-3′, primer and a reverse, 5′-GGC AAC TAG AAG GCA CAG TCG AGG C-3′, primer (Integrated DNA Technologies, Coralville, IA) amplifying a 103 bp region at the junction between the 3′-end of the GUCY2D cDNA and the 5′-end of the bovine growth hormone polyadenylation signal fragment. Before the experimental data were collected, the primers were validated according to minimum information for publication of quantitative real-time (QRT) PCR experiment (MIQE) standards [28]. PCR samples were initially heated to 95 °C for 3 min followed by 40 cycles of amplification/plate reading: 95 °C for 10 s, 58 °C for 30 s, plate reading, and 72 °C for 30 s. Once finished, a melt curve was assessed from 65 ° C to 95 °C with a plate read every 0.5 °C. Measurements averaging three replicate reactions for each retina were calculated using the $\Delta\Delta C_q$ method, with the signals normalized to glyceraldehyde 3-phosphate dehydrogenase-specific transcripts as a control and were then normalized per signal in the non-injected GCDKO eye.

GCAP1 and RD3: Myristoylated bovine GCAP1 was expressed from a Novagen pET11d vector in BLR(DE3) *Escherichia coli* strain and purified to about 95% electrophoretic homogeneity as described previously in detail [29]. In brief, inclusions bodies containing myristoylated GCAP1 were obtained by three rounds of sonication on ice and centrifugation to remove soluble proteins. GCAP1 was extracted from the inclusion bodies in the presence of urea, dialyzed to remove urea, and then FPLC-purified using butyl-Sepharose and high-resolution size-exclusion Superdex 200 (GE Healthcare Life Sciences, Pittsburgh, PA) column chromatography. Human RD3 was expressed from the pET11d vector in the BL21(DE3) CodonPlus *E. coli* strain (Stratagene/Agilent Technologies, Santa Clara, CA), extracted from the inclusion bodies, and purified to about 95% electrophoretic homogeneity as previously described in detail [30]. In brief, inclusion bodies containing RD3 were purified by three rounds of sonication/centrifugation, then RD3 was extracted from the inclusion bodies in the presence of urea, dialyzed, purified by precipitation in the presence of 250 mM NaCl, washed by centrifugation, extracted with urea second time and dialyzed, after which the insoluble material was removed by centrifugation and the supernatant containing purified RD3 was collected and used in the experiments.

Guanylyl cyclase assays: RetGC1 activity was assayed using [α-^{32}P]GTP (Perkin Elmer, Waltham, MA) as the substrate as previously described [3,7,29]. As previously described [3], membrane fractions from transfected human embryonic kidney (HEK293) cell cultures expressing RetGC1 were incubated for 40 min at 30° under ambient illumination and inactivated at 95° for 2 min. Mouse retinas for the RetGC activity measurements were excised from the dark-adapted mice under a dissecting microscope fitted with an Excalibur infrared goggles (Fogelsville, PA) [7], and the assays containing retinal samples were conducted under infrared illumination using Kodak number 11 (Eastman Kodak, Rochester, NY) infrared filters and were incubated for 12 min. The [^{32}P]cGMP product of the reaction separated with thin-layer chromatography was quantified with liquid scintillation counting [7,29].

Immunofluorescence: Mice anesthetized with a lethal dose of ketamine/xylazine injection were perfused through the heart with PBS (Fisher Scientific, Fair Lawn, NJ, product number BP399-1; 1X: 137 mM NaCl, 2.7 mM KCl, 11.9 mM phosphate buffer, pH 7.4) and then with 10% freshly prepared formaldehyde solution in PBS. The eyecups from the enucleated eyes were additionally fixed in 10% formaldehyde/PBS for 5 min and for 1 h in 5% formaldehyde/PBS at room temperature. The fixed eyecups were impregnated with 30% sucrose in PBS overnight and frozen in optimum cutting temperature (OCT) medium (Electron Microscopy Sciences, Hatfield, PA) at −70 °C. Cryosections (20 µm thick) were sliced using a Hacker-Bright (Hacker Instruments & Industries Inc., Winnsboro, SC) cryogenic microtome, dried for

1 h at room temperature, and stored in –70 °C. The sections were washed 3X in PBS/0.1 M glycine (pH 7.4) and blocked for 1 h at 30 °C in the same solution plus 5% bovine serum albumin and 0.1% Triton X-100. The sections were incubated overnight at 4 °C and then for 1 h at room temperature with the rabbit polyclonal antibody against the catalytic domain of RetGC1 [7], washed 4X for 15 min with PBS, incubated for 1 h at room temperature with the donkey anti-rabbit Alexa Fluor 568-conjugated antibody (1:400, Molecular Probes, Invitrogen, Carlsbad, CA), and washed 3X for 15 min each at room temperature. Where indicated, fluorescein-labeled peanut agglutinin (Vector Laboratories, Burlingame, CA) was added with the secondary antibody to label the cone outer segment sheaths. Sections were covered with a TOPRO3 iodide-containing or plain Vectashield mounting medium (Vector Laboratories). Confocal images were acquired using an Olympus (Center Valley, PA) FV1000 Spectral instrument controlled with FluoView FV10-ASW software (Center Valley, PA), collecting in a sequential mode emission excited by 488, 543, and 635 nm lasers, assigned respective green, red, and pseudoblue color channels.

Electroretinography: One month post-injection, the mice were dark-adapted overnight, their pupils were dilated with 1% tropicamide and 2.5% phenylephrine ophthalmic eye drops under dim red safelight illumination, and then the mice were dark-adapted for another 15 min. Full-field ERG was performed in the dark using a Maxwellian view projection-based Phoenix Research Laboratories (Pleasanton, CA) Ganzfeld ERG 2 setup. Mice were anesthetized with 50 ml/min delivery of 1.7–1.9% Isoflurane (VEDCO) using a Kent Scientific (Torrington, CT) SomnoSuite Small Animal Anesthesia System and remained on a heated pad during the entire procedure. To evoke scotopic electroretinography (ERG) responses, 1 ms 505 nm light pulses of 1.2×10^6 photons μm^{-2} at the mouse retinal surface were delivered through the infrared camera-guided corneal electrode/light-emitting diode (LED) light source in 3 min intervals, and the traces from three to four trials were averaged. Constant 505 nm illumination, equivalent to 33×10^3 photons rod^{-1} s^{-1}, was used as a background to record photopic ERG responses to 1 ms 505 nm and 365 nm pulses of 6×10^5 photons μm^{-2} at 1-s intervals, and traces were typically averaged from 15 to 25 trials for each animal.

RESULTS

R1091x but not S248W RetGC1 enables photoresponse in vivo: As expected [18], the non-injected eyes of the GCdKO mice completely lacked photoreceptor activity. However, injection of AAV vectors harboring wild-type (hWT) (9 mice) and R1091x (10 mice) GUCY2D enabled scotopic (Figure 2A) and photopic ERG (Figure 2B) responses. The scotopic ERG amplitudes varied between different animals (Figure 2C), a result likely due to the variable efficacy of subretinal injections, but the average scotopic ERG a-wave enabled by R1091x RetGC1 expression was reduced only slightly and was not statistically significantly different from that of the wild-type *GUCY2D* injected eyes (Figure 2D). In contrast, S248W GUCY2D enabled only marginally detectable cone b-wave in photopic ERG (Figure 2B) in eight of the nine tested animals and no identifiable scotopic a-wave in scotopic ERG (Figure 2A,C,D).

Guanylyl cyclase expression in the transgenic retinas: RetGC activity, undetectable in the non-injected eyes of GCdKO mice [7], was imparted by the wild-type and R1091x *GUCY2D* transgenes but remained virtually undetectable in the retinas injected with the S248W construct (Figure 3A). The wild-type and R1091x RetGC1 protein products of the AAV-delivered *GUCY2D* transgenes were robust on immunoblotting (Figure 3B), but S248W was barely detectable. Despite the lack of obvious protein expression of restoration of retinal function, S248W GUCY2D cDNA transcripts were clearly present in two AAV-S248W *GUCY2D* treated eyes (Figure 3C).

Localization of the AAV-delivered LCA1 GUCY2D mutants in the retina: In the wild-type and R1091x *GUCY2D* cDNA-treated retinas, the immunofluorescence for the AAV-mediated cyclase expression was strong in the rods and cones (Figure 4). The brightest signal in both cases was present in the outer segments. In contrast, S248W RetGC1 was occasionally detected in sparse cones but remained virtually undetectable in the rod outer segments. The overall localization patterns correlated with the presence of rod and cone ERG in the wild-type *GUCY2D* and R1091x *GUCY2D*-treated retinas and only marginal cone responses in the S248W *GUCY2D*-treated mice (Figure 2).

Preservation of S248W RetGC1 sensitivity to RD3: In the absence of a RetGC-inhibiting protein (RD3), mouse photoreceptors fail to accumulate RetGC1 and RetGC2 in the outer segments [14-16]. Therefore, the weak expression of transgenic S248W GUCY2D protein in living photoreceptors prompted us to test whether binding to RD3 was strongly compromised. We used the same HEK293 membrane preparations containing heterologously expressed S248W and wild-type GUCY2D that previously were shown to retain activity in vitro [3]. Recombinant human RD3, which inhibits RetGC1 by competing with GCAP1 [13,30], completely suppressed GCAP1-activated S248W and wild-type GUCY2D activity

in vitro in a similar dose-dependent manner and with nearly identical apparent affinities (Figure 5).

DISCUSSION

As a surprising finding from an earlier study, *GUCY2D* mutants encoded by two recessive LCA1 alleles from human patients remain partially (R1091x) or fully (S248W] active when heterologously expressed in HEK cells [3]. Our present findings help explain why S248W GUCY2D does not sustain vision when another loss-of-function LCA1 allele is present in the same patient. The affected residue is located in the extracellular domain [5,6] of the cyclase, the part mostly exposed into the intradiscal space in the outer segments, which does not participate in regulation of cyclase activity by GCAPs on the cytoplasmic side of the enzyme [17,31]. However, S248W GUCY2D fails to properly express in photoreceptors.

The downfall in S248W expression in the retina most likely occurs at or after the protein synthesis stage (Figure 3): either insufficient synthesis or an accelerated degradation rate or both. The rudimentary cone ERG accompanying the S248W GUCY2D presence in a small number of cone outer segments suggests that the mutant cyclase present in the outer segments retains its enzymatic function, consistent with its unaffected regulatory properties in vitro [3], and therefore is unlikely to undergo global protein unfolding. However, the replacement of a strongly hydrophilic side chain with a strongly hydrophobic residue could affect the folding of the extracellular domain enough to prompt GUCY2D degradation, either as the nascent polypeptide or before it reaches the outer segment. Evidently, the ability to bind RD3 (Figure 5), implicated in facilitating RetGC1 transport to the outer segment [12,14-16], does not help S248W accumulate in the outer segments once the N-terminal portion of the enzyme

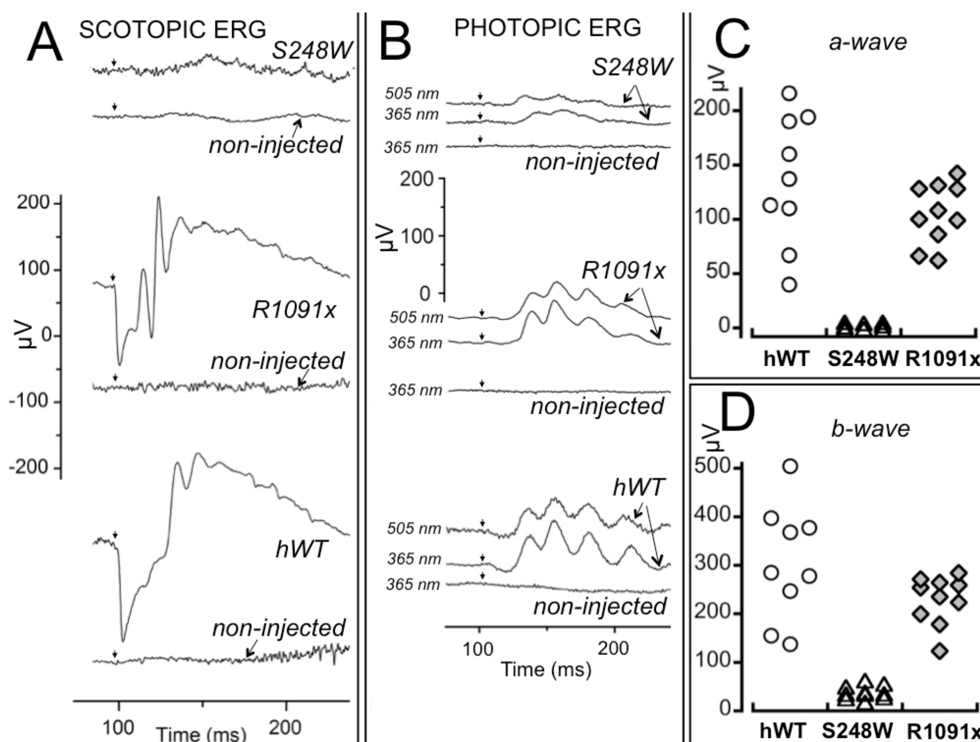

Figure 2. ERG responses to a bright flash imparted by AAV-mediated expression of human wild-type (hWT), S248W, and R1091x RetGC1. **A**: Representative scotopic electroretinography (ERG) in (top to bottom) S248W, R1091x, or wild-type GUCY2D adenoassociated virus (AAV)-injected eyes, each shown above the traces from the non-injected eyes; the small vertical arrows indicate timing of a 505 nm 1.2×10^6 photon μm^{-2} flash. **B**: Representative photopic ERG in (top to bottom) S248W, R1091x, or wild-type GUCY2D AAV-injected eyes; each group of three traces shows responses to 505 nm (upper trace) or 365 nm (middle trace) 6×10^5 photon μm^{-2} flash (small vertical arrows) delivered in the injected eyes and the 365 nm flash delivered in a non-injected eye, all in a constant background light equivalent of 33×10^3 photoisomerizations rod^{-1} s^{-1}. **C**: Maximal voltage deflection amplitudes (mean ± standard deviation, SD) of the scotopic ERG a-wave in the injected eyes among the hWT (136 ± 60 µV, n = 9), S248W (0.4 ± 4 µV, n = 9), and R1091x (105 ± 28 µV, n = 10) treated mice. Note that because the a-wave could not be identified in the ERG traces recorded from the S248W-injected mice, the amplitude of the voltage deflection at 8 ms after the flash was arbitrarily taken for comparison with the two other groups. The differences between the ERG responses in the S248W-injected eyes and the other two groups were statistically highly significant (p≤0.0001; here and from Student *t* test using unpaired data). There was no statistically significant difference between the hWT and R1091x-injected mice (p = 0.177). **D**: Maximal voltage deflection amplitudes of the scotopic b-wave in the same mouse group as panel **C** (305 ± 119, 229 ± 50, and 38 ± 14 µV for the hWT, R1091x, and S248W- injected eyes, respectively). The differences between the ERG responses in the S248W-injected eyes and the other two groups were highly statistically significant (p≤0.00013), but the difference between the hWT and the R1091x-injected mice was not (p = 0.102).

becomes compromised. The presence of S248W GUCY2D–specific immunofluorescence in cell bodies along with sparse cone outer segments (Figure 4) suggests that the affected step could be post-translational rather than cotranslational.

In this study, we used *Gucy2e*$^{-/-}$*Gucy2f*$^{-/-}$ mice, which lack RetGC1 and RetGC2 isozymes, while in patients with LCA1 RetGC2 is unaffected. However, the presence of the unaffected RetGC2 could not compensate for the defects in RetGC1 for the following reasons. RetGC2 is expressed in rods but not in cones [20,32]. In contrast, RetGC1 is the predominant (70–80% total activity) isozyme in mammalian rods and virtually the only isozyme detected in cones [7,20,23]. Consequently, even when GUCY2F remains unaffected in patients with LCA1, only the mutated GUCY2D is present in cones. Catalytic activity of RetGC requires its dimerization [33-35]; thus, in rods RetGC2 would be able to rescue S248W RetGC1, by forming a heterodimer containing the two isozymes. However, this possibility is highly unlikely. Although RetGC1 and RetGC2 can form a heterodimer when heterologously coexpressed in HEK293 cells [36], only homodimers, RetGC1:RetGC1 and RetGC2:RetGC2, are formed in living photoreceptors [36]. Therefore, we reason that the use of the double-RetGC knockout in our study provides adequate conditions for testing the S248W GUCY2D. Moreover, we suggest that using the *Gucy2e*$^{-/-}$*Gucy2f*$^{-/-}$ background in this case is preferable, because these mice retain neither biochemical RetGC2 activity nor rudimentary rod ERG [7,18,30], which are present in *Gucy2e*$^{-/-}$ single-knockout [23] and are capable of obscuring effects of the AAV-delivered S248W GUCY2D.

In a stark contrast to the S248W *GUCY2D* allele, the present in vivo study was not able to elucidate the role of

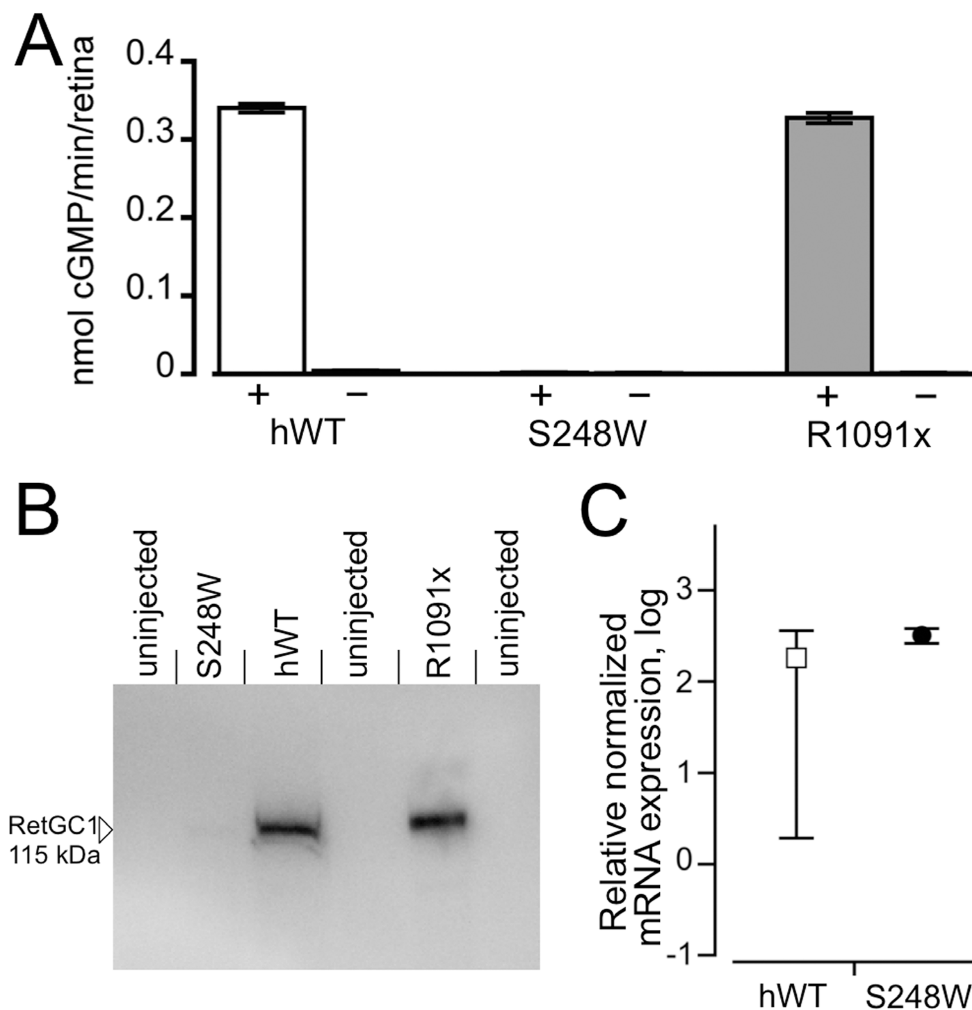

Figure 3. RetGC activity and AAV-mediated GUCY2D expression in GCdKO retinas. **A**: RetGC1 activity (mean ± standard deviation, SD) in retinal homogenates extracted from non-injected (-) eyes compared to those injected (+) with adenoassociated virus (AAV) vectors harboring wild-type (hWT), S248W, or R1091x GUCY2D cDNA. Results are the average of two independent experiments, each using two different mouse eyes. The difference in retinal membrane guanylyl cyclase 1 (RetGC1) activity between the eyes treated with AAV-S248W and the two other groups was highly statistically significant ($p \leq 0.0002$). There was no statistically significant difference between the hWT and R1091x-injected mice ($p = 0.126$). **B**: Western immunoblotting of the retinal proteins extracted from the injected and non-injected eyes probed with anti-RetGC1 antibody. Note the faint signal in the S248W lane compared to the two other GUCY2D variants. **C**: Quantitative real-time (QRT) PCR detection of GUCY2D-specific transcripts in the retinas from three wild-type (hWT) and two S248W GUCY2D-injected *Gucy2e*$^{-/-}$*Gucy2f*$^{-/-}$ knockout (GCdKO) eyes plotted as fold-increase (mean ± standard error, SE) in the GUCY2D transcript signal relative to the non-injected (Log 0) GCdKO retinas.

the R1091x allele in LCA1 pathogenesis. We found that this mutant is expressed well in photoreceptor cells, robustly accumulates in the outer segments of living rods and cones, and effectively enables their responses to light in a fashion similar to the wild-type GUCY2D transgene. A plausible explanation for the obvious paradox between the substantial activity of the R1091x GUCY2D found in a mouse retina and the established genetic link to LCA1 [1,3] is that in human photoreceptors this allele is expressed differently or the expressed protein is delivered to the outer segment through a different pathway than in the mouse. Although little is known about the molecular mechanisms of RetGC processing and trafficking to the outer segment, two RetGC isozymes in mouse rods can evidently utilize different pathways [37]. Therefore, it is also conceivable that trafficking of RetGC1 in primate rods could involve different protein interactions than in rodent photoreceptors and be less permissive for the GUCY2D lacking its C-terminal fragment. Perhaps expression of the human R1091x *GUCY2D* mutant in a GUCY2D-deficient primate animal model could resolve this paradox, but such a model is not available at this time.

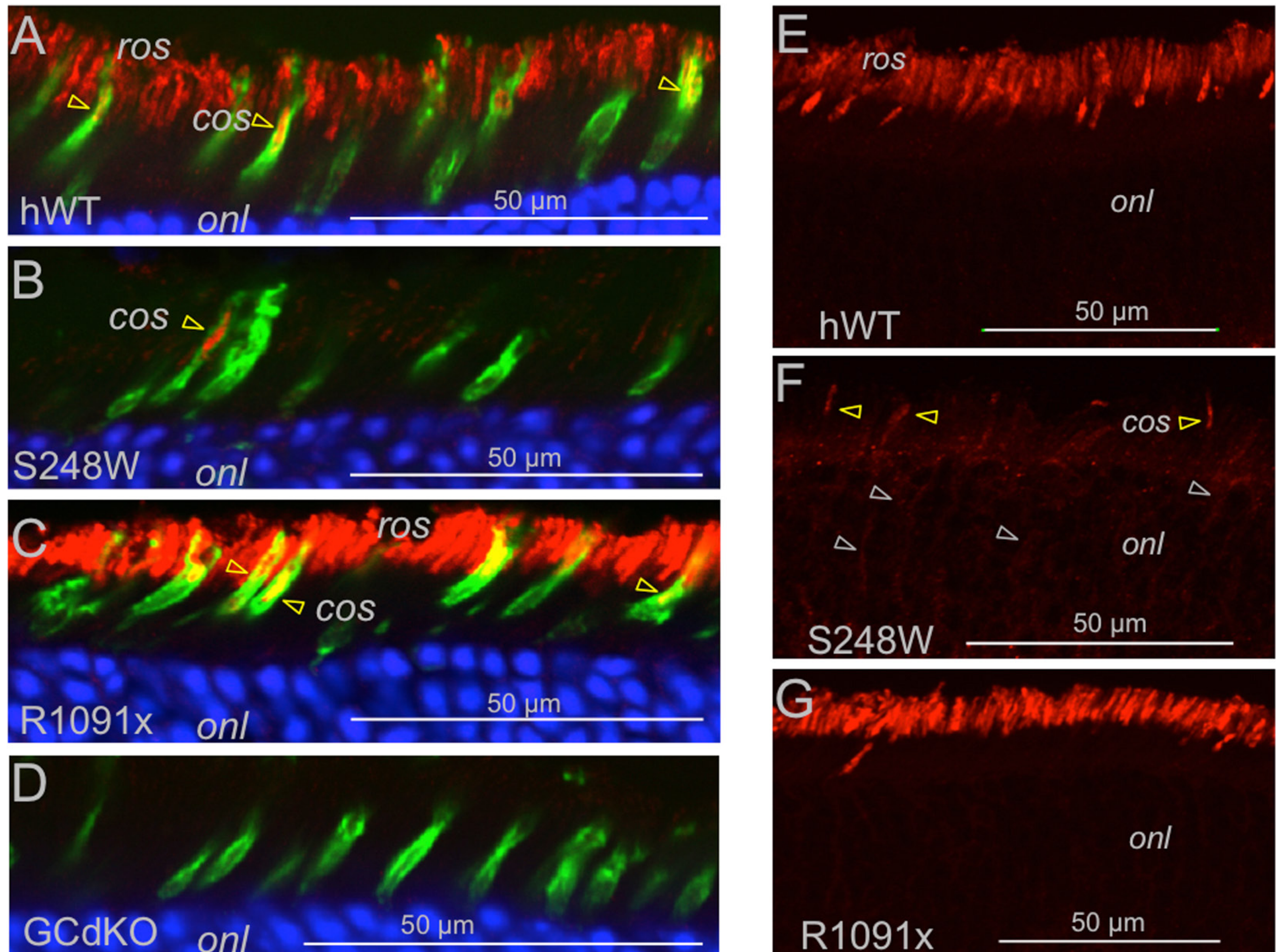

Figure 4. Localization of GUCY2D LCA1 mutants in mouse retinas. **A**–**D**: Confocal retinal membrane guanylyl cyclase 1 (RetGC1) immunofluorescence images of the GUCY2D-injected human wild-type (**A**), S248W (**B**), and R1091x (**C**) or non-injected *Gucy2e*$^{-/-}$*Gucy2* knockout (GCdKO) (**D**) retina sections probed with anti-RetGC1 antibody (red), cone outer segment sheath-staining peanut agglutinin (green), and TOPRO3 iodide (pseudoblue). The yellow arrowheads point at the cone outer segments surrounding the RetGC1 immunofluorescence. In all panels, the anti-RetGC1 fluorescence was recorded in the same experiment using identical laser settings. **E**–**G**: RetGC1 immunostaining in wild-type (**E**), S248W (**F**), and R1091x (**G**) GUCY2D-expressing retinas shown at lower magnification. Note the faint S248W GUCY2D immunofluorescence in the photoreceptor cell bodies and axons in the outer nuclear layer (gray arrowheads), along with sparse cone outer segments (yellow arrowheads) in panel **F**. Bar length = 50 µm. COS = cone outer segments; ROS = rod outer segments; ONL = outer nuclear layer.

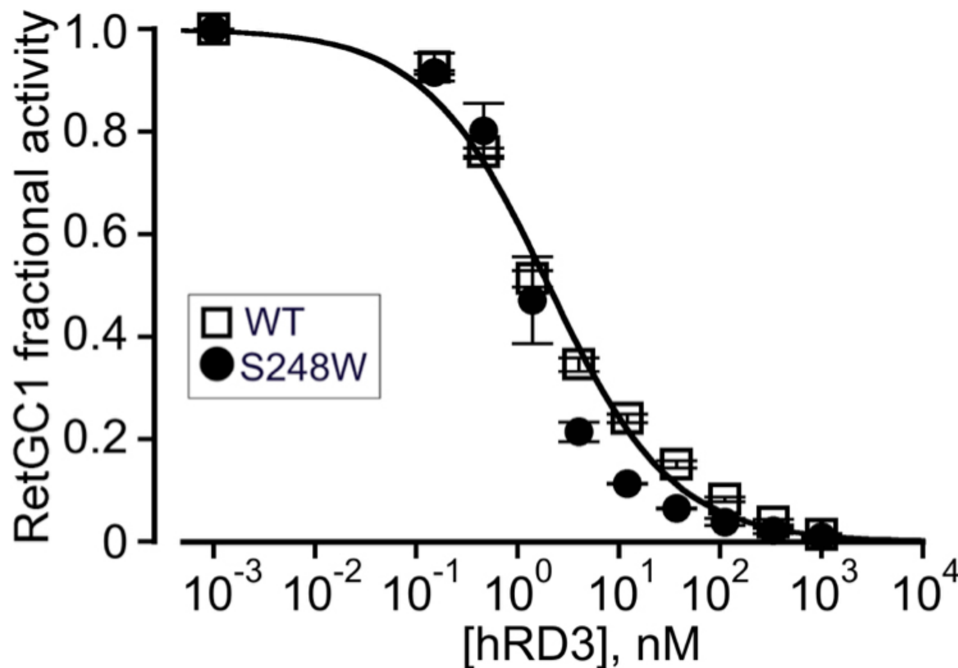

Figure 5. The S248W GUCY2D activated by GCAP1 binds human RD3 in vitro. Fractional guanylyl cyclase activity was measured in human embryonic kidney cells (HEK293) membranes containing recombinant wild-type (open square) or S248W (black circle) GUCY2D reconstituted with 1.5 µM guanylyl cyclase activating protein 1 (GCAP1) and variable concentrations of recombinant human retinal degeneration 3 (RD3) in the presence of 2 mM EGTA. The EC_{50} for RD3-dependent inhibition from the fit assuming the Hill function was 1.9 ± 0.3 nM (n = 5) and 1.4 ± 0.1 nM (n = 3), respectively (p = 0.07). Error bars = standard deviation.

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