Stem Cells and Regenerative Medicine

Disease modeling and pharmacological rescue of autosomal dominant Retinitis Pigmentosa associated with *RHO* copy number variation

Sangeetha Kandoi, Cassandra Martinez, Kevin Xu Chen, Miika Mehine, Brian C. Mansfield, Jacque L. Duncan, Deepak A. Lamba

Department of Ophthalmology, University of California San Francisco, CA, USA • Eli and Edythe Broad Center of Regeneration Medicine & Stem Cell Research, University of California San Francisco, CA, USA • Blueprint Genetics, Helsinki, Finland • Section on Cellular Differentiation, Division of Translational Medicine, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD USA

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Abstract

Retinitis pigmentosa (RP), a heterogenous group of inherited retinal disorder causes slow progressive vision loss with no effective treatments available. Mutations in the rhodopsin gene (*RHO*), account for ~40% cases of autosomal dominant RP (adRP). In this study, we describe the disease characteristics of the first ever reported mono-allelic copy number variation (CNV) in *RHO* as a novel cause of adRP. We (1) show advanced retinal degeneration in a male patient (late 60s) harboring four transcriptionally active intact copies of rhodopsin, (2) recapitulated the clinical phenotypes using retinal organoids, and (3) assessed the utilization of a small-drug like molecule, Photoregulin3 (PR3), as a clinically viable strategy to target and modify disease progression in RP patient associated with *RHO*-CNV. Patient retinal organoids showed the survival of photoreceptors with rudimentary outer segments, where rod photoreceptors displayed stunted outer segments with semi-occasional elongated cilia-like projections (microscopy); increased *RHO* mRNA expression (qRT-PCR and bulk RNA-sequencing); along with elevated levels and mislocalization of rhodopsin protein (RHO) within the cell body of rod photoreceptors (western blotting and immunohistochemistry) over the extended (300-days) culture time period. Lastly, we utilized PR3 to target *NR2E3*, an upstream regulator of *RHO*, to effectively alter the *RHO* expression and observed a partial rescue of RHO protein localization from the cell body to the inner/outer segments of rod photoreceptors in patient organoids. These results provided a proof-of-principle for personalized medicine and suggest that *RHO* expression requires a precise control. Taken together, this study supports the clinical data indicating that adRP due to *RHO*-CNV develops due to a dominant negative gain of function.

eLife assessment

This study presents an **important** finding that implicates a rhodopsin gene duplication in the progression of autosomal dominant retinitis pigmentosa in patients. The authors utilize a retinal organoid model to demonstrate a similar disease phenotype and suggest defects can be ameliorated by using photoregulin. However, the data are **incomplete** and require additional controls and analyses to support their conclusions. If validated the work will be of broad interest to vision researchers.

Introduction

Retinitis pigmentosa (RP) is a genetically heterogenous group of 'rod-cone' photoreceptor degenerative diseases that are unified by common clinical features characterized by progressive vision loss, commonly starting as night blindness(O'Neal and Luther 2023). RP affects roughly 1 in 3000-5000 individuals and is inherited as autosomal recessive, autosomal dominant (ad) or X-linked disease(Chizzolini et al. 2011). adRP can be caused by mutations in at least 24 different known genes ("RetNet: Summaries" n.d.)amongst which mutations in rhodopsin gene (*RHO*) accounts for 25% of total adRP cases(Meng, Ragi, and Tsang 2020). Mutation in the *RHO* was also the first identified cause of RP with a single-base substitution at codon 23 (P23H) leading to protein misfolding and triggering the death of the rod photoreceptors(Dryja et al. 1990). *RHO* is located on the long arm of chromosome 3 (3q22.1) and drives the expression of a 348 amino-acid G protein-coupled receptor (GPCR) with seven transmembrane domains, a luminal N terminus, and a cytoplasmic C terminus. Morphologically, the rhodopsin protein (RHO) is localized in the densely packed disc membrane of the rod photoreceptor outer segments. Currently >150 different rhodopsin mutations have been identified, all contributing through multiple mechanisms with each having distinct consequences on the protein structure and function(Athanasiou et al. 2018). Based on the experimentally studied biochemical and cellular characteristics, several mechanisms have been linked with *RHO*-mutation to cause photoreceptor degenerations including protein - misfolding, retention and instability in endoplasmic reticulum (ER), glycosylation defects, post Golgi trafficking and outer segment targeting, dimerization deficiency, altered post-translational modifications and reduced stability, disrupted vesicular trafficking and endocytosis, impaired trafficking, all leading to constitutive phototransduction activation or altered transducin interactions(Newton and Megaw 2020).

In a conference proceedings, we reported copy number variations (CNV) in *RHO* as a novel cause of adRP(Duncan et al. 2019). The current study presents a unique opportunity to better understand the pathogenic effects of two extra copies of intact wild-type *RHO* on a single allele at 3q22.1 in a male patient (late 60s) diagnosed with adRP. Transgenic mice overexpressing wild-type *Rho* have previously demonstrated photoreceptor degeneration, however the precise mechanism of degeneration is still unclear(Olsson et al. 1992). Although mice have similar genetics to humans, the distribution, subtypes, quantity of retinal cells (especially photoreceptor cells), and the developmental timeline of the retina, differs greatly(Volland et al. 2015). Therefore, access to human cells and tissues *vis-à-vis* the retinal organoid model provides a reliable, translational, and clinically relevant system to gain insights in understanding the pathogenic effects of excessive rhodopsin in photoreceptors. Induced pluripotent stem cell (iPSC)-based models have been used to model several retinal degenerations such as Retinitis Pigmentosa (Tucker et al. 2013; Giacalone et al. 2019), Usher's syndrome (Dulla et al. 2021), Leber congenital amaurosis (LCA) (Parfitt et al. 2016; Kruczek et al. 2021) and *CRX*-associated LCA7 (Chirco et al. 2021). This report

aimed at presenting the late-onset *RHO*-CNV associated with Retinitis Pigmentosa expands the spotlight on modelling a novel cause of retinal disease *via* a cutting-edge 3D 'disease-in-a-dish' platform.

Patient-specific retinal organoids serves as a versatile tool for testing various therapeutic interventions including small-drug like molecules which aims at modulating the pathways (Moore, SkowronskaLKrawczyk, and Chao 2020; Liu et al. 2021). Small molecule-based targeted therapeutics have the potential to cross the blood-brain barrier when administered systemically and can be therapeutically titrated. Amongst these, nuclear receptors are important targets as they have druggable ligand-binding sites. Approximately 15% of approved drugs, target at least 48 members of human nuclear receptors superfamily, and 10 amongst these are orphan(Zhao, Zhou, and Gustafsson 2019). Numerous orphan nuclear receptors are expressed in the retina, of which rod-specific nuclear receptor subfamily 2 group E member 3 (*NR2E3*) is a direct target of neural retina leucine zipper (*NRL*), the main rod-specifying gene(Kobayashi et al. 1999). *NR2E3* is expressed very early in post-mitotic rods and coactivates the transcription of rod-specific genes including *RHO*, with *CRX* and *NRL*(Bumsted O'Brien et al. 2004; Cheng et al. 2004; Mitton et al. 2000). Recent studies have identified photoregulins (PR), a small-drug like molecules that can target *NR2E3* and modify the disease progression in mouse models of rod photoreceptor mutation-associated RP (Nakamura et al. 2016, 2017). We tested the hypothesis that PR targeting *NR2E3* can mitigate the deleterious effects of the *RHO*-CNVs on *in vitro* patient-specific retinal organoids. Overall, we demonstrated the establishment of a human retinal organoid model of *RHO*-CNV associated with adRP to gain critical insights into the pathogenesis of disease and to provide a vital screening tool for the development of various novel therapies.

Results

RHO-CNV patient presented clinical features of adRP

The clinical and ERG data of one patient and one healthy unaffected daughter of the patient is included (**Table 1**). Complete ophthalmological examination by fundus photography and SD-OCT revealed features of RP (Hirji 2023) including bone spicule-like pigmentation changes, optic disc pallor and attenuation of retinal blood vessels (**Figure 1A, Supplementary Figure 1**), with outer retinal atrophy due to the loss of the photoreceptor layers, sparing the central foveal region (**Figure 1B**). Genetic testing of the proband by next-generation sequencing (NGS) showed a complex duplication-rearrangement of chromosome 3q22, which encompassed the entire *RHO* coding sequence, 5' and 3' regulatory regions and flanking genes. The rearrangement consisted of a 48 kb triplicated region embedded within a 188 kb duplication, resulting in three apparently intact *RHO* genes on one chromosome and a fourth, unaltered *RHO* gene on the homologous chromosome. Each of the three intact copies were flanked by *H1FOO*, *IFT122*, *EFCAB12* and *MBD4*, suggesting a duplication-inverted triplication-duplication event, in which the triplicated segment is inverted and located between correctly oriented genomic segments (**Figure 1C**). No other additional causative variants of inherited retinal degeneration candidate genes were identified. Whole genome sequencing supported the rearrangement and extra copies of *RHO* which were identified by NGS. The *RHO* copy number variants were not detected in the unaffected daughter of the patient. Clinically collected four-generation pedigree data of the family included two male family members affected with RP, indicating male-male transmission, consistent with an autosomal dominant inheritance. The proband's affected father was not examined as he was deceased but had been diagnosed clinically with RP. The clinical findings were consistent with the hypothesis that CNV in the wild-type *RHO* causes adRP.

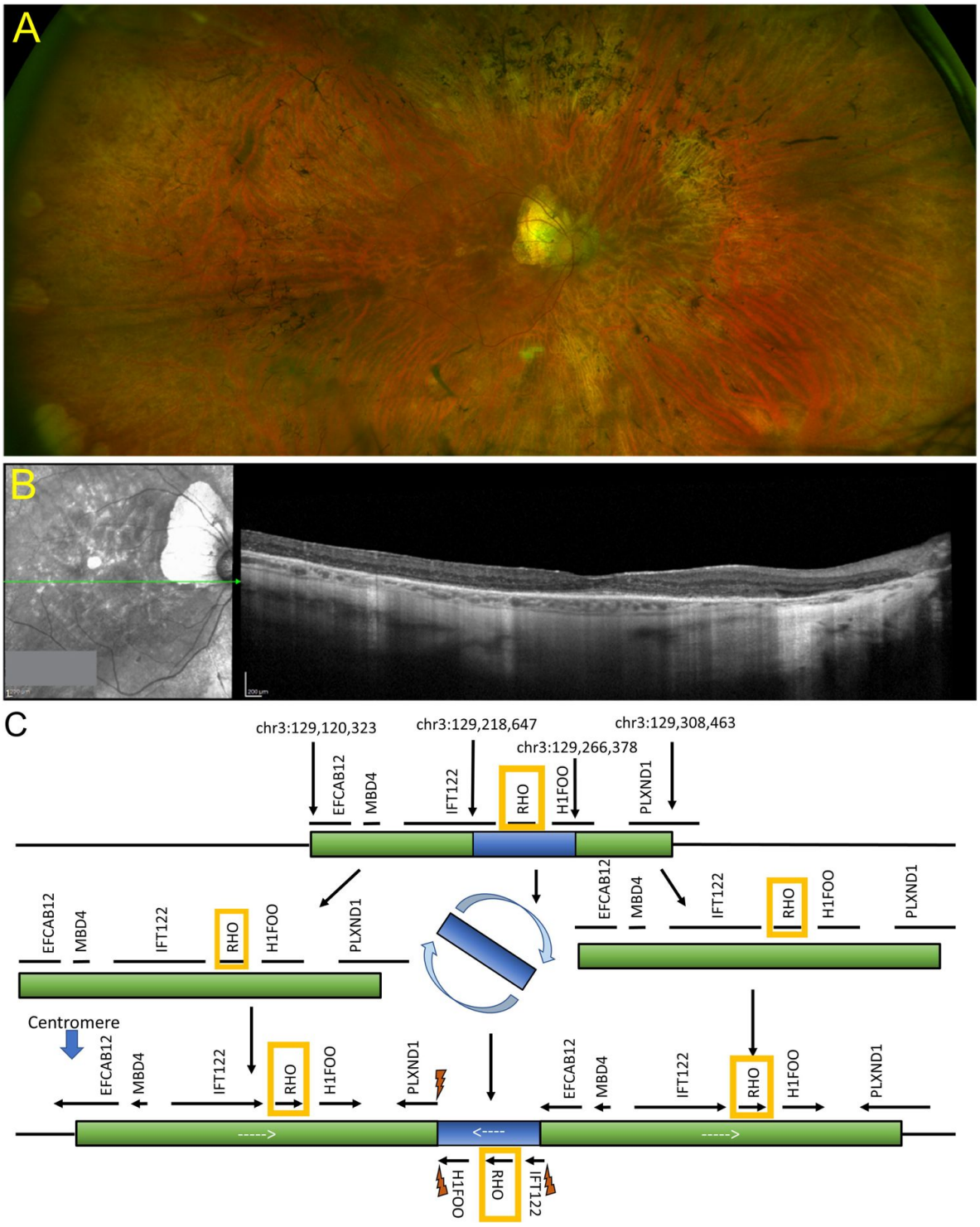

Figure 1

Retinal imaging and next generation sequencing.

(A) Ophthalmological color fundus examination of a patient clinically diagnosed of *RHO*-CNV displaying bone spicule pigmentary changes. **(B)** SD-OCT image showing extensive loss of the outer retinal layers leaving a small intact island at the fovea. **(C)** Schematic illustration of NGS using a 266-gene retinal dystrophy panel showing a complex chromosome 3q22 duplication-rearrangement resulting in an inverted 48 kb triplicated region embedded within a 188 kb duplication forming three apparently intact *RHO* genes on one allele and a fourth, unaltered *RHO* on the homologous allele. NGS=Next generation sequencing; SD-OCT= Spectral domain-optical coherence tomography.

RHO-CNV retinal organoids exhibit photoreceptor maturation defects

To assess the effects of *RHO*-CNV in human retinal organoids, we initially reprogrammed the peripheral blood mononuclear cells (PBMNCs) from one patient with four copies of *RHO* (RM) as well as the corresponding familial control (RC) into iPSCs (**Supplementary Figure 2A-B**). The iPSC lines had the colony morphology of tightly packed cells with a high nucleus-to-cytoplasm ratio, a well-defined border typical of stem cells, and expression of pluripotent markers (**Supplementary Figure 2C-D**). We then differentiated the patient and control iPSC lines into retinal organoids by following our previously published protocols(Bachu et al. 2022; Arthur et al. 2022), as per the differentiation timeline depicted in **Figure 2A**. Retinal organoids from the *RHO*-CNV patient and the control displayed a well-defined neuroepithelial lamination, indicating the arrangement of photoreceptors on the apical surface of the organoids and a dark central basal region consisting of ganglion cells by phase contrast microscopy (**Supplementary Figure 2E**). Notably, there were no clear visible anatomical changes in apical-basal retinal cell type distribution during the early differentiation timeframe, when the cone and rod photoreceptors are usually born in 45-50-days-old and 90-120-days-old human retinal organoids, respectively (data not shown). Over the prolonged differentiation culture timeframe (>200 days), the control retinal organoids displayed long hair-like protrusions which were presumptive inner and outer segments at the apical side of the retinal organoids, a critical event indicating the start of photoreceptor maturation. Conversely, the patient retinal organoids showed short initial hair-like protrusions that did not elongate at the extended culture time of 260 and 300 days in culture (**Figure 2B**). Of note, the patient retinal organoids presented semi-occasional elongated cilia-like projections within the uniformly stunted inner/outer segments by the light microscopy (**Supplementary Figure 2E, Figure 2B**). To validate these observations, we examined the ultrastructure of photoreceptors in the *RHO*-CNV organoids using transmission electron microscopy (**Figure 2C**). Upon assessing the 300-days-old organoids, we observed that while the patient organoids developed connecting cilium and inner segments similar to control organoids, they failed to extend outer segments. This data was consistent across multiple rounds of differentiation from three independent clones of patient iPSCs. Taken together, these morphological changes suggest that iPSC-retinal organoids of *RHO*-CNV patient demonstrated the survival of photoreceptors with OS dysmorphogenesis over the time course of 300-days.

RHO-CNV retinal organoids revealed conspicuous defects in rod phototransduction and ciliary transcripts

Control and patient retinal organoids were analyzed at two developmental stages: at rod photoreceptor birth (D120), and maturation (D300). For each sample, qRT-PCR was carried out by utilizing primers designed to nine key genes that regulate the development and maturation of rod photoreceptors. mRNA levels were analyzed for the expression of genes specific to early pan photoreceptors (*OTX2, CRX, RCVRN*), early rod photoreceptors (*NRL, NR2E3*), rod-specific phototransduction (*PDE6B, SAG, RHO*) and ciliary (*IFT122*) genes (**Supplementary Table 4**). To equilibrate the data to equivalent the number of photoreceptors in organoids, we compared the targeted photoreceptor gene expression by normalizing to *CRX*, a ubiquitously expressing photoreceptor gene maintained from development to adulthood(Yamamoto et al. 2020). The expression of all the target genes were detected at each time point (D120 and D300) in the retinal organoids (**Figure 3A**). Pan-photoreceptor and early rod marker genes showed similar expression levels with no noticeable variations in RC and RM organoids. In contrast, the phototransduction and ciliary genes were expressed at a higher level in the patient than in control organoids. More relevant to the current study, there was a significant ~3 $\log_2$ fold change (log2FC) increases in the *RHO* levels at D120 and D300 in the patient organoids. We also observed a small ~1 log2FC, statistically significant increase in rod/visual arrestin (*SAG*) at D300. Relatedly, a small ~1 log2FC, non-statistical increases was observed in *IFT122, a* gene reported to be triplicated in NGS along with *RHO* (**Figure 1C**).

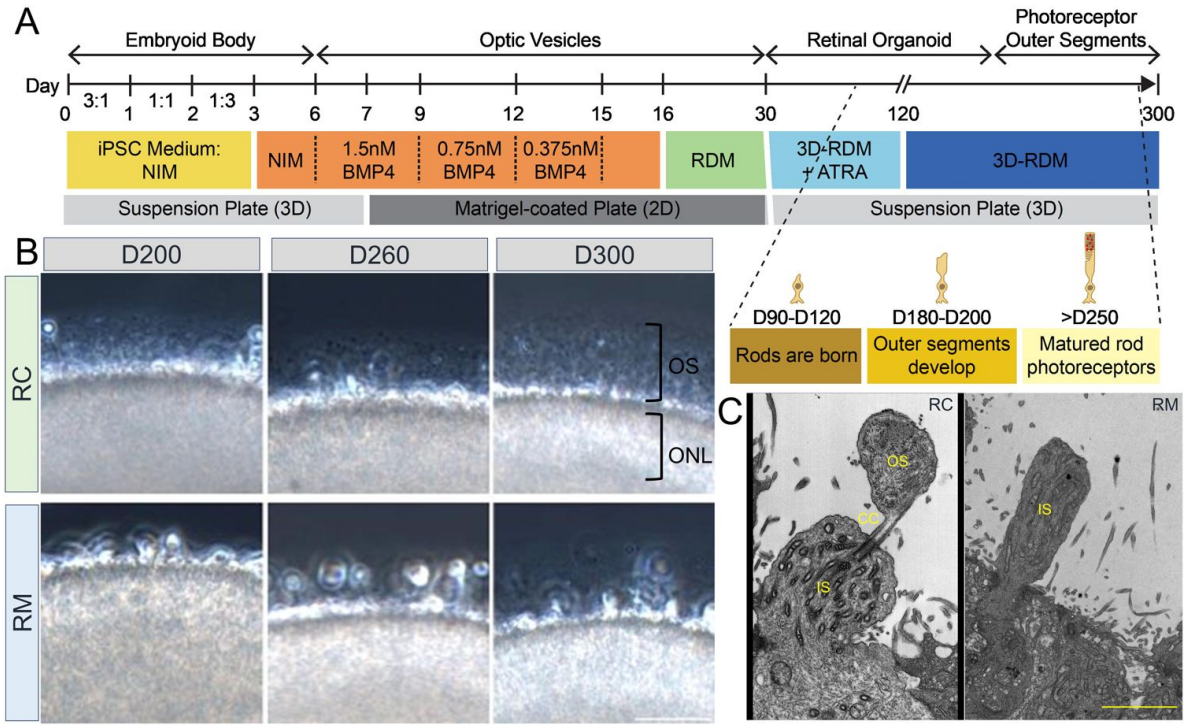

Figure 2

RHO-CNV disease modeling using iPSC-derived retinal organoids showed morphological defects.

(A) Schematic representation showing the timeline of human retinal differentiation and maturation including the birth and development of rod photoreceptors. **(B)** Phase contrast microscopy images showing OS, long hair-like protrusions from ONL of the differentiated photoreceptors present at the apical surface of control (RC) retinal organoids at day 200, 260 and 300 (top). Conversely, retinal organoids from patient (RM) showing shorter protrusions which do not extend progressively over long-term culturing indicating maturation defects (bottom). **(C)** Electron microscopy images showing ultra-magnification of distinct OS, IS and CC structures of rod photoreceptors in control organoids and the absence of OS in patient organoids. CC=Connecting cilium; IS=Inner segments; ONL= outer nuclear layer; OS=Outer segments. Scale bar = 50 µm.

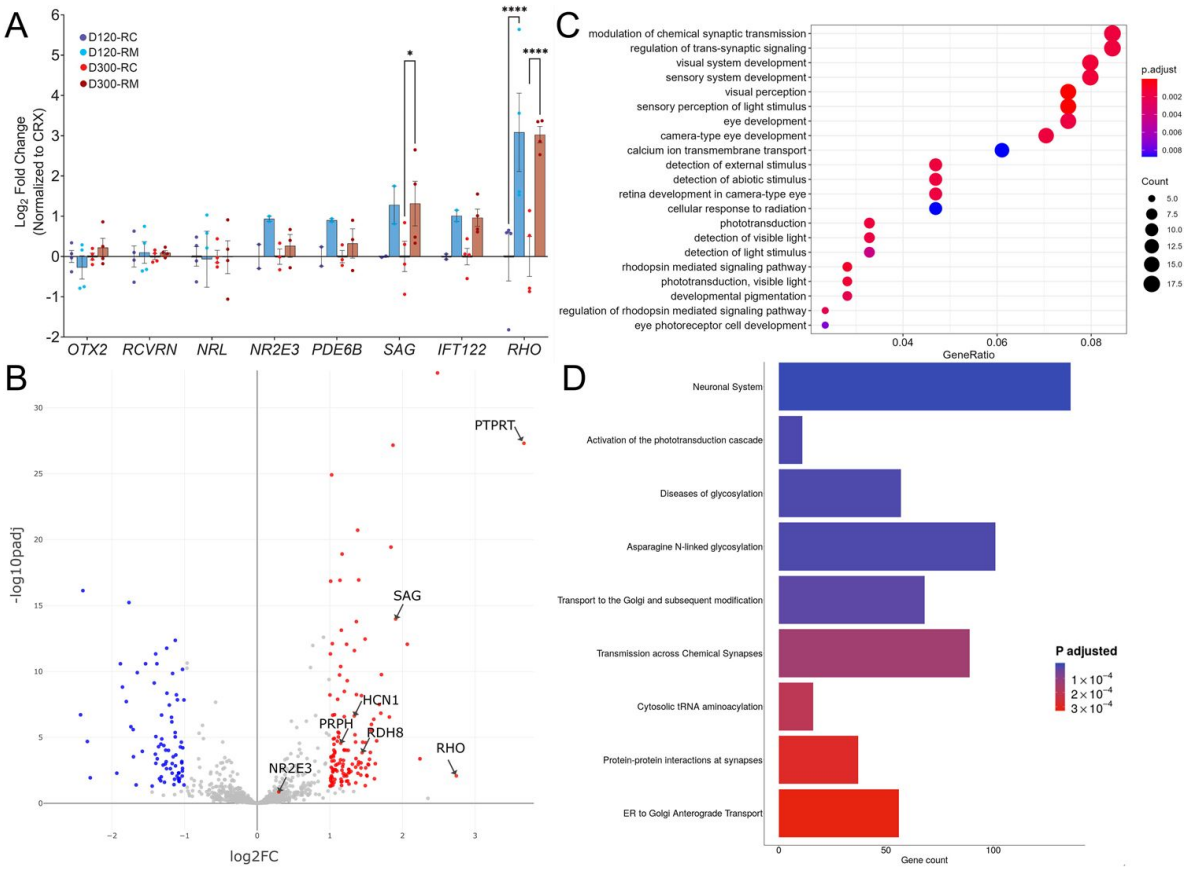

Figure 3

Transcriptomic analysis of *RHO*-CNV retinal organoids presented elevated RHO expression.

(A) qRT-PCR analysis shows ~3 Log2FC increased *RHO* mRNA levels in patient (RM) organoids at both the time-points of rod differentiation and maturation. No significant change was observed in other photoreceptor genes except for a small ~1 Log2FC increase in rod arrestin (SAG) at D300 time-point. Log2FC=Log 2 Foldchange. Statistical two-way ANOVA analysis with Fisher's LSD test and 95% confidence interval. * = p <0.05, and **** = p <0.0001. Blue bars, D120; Maroon bars, D300 **(B)** Volcano plot showing significant differentially expressed genes. Significantly upregulated genes are highlighted in red and significantly downregulated genes are highlighted in blue (adjusted p<0.01). **(C)** Dot plot showing EnrichGO analysis of biological process on the differentially expressed genes. The size of the dot represents number of differentially expressed genes in the pathway and the X-axis represent the ratio over all genes associated with the pathway. Plot shows a defect in rods and phototransduction associated pathways as well as synaptic transmission suggesting rod dysfunction. **(D)** Box plot showing the data from pathway enrichment analysis of cellular component category predominantly highlighting the defect in glycosylation and Golgi/ER modification/transport. Colors in the dot and blot plots represent relative significance (calculated p-values in scale). N=3-4 independent experiments and 12-15 organoids per experiment.

To advance a better molecular understanding on detailed genomic expression and gene regulatory networks, we performed bulk-RNA sequencing on 300-days-old retinal organoids (n=3 independent biological replicates). Patient retinal organoids demonstrated upregulated transcriptomic levels of *RHO* (~3 log2FC) and *SAG* (~2 log2FC), comparable to the qRT-PCR data (**Figure 3B**). Additionally, we also observed increased expression in disc structure support gene including *PRPH*, visual cycle genes *RDH8* and *HCN1*, and a synaptic gene, *PTPRT* in patient relative to control organoids (**Figure 3B**). Following the Gene Ontology enrichment analysis (EnrichGO) of significantly differentially up-regulated genes, we confirmed differential increases in phototransduction cascades including the visual/light perception and membrane potential/Ca+ signaling pathways in patient organoids. Additionally, increases in genes associated with the synaptic signaling pathway were also detected, suggestive of photoreceptor dysfunction (**Figure 3C**). Pathway enrichment analysis of significantly differentially expressed genes for cellular components category pointed to perturbation in pathways associated with defects in glycosylation especially N-linked glycosylation as well as ER and Golgi transport (**Figure 3D**, **Table 2**), highlighting potential pathophysiology indicative mechanisms of *RHO*-CNV associated RP. Although glycosylation is not required for the rhodopsin biosynthesis, N-linked glycosylation (at N2 and N15), a post-translational modification, is a necessary step for interacting with chaperones during ER transport. This is also an essential step towards incorporation of the heptahelical G-protein coupled receptor rhodopsin in the rod outer segments. Thus, RNAseq data suggests that the defects in rhodopsin glycosylation decreased the ability of rhodopsin to exit ER, and leads to an adRP phenotype (Tam and Moritz 2009; C.LH. Sung and Tai 1999).

RHO-CNV retinal organoids displayed mis-localized and elevated Rhodopsin protein levels

Immunofluorescence staining on the cross sections of control and the patient retinal organoids was examined for the spatial location of the photoreceptors at three time points (D120, D200, D300). Pan-photoreceptor precursor and progenitor proteins (OTX2, CRX), and rod specific proteins (NR2E3) were expressed in the apical layer of the organoids in a similar pattern both in the control and patient with preserved photoreceptors and outer nuclear layer thickness (**Supplementary Figure 3A-C**). These results corroborated our gene expression and transcriptomics data (**Figure 3A-B**) indicating no defects in rod biogenesis within the patient retinal organoids. As the organoids began to mature, the distribution of RHO protein was observed in the outer segments of control organoids starting at D200, earliest timepoint for detection in human retinal organoids using our differentiation protocol. Compared to the control, the patient organoid had mis-localized RHO protein accumulating in the photoreceptor cell soma, at all analyzed time-points (D200, and >D250) (**Figure 4A-B**). Furthermore, the patient organoids showed semi-occasional RHO in OS which were notably consistent with our results discerned by light microcopy (**Supplementary Figure 2E, Figure 2B**). Co-staining with NRL and OTX2 confirmed mislocalized RHO expression in the rod photoreceptor soma within patient organoids. We also evaluated the expression of rod-specific phototransduction protein, SAG, a cytoplasmic marker, which was increased in the outer nuclear layer of patient retinal organoids (**Figure 4C**).

To investigate protein expression levels in *RHO*-CNV organoids, we quantitated the wild-type level of RHO and SAG by western blot. Patient retinal organoid homogenates displayed a significant 16-fold and 9-fold higher fractions of ~40 kDa monomer and ~80 kDa dimer rhodopsin content respectively in patient organoids relative to controls despite loading equal amounts of protein lysates by western blot (**Figure 4D-D''**). Additionally, a significant 1.5-fold increase in ~48 kDa SAG, a rhodopsin interacting protein was also observed (**Figure 4E-E'**). These findings suggest that *RHO*-CNV organoids actively translated the excessive rhodopsin protein which presumably did not undergo the necessary post-translational modifications for the packaging and transport to the rod outer segments, ultimately leading to dysfunction and potential death of rod photoreceptors in the patient.

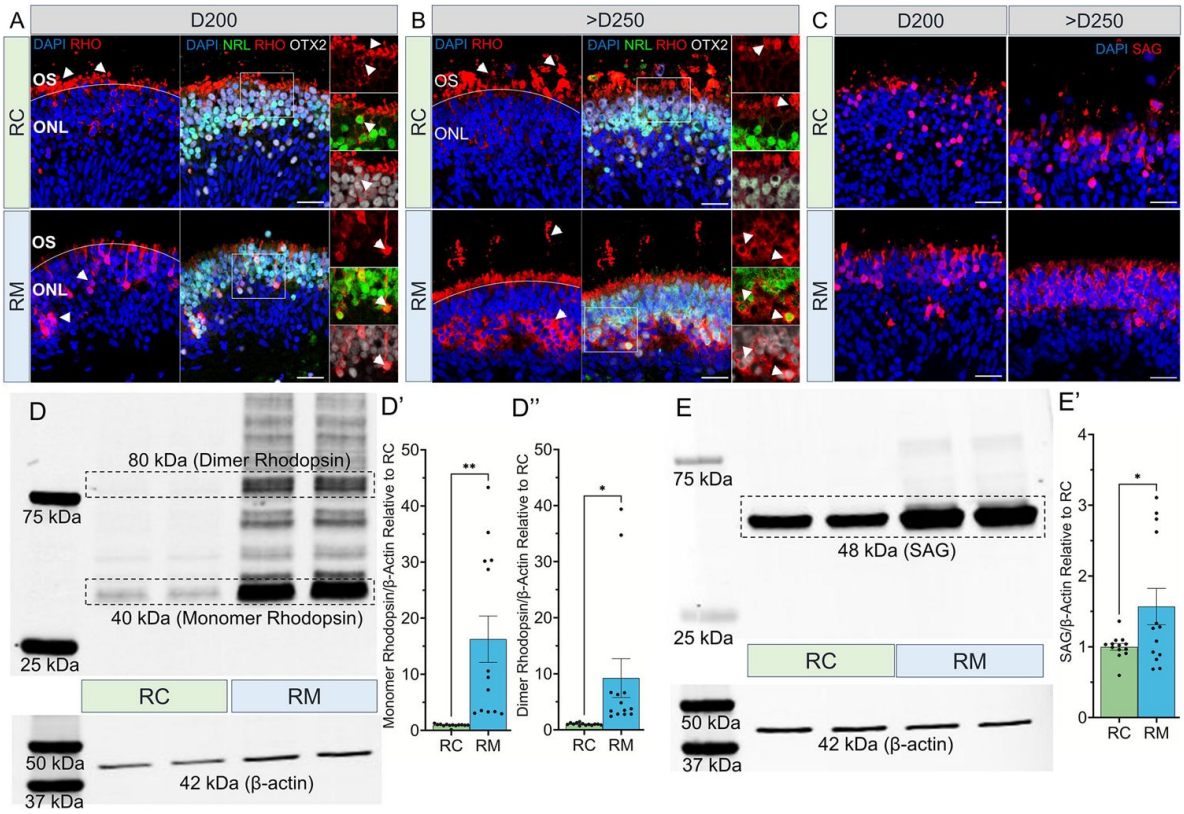

Figure 4

Rhodopsin protein mislocalization and increased levels in *RHO*-CNV retinal organoids.

(A-B) Immunofluorescence staining of RHO, NRL and OTX2 displaying the proper RHO localization (arrowheads) in the outer segments and RHO mislocalization (arrowheads) in the cell body of photoreceptors within the control (RC; top) and patient (RM; bottom) organoids respectively at two time-points, D200, and >D250. Occasional outer segments with proper RHO localization in the patient (RM) organoids were seen at >D250 time-point **(C)** SAG expression (red) followed a similar trajectory as RHO, with increased labelling in patient organoids (bottom) over control (top). DAPI (blue) labels nuclei. OS=outer segment; ONL=outer nuclear layer. Scale bar = 25 µm. **(D-E)** Blot probed for RHO and SAG showing increased levels of 40 kDa monomer and 80 kDa dimer, RHO **(D)**, and 48 kDa, SAG **(E)** in patient retinal organoids. β-actin was used as a loading control. Densitometric analysis quantifying the relative intensity of monomeric RHO **(D')**, dimeric RHO **(D'')**, and SAG **(E')** in comparisons to the control. Statistical two-tailed unpaired T-test analysis with 95% confidence level. * = p <0.05, and ** = p <0.01. N=5 independent experiment and 12-15 organoids per experiment.

PR3 treatment attenuates *RHO* expression and partially rescues RHO trafficking in *RHO*-CNV retinal organoids

Since, the excess RHO protein production due to the extra *RHO* copies is likely not being processed appropriately, we aimed to test potential therapies that may reduce RHO protein levels in rods. We targeted orphan nuclear receptor, *NR2E3*, using a small-drug like molecule to assess its effect on *RHO* regulation and expression in the human patient organoid model (**Figure 5A**). A small-molecule drug, PR3, targeted to *NR2E3*, has been previously described to regulate the rod gene expression in Rho^{P23H} mice, suggesting its potential for the use in the treatment of RP (Nakamura et al. 2017). We treated long-term cultured 300-days-old, *RHO*-CNV patient retinal organoids with varying concentrations of PR3 (0.1, 0.25 and 0.5 μM) for one week and assessed the effects on *RHO* mRNA expression and protein localization. Immunofluorescence staining of PR3-treated organoids displayed a partial rescue of RHO localization with optimal trafficking observed in the 0.25 μM PR3-treated organoids (**Figure 5B**). None of the organoids showed any evidence of toxicity post-treatment. PR3 drove a significant downregulation of *RHO* in a dose-dependent manner. Following qRT-PCR analysis, we observed a 2-to-5 log2FC decrease in *RHO* expression, along with smaller decreases in other rod-specific genes including *NR2E3*, *GNAT1 and PDE6B* (**Figure 5C**). We did not see any significant effects of PR3 on blue-, green- and red-cone opsin genes (**Supplementary Figure 4**). Upon comparison of PR3-treated patient organoids with control (RC) organoids. *RHO* expression levels in the patient organoids treated with 0.1 and 0.25 μM PR3 was not significantly different from control organoids. However, 0.5 μM PR3 resulted in significantly decreased *RHO* expression, much lower than the *RHO* levels observed in control organoids (**Figure 5D**). These results set the platform for translational therapeutic studies for achieving the *RHO* expression levels at par to the control-matched organoids.

We further carried out bulk RNA-sequencing analysis to comprehensively characterize three different groups of organoids, 0.25 μM PR3-treated and vehicle-treated patient organoids and control (RC) organoids from three independent differentiation experiments. Consistent with the qRT-PCR gene expression analysis, the results showed a significant downregulation in *RHO* and other rod phototransduction genes including *SAG* and *GNAT1* (**Figure 6A**). Additionally, we confirmed that PR3 did not have any adverse effects on cone opsin transcripts. Principal component analysis (PCA) and normalized read counts analysis of sequenced data for rod (*RHO, SAG*, *GNAT1*, *NR2E3*) and other genes demonstrated that the PR3-treated organoids were more alike to control organoids than the vehicle treated patient organoids (**Figure 6B-C**, **Supplementary Figure 5B**). Upon KEGG analysis of differentially expressed genes associated with the rod phototransduction pathway, we observed that several rod pathway components which were upregulated in patient organoids were potentially salvaged following PR3 treatment (**Figure 6D**). EnrichGO analysis of significantly downregulated genes in visual perception/phototransduction pathways confirmed the specific activity of PR3 in retina (**Figure 6E**). While analysis for significantly upregulated genes showed changes in cilium organization, axoneme assembly, ER to Golgi transport and glycosylation, all of which are critically required events for outer segment formation and protein trafficking (**Figure 6F**, **Supplementary Figure 5A-B**) suggestive rod photoreceptor maturation recovery. Thus, the data presented strongly suggests that PR3, a *NR2E3* modulator, could potentially rescue rod photoreceptor homeostasis in *RHO*-CNV patients.

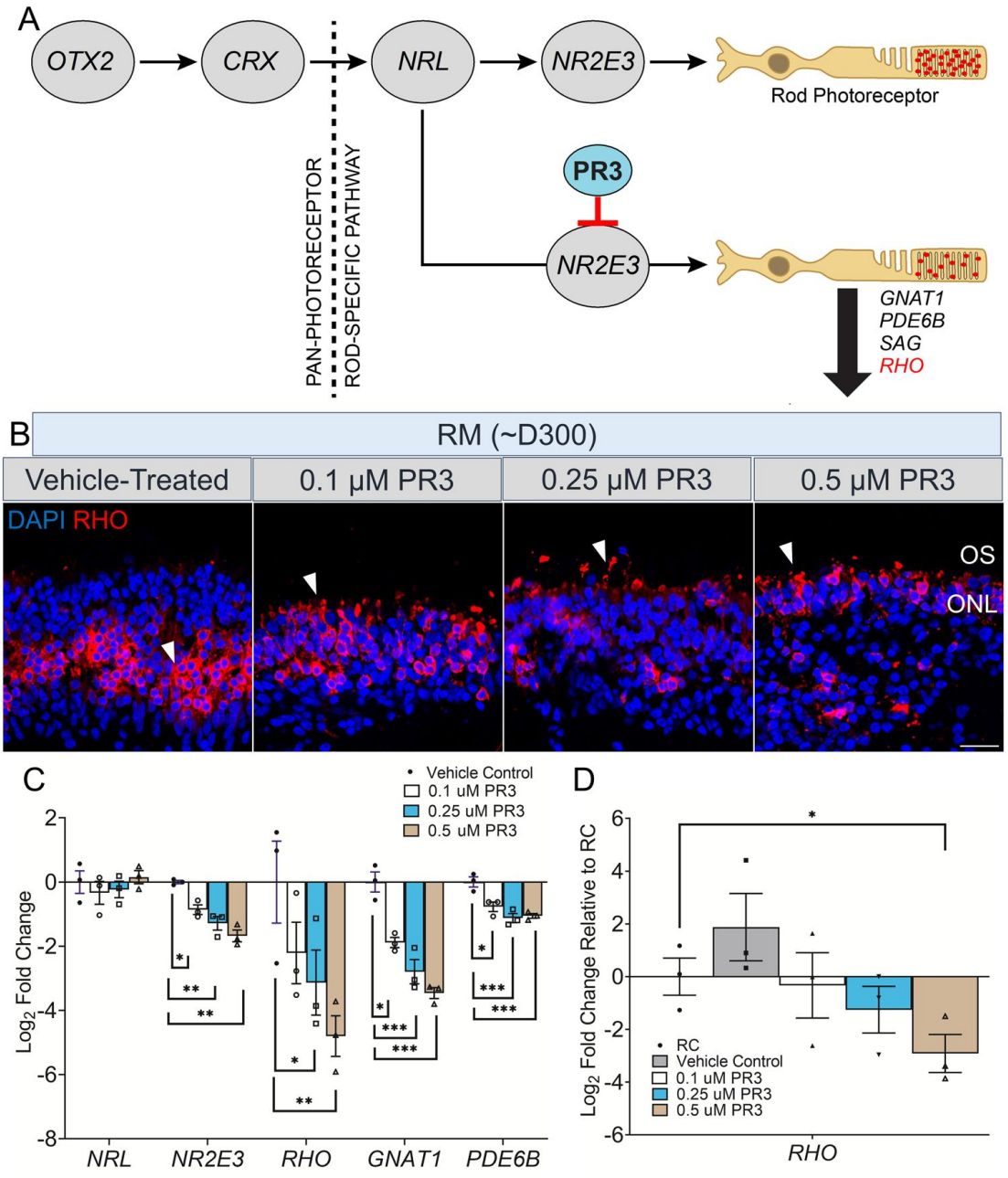

Figure 5

Partial rescue of rhodopsin localization and expression levels in PR3 treated *RHO*-CNV retinal organoids.

(A) Cartoon illustration showing gene expression during the stepwise development and maturation of rod photoreceptors. Small molecule, PR3 acts on *NR2E3* downregulating the expression of *GNAT1, PDE6B, SAG* and *RHO*. **(B)** Immunostaining of ~300-days-old PR3-treated retinal organoid sections from patient (RM) showing the trafficking of RHO protein (arrowheads) towards outer segments at all three doses of PR3, 0.1, 0.25 and 0.5 μM. The most appropriate RHO localization to OS is seen at 0.25 μM PR3 (arrowheads). DAPI (blue) labels nuclei. OS=outer segment; ONL=outer nuclear layer. Scale bar =25 μm. **(C)** qRT-PCR analysis shows ~2-5 Log2FC decrease in *RHO* mRNA levels in a dose-dependent manner for PR3 treated-patient (RM) organoids. No significant change was observed in *NRL*, but a small decrease was observable in *GNAT1, PDE6B* and *NR2E3* (one-way ANOVA analysis with Sidak test and 95% confidence interval). **(D)** qRT-PCR analysis showing a comparison of *RHO* mRNA levels in PR3-treated patient organoids to control (RC) organoids (unpaired t-test). Log2FC=Log2 Fold change. * = p <0.05, ** = p <0.01and *** = p <0.001. N=3-4 independent experiments and 12-15 organoids per experiment.

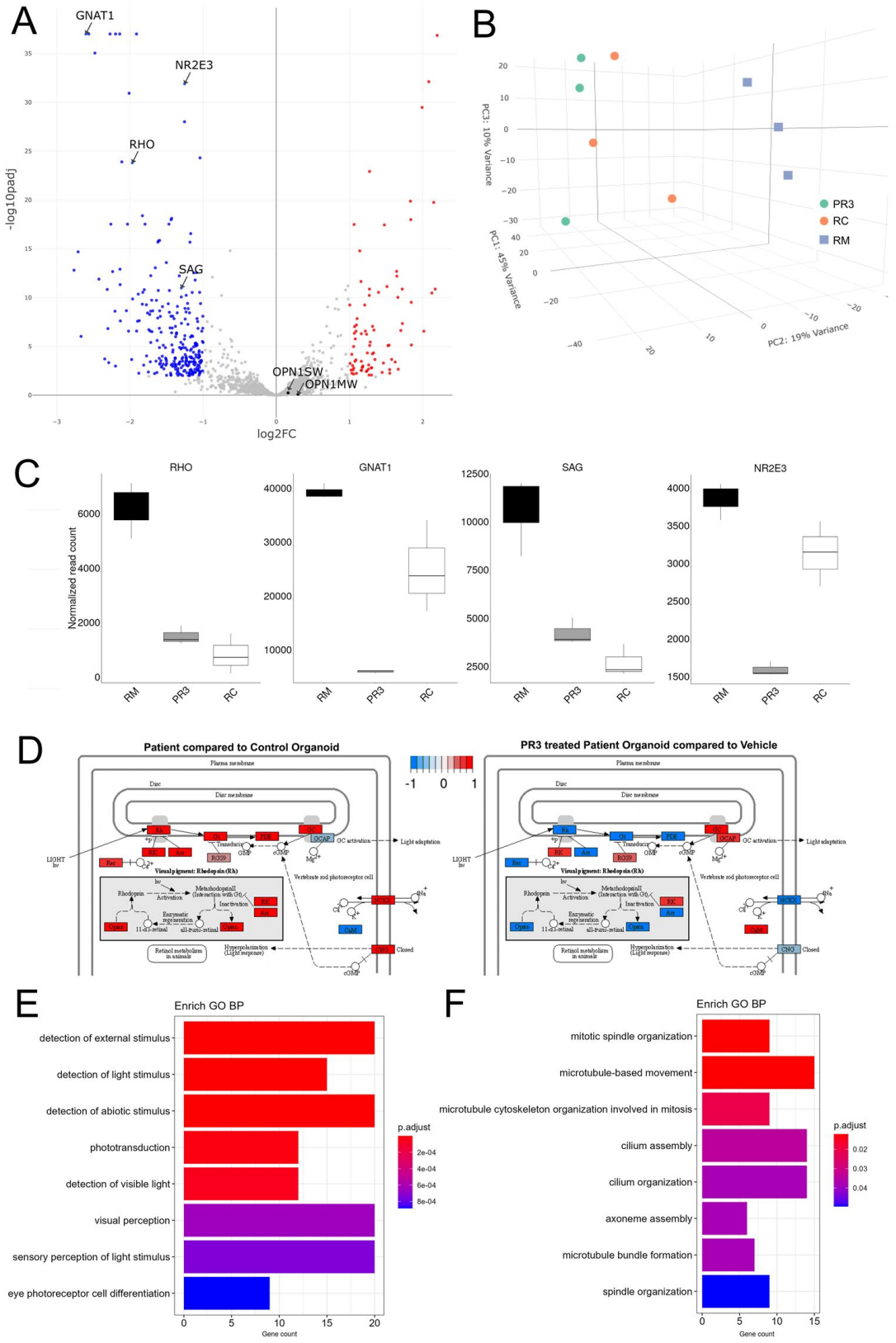

Figure 6:

RNA sequencing analysis of *RHO*-CNV organoids following PR3 treatment.

(A) Volcano plot showing significant differentially expressed genes following 1 week of PR3 treatment in D300+ patient organoids with cutoffs at p<0.01 and ~1log2FC. **(B)** 3D principal component analysis (PCA) plot showing the tightly clustered independent biological replicates. Additionally, PR3 treated patient organoids were spatially closer to control (RC) compared to patient (RM) organoids. **(C)** Normalized read count plots showing relative expression of *RHO, GNAT1, SAG* and *NR2E3*. **(D)** KEGG analysis of differentially expressed genes showing dysregulation of key phototransduction pathway comparing RC with RM organoids and the recovery following PR3 treatment in RM organoids. Down- and up-regulated genes are indicated in blue and red respectively. **(E, F)** Box plots showing EnrichGO analysis of differentially expressed genes that are either downregulated (**E**) or upregulated (**F**) by comparing PR3 treated to vehicle treated organoids. N=3 independent experiments and 12-15 organoids per experiment. Log2FC=Log2 Fold change.

Discussion

To our knowledge, there have been no previous reports of RP associated with multiple copies of wild-type *RHO* in humans. However, retinal degeneration phenotypes have been reported in mice bearing extra copies of *Rho*. A 10-30% increase in rhodopsin expression in mice caused gradual degeneration, while a 3-5-fold increase resulted in severe and rapid photoreceptor loss (Olsson et al. 1992). More recent studies show that the retinal degeneration phenotype correlates closely to the amount of *RHO* overexpression (Tan et al. 2001; Wen et al. 2009). The current study reports the clinical retinal phenotype of a patient with *RHO*-CNV, and photoreceptor abnormalities in iPSC-derived retinal organoids from the patient.

There is an increasing interest in gene augmentation therapeutic strategies for retinal degeneration patients with biallelic mutations in *RPE65*, since the approval of Voretigene neparvovec. Although visual outcomes in most treated patients have been very encouraging with significant improvement in visual field and light sensitivity(Maguire et al. 2021), there have been some recent reports of progressive pericentral atrophy(Gange et al. 2022). One potential cause of retinal pigmented epithelium (RPE) atrophy following *RPE65* gene augmentation could be overexpression of *RPE65* in the AAV-*RPE65* infected cells, due to the utilization of strong promoters such as CAG. Furthermore, the current preclinical approaches to target adRP due to *RHO* are primarily based on gene therapies, with three competing approaches; one to knockdown the mutant *RHO* by replacing with a wild-type *RHO*; second to merely overexpress wild-type RHO to overcome the mutant protein effect (Massengill and Lewin 2021), and third to overexpress NR2E3, the upstream regulator of *RHO*(S. Li et al. 2021). Overexpression of wild-type RHO approach could have unintended adverse consequences on photoreceptor survival if the relationship between RHO expression and rod survival is not clearly understood. Our current study on *RHO*-CNV associated with adRP raises the awareness of toxicities and complications that might arise from either of the gene augmentation therapy approaches.

Our data strongly supports the notion that the RP phenotype in the patient is likely due to RHO accumulation and mislocalization in the patient's photoreceptors. Based on studies in animal models, *RHO* mutations could lead to death of rod photoreceptors by several mechanisms, including: (a) overwhelming the transport machinery, thereby mislocalizing and driving cell dysfunction and death, as observed with Q344R/P/ter *RHO* mutations in mice(C. H. Sung et al. 1994); (b) reducing the supply of phototransduction deactivating proteins for chromophore-attached RHO, leading to constitutive activation and degeneration, as proposed in some forms of *RHO* mutations such as G90D and T94I(Dizhoor et al. 2008); (c) reducing RHO glycosylation in the Golgi leading to RHO instability and photoreceptor degeneration, as in T17M mutation(Murray et al. 2015), or by (d) RHO misfolding, driving ER stress/unfolded protein response (UPR)(Olsson et al. 1992; Chiang et al. 2015). Based on the RNAseq data of our current study, it is likely that RHO overexpression overloads the rod ER significantly reducing the efficiency of RHO glycosylation. Previous studies have shown that RHO undergoes N-linked glycosylation at two distinct sites, Asn-2 and Asn-15. This glycosylation is believed to be critical for protein folding through interactions with chaperones as well as for the transport of rhodopsin to the outer

segments. Interestingly, studies in *Xenopus* suggest that this glycosylation is also responsible for nascent disc assembly(Murray, Fliesler, and AlLUbaidi 2009). Additionally, we specifically analyzed the ER stress-UPR pathway in the patient organoids and did not detect any differences compared to control (*data not shown*). One shortcoming of the current study is the lack of comparison of the organoid phenotype to an isogenic line. While the creation of isogenic lines and comparing them with disease organoid models is an ideal approach, carrying out large deletions by CRISPR-cas9 such as those required to fix the duplicated 188 kb and 48 kb inverted triplicated region in our *RHO*-CNV patient line is challenging effort. Large CRISPR edits generally have very low-efficiency (Eleveld et al. 2021; Canver et al. 2014). Thus, our study utilized an unaffected first-degree relative as a control.

Mislocalization of RHO to the soma was the most striking phenotype discerned in our patient organoids. While there were some breakthroughs of RHO in putative OS, most of the expression especially in mature (>D250) was stuck in the soma. Similar trafficking defects have been documented in post-mortem patient eye samples. RHO protein was found to be localized in the cell soma as well as synaptic processes and neurite extensions in RHO adRP patient samples as well as other RP patients (Z. Y. Li, Kljavin, and Milam 1995). This was also observed in transgenic pigs expressing human mutant RHO (Pro347Leu) (Z. Y. Li et al. 1998) as well as Xenopus expressing Q350ter (Tam et al. 2006).

A remarkable feature of adRP is the late-stage retinal disease manifesting slow progressive degeneration with initial OS disruption prior to photoreceptor death. In our results, the *RHO*-CNV patient-matched retinal organoid model rigorously mimicked the clinically degenerative OS and intact photoreceptor phenotype. Interestingly the mislocalized RHO was not degraded over extended culture time of 300-days. Transgenic Rho mutant mice such as P23H mice have demonstrated the degradation of mislocalized RHO at 3 months of age (Price et al. 2011). These findings suggest that the death of the photoreceptors and vision loss in *RHO*-CNV patient is conversely different from patients with other adRP mutations including the P23H mutations. Hence it is essential to understand the underlying mechanism of rod photoreceptor death with similar mislocalized RHO cellular phenotype in patient-specific P23H and *RHO*-CNV retinal organoid models. Proof-of-principle studies utilizing PR3 have been established to alleviate the retinal pathology. Hence further studies comparing the P23H and *RHO*-CNV retinal organoid models could serve as a valuable cellular platform to evaluate the efficacy of potential genome editing and small molecule therapeutic strategies.

Nuclear receptors are ideal therapeutic targets because their activities can be readily induced or repressed with small molecules. This allows for fine tuning of the biological functions of the receptor to alter disease pathogenesis. A few drugs targeting these receptors are already in the clinic (Dhiman, Bolt, and White 2018). It is interesting that targeting a reduction in wild-type RHO expression has also been proposed as a pan-RP therapy. Several groups have explored reduction in the expression of RHO as even small decreases can slow RP progression (Lewin et al. 1998). The photoregulin group of compounds identified through a screen to target the *NR2E3* orphan nuclear receptor were shown to repress rhodopsin expression with minor effects on cone opsins in mice (Nakamura et al. 2016). Additionally, upon delivery to mice by intra-peritoneal injection, PR3 has been found to be safe and effective in mitigating retinal degeneration, and improving visual function in the P23H rhodopsin mouse model(Nakamura et al. 2017), suggesting photoregulins an attractive preclinical candidates to treat humans with *RHO*-related adRP. Since excessive *RHO* leads to adRP, a tight regulation of *RHO* may be necessary to treat photoreceptor degeneration. In our current study, PR3 had a robust effect on rhodopsin expression with no effects on cone opsins.

In conclusion, we have established a disease-in-a-dish model for *RHO*-CNV associated adRP, and a potential therapeutic option for managing the devastating outcomes of the disorder.

Conclusion

This is the first reported study of characterizing the *RHO*-CNV, a novel cause of adRP. This case of CNV has expanded the profile of highly mutated *RHO* by correlating the clinical behavior to disease modeling using retinal organoids. We have used the organoid model for targeted drug testing with PR3, small molecule inhibitor of *NR2E3*. Taken together, the results of this study demonstrate that *RHO* expression requires precise control for rod photoreceptor functioning and maintenance.

Methods

Clinical and Molecular Diagnosis

Both the proband (Male patient, late 60s) and his unaffected daughter (early 30s) provided written informed consent, which was approved by the UCSF Institutional Review Board (IRB # 18-26409) and adhered to the tenets set forth in the Declaration of Helsinki. The participants gave consent to participate in the current study and have the results of this research work published. The proband (unidentified Lab ID # RM) and his asymptomatic daughter (unidentified Lab ID # RC) were examined at the University of California San Francisco (UCSF, CA, USA) including the pedigree data collection, clinical examination, and genetic diagnosis. Retinal examination included visual acuity testing, fundus photography, spectral domain optical coherence tomography (SD-OCT), full-field electroretinogram testing (ERG). For genetic diagnosis, peripheral blood (PB) samples collected from the proband and the unaffected daughter of the proband were screened by targeted Next Generation Sequencing (NGS) at Blueprint Genetic, a College of American Pathologists–and Clinical Laboratory Improvement Amendments– certified laboratory. The details of the methodology are available in our previous publication(Tuupanen et al. 2022). In brief, the sequencing was conducted using a retinal dystrophy NGS panel consisting of 266 genes, which was derived from an in-house tailored whole exome sequencing assay. The sequence reads were mapped to the human reference genome (GRCh37/hg19). CNV analysis was conducted using CNVkit (Talevich et al. 2016). Following a manual review of the identified RHO-CNV, discordant read-pairs suggesting a duplication-inverted triplication-duplication event was detected(Carvalho et al. 2011).

Generating iPSC lines

Peripheral blood samples collected from the human subjects (unidentified Lab ID # RC and RM) were reprogrammed into iPSCs as per the schematic outlined in **Supplementary Figure 2A**. Blood samples were processed by density-gradient centrifugation using Ficoll-paque™ PLUS (17-1440-02, GE Healthcare Biosciences, Sweden) to isolate the peripheral blood mononuclear cells (PBMNCs) (**Supplementary Figure 2B)**. A small fraction of ~1-2 × 10^6 PBMNCs was cultured in 1-well of a 12-well suspension plate with peripheral blood mononuclear cells expansion medium (**Supplementary Table 1**) by changing half media every day. Date of seeding the PBMNCs in culture was designated as Day (D) −4. Four days later, ~0.2-0.4 × 10^6 PBMNCs were reprogrammed using CytoTune™-iPS 2.0 Sendai Reprogramming Kit (A16517, Thermo Fisher Scientific, USA), at 2.5:2.5:1.5 (KOS:c-myc: Klf4) multiplicity of infection. 24h post-transfection on D1, a full media change was done. Two days post transfection on D3, cells were plated onto 1-2 wells of a Matrigel (354234, Corning, USA, diluted as 1 µg/mL in DMEM/F12)-coated 6-well plate with reprogramming medium (**Supplementary Table 1**). Half media change was done every other day from D3 until D6. For all the suspension cultures, medium change was done by centrifugation at 200x *g* for 10 minutes and resuspending the cell pellet with the respective medium and culturing back into the plates. On D8, cells were transitioned to iPSC medium (**Supplementary Table 1**) with further media changes done on every other day. About 14-21 days post transfection, colonies with typical

characteristics of iPSC morphology were manually picked. Pure, compact, and tightly packed iPSC clones were lifted using dissociating solution (**Supplementary Table 1**) for further expansion, characterization (**Supplementary Figure 2C-G)**, and retinal organoid differentiation.

Retinal Organoid Differentiation

Retinal organoids were differentiated (three to six independent differentiation from each clones) using three different clones from each iPSC lines *via* the three step embryoid body (EB) approach following our previously published protocols and as per the schematic depicted in **Figure 3A** (Bachu et al. 2022; Arthur et al. 2022). Briefly, iPSC colonies were lifted and cultured in 6-well suspension plate. Small iPSC colonies self-aggregated as EBs within 24h were gradually transitioned to complete Neural Induction Medium (NIM) (**Supplementary Table 1**) from D0 to D3. On D6, EBs were treated with 1.5 nM BMP4 (120-05ET, PeproTech, USA). By D7, the EBs were transferred onto a Matrigel-coated plate to facilitate the adherence of EBs to the plate with NIM and 1.5 nM BMP4. Brief exposure of BMP4 in the differentiation process included the treatment of EBs with 1.5 nM (from D6-D8) to 0.75 nM (D9-D11) and 0.375 nM (D12-14). On D15, BMP4 was completely removed and EBs were fed with just NIM. From D16 through D30, the adherent EBs were fed Retinal Differentiation Medium (RDM) (**Supplementary Table 1**) at the intervals of every 2 days. Regions of the plate displaying clear, shiny borders indicative of retina-like morphology were manually lifted using a sterile P100 pipette tip. Lifted neural retina was transferred to a 6-well suspension plates and allowed to self-acquire the 3D organoid configuration within the next 2 days. From D31, the developing retinal organoids were fed 3D-RDM medium (**Supplementary Table 1**) along with 1 µM All-trans retinoic acid (R2625, Millipore Sigma) every 3days until D120. From D120 onwards, organoids were fed with just 3D-RDM medium without All-trans retinoic acid every 3 days until the completion of the study. Organoids were periodically assessed for morphological characteristics using phase contrast microscopy (Olympus IX70) at regular intervals. Various time-points of rod photoreceptor differentiation and maturation in the retinal organoids were utilized for phenotypic characterization and drug assessments (**Supplementary Figure 3B**).

Immunohistochemistry and Imaging

Retinal organoids and confluent colonies of iPSCs were fixed in 4% paraformaldehyde (PFA) (157-8, Electron Microscopy Sciences, USA) in 1X PBS for 20 minutes. Organoids were cryopreserved in 15% through 30% sucrose (made in 1X PBS), and frozen in 2:1 mixture (20% sucrose: OCT, Sakura, USA). 10 µm sections of retinal organoids were collected on Super frost® Plus microscopic slides (12-550-15, Fisher Scientific, USA) using Leica CM3050 S cryostat. Immunohistochemistry was done as described previously. Organoid sections were pap-pen and PFA-fixed iPSC clones were permeabilized at room temperature for 15 mins with 0.1% Triton® X-100 (0694-1L, VWR Life Science, USA) in 10% Normal Donkey Serum (NDS, S30-100ML, EMD Millipore Corp, USA; made in 1X PBS). Following to that, sections were incubated for 1 hour in 10% NDS. Primary antibodies (**Supplementary Table 3A**) diluted in 10% NDS were added to the slides and incubated overnight (12-18 h) at 4°C. The slides were washed thrice in 1X PBS at 5 mins intervals and further incubated for 1 hour at room temperature with fluorescently conjugated secondary antibodies (**Supplementary Table 3B**) diluted at 1:250 in 10% NDS. Cell nuclei were counter stained with DAPI (1ug/mL, Roche, USA) for 10 minutes. The slides were then washed thrice in 1X PBS at 5 mins interval and cover slipped using Fluoromount G (Electron Microscopy Sciences, USA). Images were acquired using ZEN software on LSM700 confocal microscope (Zeiss, Inc.) and processed by Image J (NIH, USA).

RNA extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from retinal organoids using RNeasy® Mini Kit (74104, Qiagen, USA) as per the manufacturer's instructions. The quality and purity of the extracted RNA was assessed by NanoDrop One (Thermo Fisher Scientific, USA). cDNA was synthesized using iScript cDNA

Synthesis Kit (1708891, Bio-Rad, USA). Real-time qRT-PCR was performed on CFX96 system (Bio-Rad, USA) using iTaq™ Universal SYBR® Green Supermix (64047467, Bio-Rad, USA). Primer sequences used for qRT-PCR is listed in **Supplementary Table 4**. The amplification reactions set were: 95°C for 30 s, 40 cycles at 95°C for 5 s, 60°C for 25 s, 95°C for 5 s and final extension at 95°C for 5 s. The Ct values of the target genes were first normalized to the endogenous control β-actin. The corrected Ct values were then utilized to compare and validate the control and patient retinal organoids. Log_2 fold change (log2FC) in gene expression of all targets were then calculated.

Bulk RNA Sequencing

The quality control, RNA library preparations and sequencing reactions of the extracted RNA from retinal organoids were performed at Novogene Corporation Inc (CA, USA). FASTQ files received from Novogene following QC were quantified using Salmon package (V 1.9.0) by pseudo-aligning against homo sapiens hg19 genomic assembly in Galaxy(Afgan et al. 2016). Low-counts (<10) were filtered out and batch correction was carried out using Combat package in DEBrowser (v1.24.1) R package (Kucukural et al. 2019). Differential expression analysis was carried out using DESeq2 in DEbrowser. Genes with an adjusted p-value < 0.05 were assigned as differentially expressed. Gene ontology enrichment analysis and KEGG pathway analysis of differentially expressed genes was carried out in DEBrowser and Beavr (Perampalam and Dick 2020)packages. Differential expression analysis and pathway enrichment data have been uploaded as supplementary files (**Table 2**).

Transmission Electron Microscopy (TEM)

Retinal organoids (300-days-old) were washed in 1X PBS three times at 10 mins intervals and fixed with Karnovsky's fixative (4% paraformaldehyde/2% glutaraldehyde in 0.1 M PBS, pH 7.4) overnight at 4°C. For TEM, organoids were washed in 1X PBS and stained in 1% osmium tetroxide (19180, Electron Microscopy Sciences, USA; made in distilled water) for 1 hour at room temperature followed by staining with 2% uranyl acetate (22400, Electron Microscopy Sciences, USA; made in distilled water). After three washes in distilled water, organoids were then dehydrated in a graded series of ice-cold ethanol (50%, 70%, 95% and 100%) for 20 mins each. Organoids were incubated in propylene oxide (00235-1, Polysciences Inc, USA) twice for 5 mins, followed by a 5-mins incubation in 1:1 mix of propylene oxide: Epon 812 (13940, Electron Microscopy Sciences, USA). Organoids were allowed to infiltrate in Epon 812 overnight at room temperature. Next day, the organoids were embedded in PELCO silicone rubber molds (105, Ted Pella Inc, USA) using Epon 812, and polymerized for 48 hours at 60°C. Ultrathin (70 nm) sections were collected and imaged using a Philips Tecnai 10 electron microscope at the VA Medical Center, San Francisco, USA.

Western Blotting

Retinal organoids were collected and washed in cold 1X PBS twice at 5 mins intervals. Protein was extracted by homogenizing the organoids using hand-held pestle (1415-5390, USA Scientific) in ice-cold RIPA buffer (**See supplementary Table 2A**) with 1% complete™ Protease inhibitor cocktail (11697498001, Roche Diagnostics, GmbH). Lysates were incubated at 4°C for 10 minutes and then centrifuged at 14,000 *xg* for 15 minutes at 4°C. Supernatant was collected and the protein concentration was measured using Pierce™ BCA Protein Assay Kit (23227, Thermo Scientific, USA) as per the manufacturer's instructions. Equal amounts (20 µg) of protein lysates were mixed with 4x Laemmli sample buffer (161-0747, Bio-Rad, USA) and 10% dithiothreitol (DTT, R0861, Thermo Scientific, USA). Samples were resolved on a precast gel (12% Mini-Protean TGX Gels, 456-1044, Bio-Rad, USA) for 20 mins at 70 V until the loading dye entered the resolving layer, then increased to 150 V for 40-60 mins or until the run was completed (**See supplementary Table 2A**). After electrophoresis, the proteins were transferred onto an Immobilon®-FL PVDF membrane (IPFL20200, Merck Millipore, USA) ice-cold transfer buffer (**See supplementary Table 2A**) at 100V for 90 minutes. The blots were blocked with blocking solution for 30 minutes. The blots were

incubated overnight at 4°C with primary antibodies diluted in blocking solution (**See supplementary Table 3A for primary antibody details**). Thereafter, blots were washed thrice with TBS-T buffer (10 mins interval each) and incubated for 1 hour at room temperature with host-specific secondary antibodies diluted in blocking solution (**See supplementary Table 3B for secondary antibody details**). The blots were washed another three times with TBS-T buffer (10 mins interval each) and visualized using LiCor Odyssey XF scanner. For quantification of protein expression, the background was subtracted for each band and normalized to internal control band using Image J software. Statistical calculations were performed using multiple technical and five independent biological replicates.

PR3 treatment on Retinal Organoids

Patient-specific *RHO*-CNV retinal organoids (~300-days-old) were treated with small-drug like molecule Photoregulin3 (PR3, SML2299-5MG, Sigma, 5 mM stock made in DMSO) supplemented in 3D-RDM for 1 week by changing medium every other day. Three different concentrations of PR3 utilized in this study were 0.1, 0.25, and 0.5 μM. DMSO was used as a vehicle control. One-week, post-PR3 treatment, retinal organoids were collected and assessed by qRT-PCR, bulk RNA sequencing and imaging experiments.

Statistical Analysis

All the data were obtained from three to six independent retinal differentiation experiments. The quantified data values were provided as mean ± SEM. The intergroup differences for all the analysis were determined with the GraphPad Prism v9, using a two-tailed student's t-test or ANOVA. Significant differences were indicated by p values listed in the figures. For imaging studies, at least 3 sections were averaged to account for regional variability in iPSC reprograming and retinal organoid differentiation.

Data Availability

All data produced in the present study will be available upon reasonable request to the authors.

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Supplementary Figures

Supplementary Figure 1: *RHO*-CNV identification. Fundus photography showing the retina of control (RC) and irregular pigmentation pattern in a patient (RM). All the clinical hallmarks of RP including black-spicule pigmentation, attenuated blood vessel and optic disc pallor seen in patient are indicative of RP.

Supplementary Figure 2: Patient-specific iPSC reprogramming and retinal organoids. (A) Timeline depicting the sequence of events for reprogramming the PBMNCs to iPSCs *via* Cytotune™ iPS 2.0 Sendai Reprogramming kit. **(B, C)** Representative phase contrast microscopy image of PBMNCs and a stably generated iPSC colony. **(D)** Confocal microscopic images of an iPSC colony expressing pluripotent markers, SOX2, OCT3/4, NANOG and a co-localized image of all three pluripotent markers. DAPI labels nuclei. Scale bar = 25 μm. **(E)** Low-power bright-field microscopic images showing full view of a retinal organoid from control (RC) and patient (RM) at different time-points of photoreceptor maturation. Patient organoids showed much shorter and less dense protrusions in comparison to control. Scale bar = 50 μm. iPSC=induced pluripotent stem cells; OS=outer segment; ONL=outer nuclear layer; PBMNC=Peripheral blood mononuclear cells.

Supplementary Figure 3: Rod biogenesis and development in *RHO*-CNV retinal organoids did not differ from control. Confocal microscopic images of control (RC) and patient (RM) organoids labeling the early pan-photoreceptor markers, OTX2 and CRX; and early rod-specific post-mitotic photoreceptor marker, NR2E3 showing no difference in expression during **(A)** rod biogenesis at D120 **(B)** early rod maturation at D200, and **(C)** late rod maturation at D300. DAPI (blue) labels nuclei. Scale bar = 25 μm.

Supplementary Figure 4: PR3 treated *RHO*-CNV retinal organoids do not affect cone opsin expression. (A) qRT-PCR analysis shows no effect on cone opsin (*BCO*, *GCO*, *LCO*) mRNA levels at all the doses of PR3 treated-patient (RM) retinal organoids. Statistical analysis (ANOVA) revealed no significant differences. N=3 independent experiments and 12-15 organoids per experiment.

Supplementary Figure 5: Recovery of glycosylation and ER-Golgi transport pathways in PR3-treated *RHO*-CNV organoids. (A) Box plot showing the data from pathway enrichment analysis of cellular component category of differentially expressed genes following PR3 treatment showing the recovery in the dysregulated glycosylation as well as ER pathways **(B)** Normalized read count plots showing relative expression of *MAN1A2*, *MIA3*, *TFG*, *GBF1*, *COG3*, *SEC16B*, *SEC13*, *ARF4*, and *ALG12* in RC, RM and PR3-treated RM organoids. N=3 independent experiments and 12-15 organoids per experiment.

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Article and author information

Sangeetha Kandoi
Department of Ophthalmology, University of California San Francisco, CA, USA, Eli and Edythe Broad Center of Regeneration Medicine & Stem Cell Research, University of California San Francisco, CA, USA

Cassandra Martinez
Department of Ophthalmology, University of California San Francisco, CA, USA, Eli and Edythe Broad Center of Regeneration Medicine & Stem Cell Research, University of California San Francisco, CA, USA

Kevin Xu Chen
Eli and Edythe Broad Center of Regeneration Medicine & Stem Cell Research, University of California San Francisco, CA, USA

Miika Mehine
Blueprint Genetics, Helsinki, Finland

Brian C. Mansfield
Section on Cellular Differentiation, Division of Translational Medicine, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD USA

Jacque L. Duncan
Department of Ophthalmology, University of California San Francisco, CA, USA

Deepak A. Lamba
Department of Ophthalmology, University of California San Francisco, CA, USA, Eli and Edythe Broad Center of Regeneration Medicine & Stem Cell Research, University of California San Francisco, CA, USA
For correspondence: deepak.lamba@ucsf.edu

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Editors

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Reviewer #1 (Public Review):

Summary:
In the manuscript titled "Disease modeling and pharmacological rescue of autosomal dominant Retinitis Pigmentosa associated with RHO copy number variation" the authors describe the use of patient iPSC-derived retinal organoids to evaluate the pathobiology of a RHO-CNV in a family with dominant retinitis pigmentosa (RP). They find significantly increased expression of rhodopsin, especially within the photoreceptor cell body, and defects in photoreceptor cell outer segment formation/maturation. In addition, they demonstrate how an inhibitor of NR2E3 (a rod transcription factor required for inducing rhodopsin expression), can be used to rescue the disease phenotype.

Strengths:
The manuscript is very well written, the illustrations and data presented are compelling, and the authors' interpretation/discussion of their findings is logical.

Weaknesses:
A weakness, which the authors have addressed in the discussion section, is the lack of an isogenic control, which would allow for direct analysis of the RHO-CNV in the absence of the other genetic sequence contained within the duplicated region. As the authors suggest, CRISPR correction of a large CNV in the absence of inducing unwanted on-target editing

events in patient iPSCs is often very challenging. Given that they have used a no-disease iPSC line obtained from a family member, controlled for organoid differentiation kinetics/maturation state, and that no other complete disease-causing gene is contained within the duplicated region, it is unlikely that the addition of an isogenic control would yield significantly different results.

Aims and conclusions:
This reviewer is of the opinion that the authors have achieved their aims and that their results support their conclusions.

Discussion:
The authors have provided adequate discussion on the utility of the methods and data as well as the impact of their work on the field.

Reviewer #2 (Public Review):

Summary:
The manuscript by Kandoi et al. describes a new 3D retinal organoid model of a mono-allelic copy number variant of the rhodopsin gene that was previously shown to induce autosomal dominant retinitis pigmentosa via a dominant negative mechanism in patients. With advancements in the low-cost genomics application to detect copy number variations, this is a timely article that highlights a potential disease mechanism that goes beyond the retina field. The evidence is relatively strong that the rod photoreceptor phenotype observed in an adult patient with RP in vivo is similar to that phenotype observed in human stem cell-derived retinal organoids. Increases in RHO expression detected by qPCR, RNA-seq, and IHC support this phenotype. Importantly, the amelioration of photoreceptor rhodopsin mislocalization and related defects using the small molecule drug photoregulin demonstrates an important potential clinical application.

Overall, the authors succeeded in providing solid evidence that copy number variation via a genomic RHO duplication leads to abnormalities in rod photoreceptors that can be partially blocked by photoregulin. However, there are several points that should be addressed that will enhance this paper.

Strengths:
- The use of patient-derived organoids from patients that have visual defects is a major strength of this work and adds relevance to the disease phenotype.
- The rod phenotype assessed by qPCR, RNA-seq, and IHC supports a phenotype that shares similarities with the patient.
- The use of a small molecule drug that selectively targets rod photoreceptors, as opposed to cones, is a noteworthy strength.

Weaknesses:
1. The chromosomal segment that was duplicated had 3 copies of RHO in addition to three copies of each of the flanking genes (IFT122, HIF100, PLXND1). Discussion of the involvement of these genes would be helpful. Would duplication of any of these genes alone cause or contribute to adRP? As an example, a missense mutation in IFT122 was previously implicated in photoreceptor loss (PMID: 33606121 PMCID: PMC8519925).

2. Related to #1, have the authors considered inserting extra copies of RHO (and/or the flanking genes) of these at a genomic safe harbor site? Although not required, this would allow one to study cells with isogenic-matched genetic backgrounds and would partially address the technical challenge of repairing a 188kb duplication, which as the authors note would be difficult to do. Demonstrating that excess copy numbers in different genetic backgrounds would be a huge contribution to the field. At a minimum, a discussion of the role of the nearby genes should be included.

3. In the patient, the central foveal region was spared suggesting that cones were normal. Was there a similar assessment that cones are unaffected in retinal organoids?

4. Pathway analysis indicated that glycosylation was perturbed and this was proposed as an explanation as to why rhodopsin was mislocalized. Have the authors verified that there is an actual decrease in glycosylation?

5. Line 182: by what criteria are the authors able to state that " there were no clear visible anatomical changes in apical-basal retinal cell type distribution during the early differentiation timeframe (data not shown)." Was this based on histological staining with antibodies, nuclear counter-staining, or some other evaluation?

6. Figure 2C - the appearance of the inner segments in RC and RM looks very different from one another. Have the authors ruled out the possibility that the RC organoid cell isn't a cone? In addition, the RM structure has what appears to be a well-defined OLM which would suggest well-formed Muller glia. Do these structures also exist in RC organoids? Typically the OLM does form in older organoids. In addition, was this representative in numerous EM preparations?

7. What criteria were used to assess cell loss? Has any TUNEL labeling been performed to confirm cell loss? From the existing data, it seems that rod outer segments appear to be affected in organoids. However, it's not clear if the photoreceptors themselves actually die in this model.

8. Figure 5B. The RHO staining in the vehicle-treated sample is perturbed relative to the PR3 treatments as indicated in the text. In the vehicle-treated sample, the number of DAPI-positive cells that are completely negative proximal to the inner segments suggests that there might be non-rod cells there. Have the authors confirmed whether these are cones? Labels would be helpful in the left vehicle panel as the morphology looks very different than the treated samples.

9. It is interesting that in addition to increases in RHO, and photo-transduction, there are also increases in PTPRT which is related to synaptic adhesion. Is there evidence of ectopic neurites that result from PTPRT over-expression?

Reviewer #3 (Public Review):

This manuscript reports a novel pedigree with four intact copies of RHO on a single chromosome which appears to lead to overexpression of rhodopsin and a corresponding autosomal dominant form of RP. The authors generate retinal organoids from patient- and control-derived cells, characterize the phenotypes of the organoids, and then attempt to 'treat' aberrant rhodopsin expression/mislocalization in the patient organoids using a small molecule called photoregulin 3 (PR3). While this novel genetic mechanism for adRP is interesting, the organoid work is not compelling. There are multiple problems related to the technical approaches, the presentation of the results, and the interpretations of the data. I will present my concerns roughly in the order in which they appear in the manuscript.

Major concerns:
(1) Individual human retinal organoids in culture can show a wide range of differentiation phenotypes with respect to the expression of specific markers, percentages of given cell types, etc. For this reason, it can be very difficult to make rigorous, quantitative comparisons between 'wild-type' and 'mutant' organoids. Despite this difficulty, the author of the present manuscript frequently presents results in an impressionistic manner without quantitation. Furthermore, there is no indication that the investigator who performed the phenotypic analyses was blind with respect to the genotype. In my opinion, such blinding is essential for the analysis of phenotypes in retinal organoids.

To give an example, in lines 193-194 the authors write "we observed that while the patient organoids developing connecting cilium and the inner segments similar to control organoids, they failed to extend outer segments". Outer segments almost never form normally in human retinal organoids, even when derived from 'wild-type' cells. Thus, I consider it wholly inadequate to simply state that outer segment formation 'failed' without a rigorous, quantitative, and blinded comparison of patient and control organoids.

(2) The presentation of qPCR results in Figure 3A is very confusing. First, the authors normalize expression to that of CRX, but they don't really explain why. In lines 210-211, they write "CRX, a ubiquitously expressing photoreceptor gene maintained from development to adulthood." Several parts of this sentence are misleading or incomplete. First, CRX is not 'ubiquitously expressed' (which usually means 'in all cell types') nor is it photoreceptor-specific: CRX is expressed in rods, cones, and bipolar cells. Furthermore, CRX expression levels are not constant in photoreceptors throughout development/adulthood. So, for these reasons alone, CRX is a poor choice for the normalization of photoreceptor gene expression.

Second, the authors' interpretation of the qPCR results (lines 216-218) is very confusing. The authors appear to be saying that there is a statistically significant increase in RHO levels between D120 and D300. However, the same change is observed in both control and patient organoids and is not unexpected, since the organoids are more mature at D300. The key comparison is between control and patient organoids at D300. At this time point, there appears to be no difference between control and patient. The authors don't even point this out in the main text.

Third, the variability in the number of photoreceptor cells in individual organoids makes a whole-organoid comparison by qPCR fraught with difficulty. It seems to me that what is needed here is a comparison of RHO transcript levels in isolated rod photoreceptors.

(3) I cannot understand what the authors are comparing in the bulk RNA-seq analysis presented in the paragraph starting with line 222 and in the paragraph starting with line 306. They write "we performed bulk-RNA sequencing on 300-days-old retinal organoids (n=3 independent biological replicates). Patient retinal organoids demonstrated upregulated transcriptomic levels of RHO... comparable to the qRT-PCR data." From the wording, it suggests that they are comparing bulk RNA-seq of patients and control organoids at D300. However, this is not stated anywhere in the main text, the figure legend, or the Methods. Yet, the subsequent line "comparable to the qRT-PCR data" makes no sense, because the qPCR comparison was between patient samples at two different time points, D120 and D300, not between patient and control. Thus, the reader is left with no clear idea of what is even being compared by RNA-seq analysis.

Remarkably, the exact same lack of clarity as to what is being compared is found in the second RNA-seq analysis presented in the paragraph starting with line 306. Here the authors write "We further carried out bulk RNA-sequencing analysis to comprehensively characterize three different groups of organoids, 0.25 μM PR3-treated and vehicle-treated patient organoids and control (RC) organoids from three independent differentiation experiments. Consistent with the qRT-PCR gene expression analysis, the results showed a significant downregulation in RHO and other rod phototransduction genes." Here, the authors make it clear that they have performed RNA-seq on three types of samples: PR3-treated patient organoids, vehicle-treated patient organoids, and control organoids (presumably not treated). Yet, in the next sentence, they state "the results showed a significant downregulation in RHO", but they don't state what two of the three conditions are being compared! Although I can assume that the comparison presented in Fig. 6A is between patient vehicle-treated and PR3-treated organoids, this is nowhere explicitly stated in the manuscript.

(4) There are multiple flaws in the analysis and interpretation of the PR3 treatment results. The authors wrote (lines 289-2945) "We treated long-term cultured 300-days-old, RHO-CNV patient retinal organoids with varying concentrations of PR3 (0.1, 0.25 and 0.5 μM) for one week and assessed the effects on RHO mRNA expression and protein localization. Immunofluorescence staining of PR3-treated organoids displayed a partial rescue of RHO localization with optimal trafficking observed in the 0.25 μM PR3-treated organoids (Figure 5B). None of the organoids showed any evidence of toxicity post-treatment."

There are multiple problems here. First, the results are impressionistic and not quantitative. Second, it's not clear that the investigator was blinded with respect to the treatment condition. Third, in the sections presented, the organoids look much more disorganized in the PR3-treated conditions than in the control. In particular, the ONL looks much more poorly formed. Overall, I'd say the organoids looked considerably worse in the 0.25 and 0.5 microM conditions than in the control, but I don't know whether or not the images are representative. Without rigorously quantitative and blinded analysis, it is impossible to draw solid conclusions here. Lastly, the authors state that "none of the organoids showed any evidence of toxicity post-treatment," but do not explain what criteria were used to determine that there was no toxicity.

(5) qPCR-based quantitation of rod gene expression changes in response to PR3 treatment is not well-designed. In lines 294-297 the authors wrote "PR3 drove a significant downregulation of RHO in a dose-dependent manner. Following qRT-PCR analysis, we observed a 2-to-5 log2FC decrease in RHO expression, along with smaller decreases in other rod-specific genes including NR2E3, GNAT1 and PDE6B." I assume these analyses were performed on cDNA derived from whole organoids. There are two problems with this analysis/interpretation. First, a decrease in rod gene expression can be caused by a decrease in the number of rods in the treated organoids (e.g., by cell death) or by a decrease in the expression of rod genes within individual rods. The authors do not distinguish between these two possibilities. Second, as stated above, the percentage of cells that are rods in a given organoid can vary from organoid to organoid. So, to determine whether there is downregulation of rod gene expression, one should ideally perform the qPCR analysis on purified rods.

(6) In Figure 4B 'RM' panels, the authors show RHO staining around the somata of 'rods' but the inset images suggest that several of these cells lack both NRL and OTX2 staining in their nuclei. All rods should be positive for NRL. Conversely, the same image shows a layer of cells scleral to the cells with putative RHO somal staining which do not show somal staining, and yet they do appear to be positive for NRL and OTX2. What is going on here? The authors need to provide interpretations for these findings.

Author Response

> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *In the manuscript titled "Disease modeling and pharmacological rescue of autosomal dominant Retinitis Pigmentosa associated with RHO copy number variation" the authors describe the use of patient iPSC-derived retinal organoids to evaluate the pathobiology of a RHO-CNV in a family with dominant retinitis pigmentosa (RP). They find significantly increased expression of rhodopsin, especially within the photoreceptor cell body, and defects in photoreceptor cell outer segment formation/maturation. In addition, they demonstrate how an inhibitor of NR2E3 (a rod transcription factor required for inducing rhodopsin expression), can be used to rescue the disease phenotype.*
>
> *Strengths:*

The manuscript is very well written, the illustrations and data presented are compelling, and the authors' interpretation/discussion of their findings is logical.

Weaknesses:

A weakness, which the authors have addressed in the discussion section, is the lack of an isogenic control, which would allow for direct analysis of the RHO-CNV in the absence of the other genetic sequence contained within the duplicated region. As the authors suggest, CRISPR correction of a large CNV in the absence of inducing unwanted on-target editing events in patient iPSCs is often very challenging. Given that they have used a no-disease iPSC line obtained from a family member, controlled for organoid differentiation kinetics/maturation state, and that no other complete disease-causing gene is contained within the duplicated region, it is unlikely that the addition of an isogenic control would yield significantly different results.

Aims and conclusions:

This reviewer is of the opinion that the authors have achieved their aims and that their results support their conclusions.

Discussion:

The authors have provided adequate discussion on the utility of the methods and data as well as the impact of their work on the field.

We thank the reviewer for their insightful, and encouraging review of our work that has taken several years to get to current stage.

Reviewer #2 (Public Review):

Summary:

The manuscript by Kandoi et al. describes a new 3D retinal organoid model of a mono-allelic copy number variant of the rhodopsin gene that was previously shown to induce autosomal dominant retinitis pigmentosa via a dominant negative mechanism in patients. With advancements in the low-cost genomics application to detect copy number variations, this is a timely article that highlights a potential disease mechanism that goes beyond the retina field. The evidence is relatively strong that the rod photoreceptor phenotype observed in an adult patient with RP in vivo is similar to that phenotype observed in human stem cell-derived retinal organoids. Increases in RHO expression detected by qPCR, RNA-seq, and IHC support this phenotype. Importantly, the amelioration of photoreceptor rhodopsin mislocalization and related defects using the small molecule drug photoregulin demonstrates an important potential clinical application.

Overall, the authors succeeded in providing solid evidence that copy number variation via a genomic RHO duplication leads to abnormalities in rod photoreceptors that can be partially blocked by photoregulin. However, there are several points that should be addressed that will enhance this paper.

Strengths:

- *The use of patient-derived organoids from patients that have visual defects is a major strength of this work and adds relevance to the disease phenotype.*

- *The rod phenotype assessed by qPCR, RNA-seq, and IHC supports a phenotype that shares similarities with the patient.*
- *The use of a small molecule drug that selectively targets rod photoreceptors, as opposed to cones, is a noteworthy strength.*

We thank the reviewers for highlighting the key strengths of the paper.

Weaknesses:

1. *The chromosomal segment that was duplicated had 3 copies of RHO in addition to three copies of each of the flanking genes (IFT122, HIF100, PLXND1). Discussion of the involvement of these genes would be helpful. Would duplication of any of these genes alone cause or contribute to adRP? As an example, a missense mutation in IFT122 was previously implicated in photoreceptor loss (PMID: 33606121 PMCID: PMC8519925).*

Thank you for your comment. It is an interesting question on the contribution of the other duplicated genes. Of these, IFT122 is particularly interesting as pointed out. We did a thorough survey through literature and our genetic testing partner's database, BluePrint Genetics. We did not find any human retinal degeneration cases with variants in IFT122. IFT122 has been shown to cause recessive phenotype in dogs and in complete knockout zebrafish model but dominant or overexpression has not been shown to have a phenotype. Interestingly, recessive biallelic IFT122 mutation can cause Cranioectodermal Dysplasia (Sensenbrenner syndrome, PMID: 24689072) and none of these patient exhibited retinal dystrophy. HIF100 is an epigenetic modifier gene while PLXND1 is expressed in endothelial cells. We will include a discussion on this in the revised manuscript.

1. *Related to #1, have the authors considered inserting extra copies of RHO (and/or the flanking genes) of these at a genomic safe harbor site? Although not required, this would allow one to study cells with isogenic-matched genetic backgrounds and would partially address the technical challenge of repairing a 188kb duplication, which as the authors note would be difficult to do. Demonstrating that excess copy numbers in different genetic backgrounds would be a huge contribution to the field. At a minimum, a discussion of the role of the nearby genes should be included.*

Thank you for your suggestion. We plan to test the relative role of 1-3 extra copies of RHO driven off a NRL promoter in order to drive it only in rods in our future mechanistic analysis studies. We will include a discussion on the potential role of the other genes in the revised manuscript.

1. *In the patient, the central foveal region was spared suggesting that cones were normal. Was there a similar assessment that cones are unaffected in retinal organoids?*

We will include this data in our revised manuscript but overall did not see a cone defect in RHO CNV organoids. Additionally, although it is true that the central foveal region was relatively spared in this patient, the cones are definitely not normal. The macular cones that remain have been damaged by chronic edema, and photoreceptor and RPE atrophy has progressed into the macula, sparing only the foveal cones.

1. *Pathway analysis indicated that glycosylation was perturbed and this was proposed as an explanation as to why rhodopsin was mislocalized. Have the authors verified that there is an actual decrease in glycosylation?*

These studies are ongoing. We are currently looking into the details of cellular pathophysiology focusing on RHO trafficking in RHO-CNV including role of glycosylation and other post-translational modifications defects.

1. *Line 182: by what criteria are the authors able to state that " there were no clear visible anatomical changes in apical-basal retinal cell type distribution during the early differentiation timeframe (data not shown)." Was this based on histological staining with antibodies, nuclear counter-staining, or some other evaluation?*

This was based on both IHC for various cell type markers and nuclear (DAPI) staining.

1. *Figure 2C - the appearance of the inner segments in RC and RM looks very different from one another. Have the authors ruled out the possibility that the RC organoid cell isn't a cone? In addition, the RM structure has what appears to be a well-defined OLM which would suggest well-formed Muller glia. Do these structures also exist in RC organoids? Typically the OLM does form in older organoids. In addition, was this representative in numerous EM preparations?*

For clarification on EM data, we will include additional images in the revision as supplementary data. We have not carefully compared OLM between the patient and control organoids but do observe them in both conditions in the older organoids. The EM preparations were made from multiple organoids from two different batches with consistent results.

1. *What criteria were used to assess cell loss? Has any TUNEL labeling been performed to confirm cell loss? From the existing data, it seems that rod outer segments appear to be affected in organoids. However, it's not clear if the photoreceptors themselves actually die in this model.*

TUNEL was used to assess cell loss and it was not significantly different between the control and patient organoids at the timepoints examined. We did not expect a change as the disease in the patient developed over decades.

1. *Figure 5B. The RHO staining in the vehicle-treated sample is perturbed relative to the PR3 treatments as indicated in the text. In the vehicle-treated sample, the number of DAPI-positive cells that are completely negative proximal to the inner segments suggests that there might be non-rod cells there. Have the authors confirmed whether these are cones? Labels would be helpful in the left vehicle panel as the morphology looks very different than the treated samples.*

Thank you very much for the various suggestions and these will be included in the revised manuscript version. A number of the cells in the negative regions are OTX2+/NRL- and likely to be cones (Figure 4 A and B). Unfortunately, we do not have a very good cone nuclear marker as RXRγ does not consistently stain mature cones.

1. *It is interesting that in addition to increases in RHO, and photo-transduction, there are also increases in PTPRT which is related to synaptic adhesion. Is there evidence of ectopic neurites that result from PTPRT over-expression?*

You are absolutely correct that PTPRT data is very interesting. PTPRT requires similar PTMs like RHO in photoreceptors for its synaptic localization. We did not specifically look at ectopic neurites and test that in the revision. It will interesting to follow-up on its expression pattern to see if it gets processed or localized normally if we can find a working antibody. It is also possible that the gene-expression increase due to feedback upregulation secondary to improper protein processing.

Reviewer #3 (Public Review):

This manuscript reports a novel pedigree with four intact copies of RHO on a single chromosome which appears to lead to overexpression of rhodopsin and a corresponding autosomal dominant form of RP. The authors generate retinal organoids from patient- and control-derived cells, characterize the phenotypes of the organoids, and then attempt to 'treat' aberrant rhodopsin expression/mislocalization in the patient organoids using a small molecule called photoregulin 3 (PR3). While this novel genetic mechanism for adRP is interesting, the organoid work is not compelling. There are multiple problems related to the technical approaches, the presentation of the results, and the interpretations of the data. I will present my concerns roughly in the order in which they appear in the manuscript.

Major concerns:

(1) Individual human retinal organoids in culture can show a wide range of differentiation phenotypes with respect to the expression of specific markers, percentages of given cell types, etc. For this reason, it can be very difficult to make rigorous, quantitative comparisons between 'wild-type' and 'mutant' organoids. Despite this difficulty, the author of the present manuscript frequently presents results in an impressionistic manner without quantitation. Furthermore, there is no indication that the investigator who performed the phenotypic analyses was blind with respect to the genotype. In my opinion, such blinding is essential for the analysis of phenotypes in retinal organoids. To give an example, in lines 193-194 the authors write "we observed that while the patient organoids developing connecting cilium and the inner segments similar to control organoids, they failed to extend outer segments". Outer segments almost never form normally in human retinal organoids, even when derived from 'wild-type' cells. Thus, I consider it wholly inadequate to simply state that outer segment formation 'failed' without a rigorous, quantitative, and blinded comparison of patient and control organoids.

We agree it is challenging to generate outer segments in retinal organoids but we are not the first to show this. This has been demonstrated by multiple independent labs (Mayerl et al (PMID: 36206764), Wahlin et al (PMID: 28396597), West at al (PMID: 35334217) including ours (Chirco et al (PMID: 34653402). To clarify, we did not observe any OS like tissue in the patient organoids across multiple EM preps of a number of organoids from two independent 300+ day experiments which matched the phase microscopy data presented in Fig2B.

(2) The presentation of qPCR results in Figure 3A is very confusing. First, the authors normalize expression to that of CRX, but they don't really explain why. In lines 210-211, they write "CRX, a ubiquitously expressing photoreceptor gene maintained from development to adulthood." Several parts of this sentence are misleading or incomplete. First, CRX is not 'ubiquitously expressed' (which usually means 'in all cell types') nor is it

> *photoreceptor-specific: CRX is expressed in rods, cones, and bipolar cells. Furthermore, CRX expression levels are not constant in photoreceptors throughout development/adulthood. So, for these reasons alone, CRX is a poor choice for the normalization of photoreceptor gene expression.*

As you are aware, all housekeeping genes have shortcomings when used for normalizing PCR data. We went with CRX as within the timepoints chosen, it is not expected to change much and thus represent a good equalizer for relative photoreceptor numbers between the organoids and conditions. While we agree that CRX is weakly expressed in bipolar cells (Yamamoto et al 2020), it is not expected to bias the data too much as we have not seen nor have other reported a huge relative difference in bipolar cell number in organoids. We also confirm this by showing equivalent expression of OTX2, RCVRN and NRL between all conditions.

> *Second, the authors' interpretation of the qPCR results (lines 216-218) is very confusing. The authors appear to be saying that there is a statistically significant increase in RHO levels between D120 and D300. However, the same change is observed in both control and patient organoids and is not unexpected, since the organoids are more mature at D300. The key comparison is between control and patient organoids at D300. At this time point, there appears to be no difference between control and patient. The authors don't even point this out in the main text.*

Thank you for the comment and we apologize if this confused you. However, as can been seen in the graph in Figure 3A, we do compare expression of genes including RHO between control and patient organoids at two different time points. There are four conditions: D120-RC, D120-RM, D300-RC and D300-RM with individual data points and error bars for each condition. There is a statistically significant increase at both time points upon comparing the control and patient organoids for RHO. We compared RHO expression between patient organoids at the two time points and it was not statistically different.

> *Third, the variability in the number of photoreceptor cells in individual organoids makes a whole-organoid comparison by qPCR fraught with difficulty. It seems to me that what is needed here is a comparison of RHO transcript levels in isolated rod photoreceptors.*

We agree that this makes it challenging. This was the exact reasoning for using CRX for normalization since it is predominantly present in photoreceptors. This was validated by the data showing no difference in expression of photoreceptor markers OTX2, RCVRN or NRL between the organoids.

> *(3) I cannot understand what the authors are comparing in the bulk RNA-seq analysis presented in the paragraph starting with line 222 and in the paragraph starting with line 306. They write "we performed bulk-RNA sequencing on 300-days-old retinal organoids (n=3 independent biological replicates). Patient retinal organoids demonstrated upregulated transcriptomic levels of RHO... comparable to the qRT-PCR data." From the wording, it suggests that they are comparing bulk RNA-seq of patients and control organoids at D300. However, this is not stated anywhere in the main text, the figure legend, or the Methods. Yet, the subsequent line "comparable to the qRT-PCR data" makes no sense, because the qPCR comparison was between patient samples at two different time points, D120 and D300, not between patient and control. Thus, the reader is left with no clear idea of what is even being compared by RNA-seq analysis.*

We apologize if the conditions were not obvious and will clarify this in the revised version. The conditions compared are control and patient organoids at D300. Regarding comparison

to RT-PCR, as stated above, the comparison shown is between patient and control organoids at two different timepoints.

> *Remarkably, the exact same lack of clarity as to what is being compared is found in the second RNA-seq analysis presented in the paragraph starting with line 306. Here the authors write "We further carried out bulk RNA-sequencing analysis to comprehensively characterize three different groups of organoids, 0.25 μM PR3-treated and vehicle-treated patient organoids and control (RC) organoids from three independent differentiation experiments. Consistent with the qRT-PCR gene expression analysis, the results showed a significant downregulation in RHO and other rod phototransduction genes." Here, the authors make it clear that they have performed RNA-seq on three types of samples: PR3-treated patient organoids, vehicle-treated patient organoids, and control organoids (presumably not treated). Yet, in the next sentence, they state "the results showed a significant downregulation in RHO", but they don't state what two of the three conditions are being compared! Although I can assume that the comparison presented in Fig. 6A is between patient vehicle-treated and PR3-treated organoids, this is nowhere explicitly stated in the manuscript.*

Thank you for the comment and we will explicitly state various comparisons in the revised version.

> *(4) There are multiple flaws in the analysis and interpretation of the PR3 treatment results. The authors wrote (lines 289-2945) "We treated long-term cultured 300-days-old, RHO-CNV patient retinal organoids with varying concentrations of PR3 (0.1, 0.25 and 0.5 μM) for one week and assessed the effects on RHO mRNA expression and protein localization. Immunofluorescence staining of PR3-treated organoids displayed a partial rescue of RHO localization with optimal trafficking observed in the 0.25 μM PR3-treated organoids (Figure 5B). None of the organoids showed any evidence of toxicity post-treatment."*
>
> *There are multiple problems here. First, the results are impressionistic and not quantitative. Second, it's not clear that the investigator was blinded with respect to the treatment condition. Third, in the sections presented, the organoids look much more disorganized in the PR3-treated conditions than in the control. In particular, the ONL looks much more poorly formed. Overall, I'd say the organoids looked considerably worse in the 0.25 and 0.5 microM conditions than in the control, but I don't know whether or not the images are representative. Without rigorously quantitative and blinded analysis, it is impossible to draw solid conclusions here. Lastly, the authors state that "none of the organoids showed any evidence of toxicity post-treatment," but do not explain what criteria were used to determine that there was no toxicity.*

Thank you for your critical insight. The RHO localization data is qualitative as it is very difficult to accurately quantify rhodopsin trafficking within the cell in the organoid. Thus, for quantitative comparison, we have provided expression level changes. Regarding toxicity, we analyzed the organoids by morphology and TUNEL and did not observe significant difference between the conditions. This closely mimics mouse data on PR3 which suppressed rod function in mice following IP injection without any obvious toxicity.

> *(5) qPCR-based quantitation of rod gene expression changes in response to PR3 treatment is not well-designed. In lines 294-297 the authors wrote "PR3 drove a significant downregulation of RHO in a dose-dependent manner. Following qRT-PCR analysis, we observed a 2-to-5 log2FC decrease in RHO expression, along with smaller decreases in other rod-specific genes including NR2E3, GNAT1 and PDE6B." I assume these analyses were performed on cDNA derived from whole organoids. There are two*

> *problems with this analysis/interpretation. First, a decrease in rod gene expression can be caused by a decrease in the number of rods in the treated organoids (e.g., by cell death) or by a decrease in the expression of rod genes within individual rods. The authors do not distinguish between these two possibilities. Second, as stated above, the percentage of cells that are rods in a given organoid can vary from organoid to organoid. So, to determine whether there is downregulation of rod gene expression, one should ideally perform the qPCR analysis on purified rods.*

The reviewer is correct in pointing the potential reasons for reduction in RHO levels following PR3 treatment. Thus, we have provided NRL expression levels in the graph to show that this key rod-specific gene does not change suggesting equivalent number of rod photoreceptor cells. The suggestion of using purified rods is not practical here, as we do not have any way to sort human rods due to the lack of a rod-specific cell surface marker.

> *(6) In Figure 4B 'RM' panels, the authors show RHO staining around the somata of 'rods' but the inset images suggest that several of these cells lack both NRL and OTX2 staining in their nuclei. All rods should be positive for NRL. Conversely, the same image shows a layer of cells scleral to the cells with putative RHO somal staining which do not show somal staining, and yet they do appear to be positive for NRL and OTX2. What is going on here? The authors need to provide interpretations for these findings.*

Since RHO is a cytoplasmic marker and photoreceptor are tightly packed, it is difficult to make a 1:1 comparison to NRL/OTX2 nuclear marker to RHO. Additionally, as the RHO+ cytoplasm moves towards scleral surface, it is expected to pass adjacent to other nuclei. Few of the rods do still have normal Rhodopsin trafficking and it is likely these will not have somal RHO similar to control conditions. We do rarely observe these cells as highlighted by the occasional RHO in IS/OS of RM organoids in the figure. We do agree that the NRL staining in the figure 4B (>D250) is not extremely crisp and we will include an updated figure in the revised version.