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Stress resilience-enhancing drugs preserve tissue structure and function in degenerating retina via phosphodiesterase inhibition

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Chronic, progressive retinal diseases, such as age-related macular degeneration (AMD), diabetic retinopathy, and retinitis pigmentosa, arise from genetic and environmental perturbations of cellular and tissue homeostasis. These disruptions accumulate with repeated exposures to stress over time, leading to progressive visual impairment and, in many cases, legal blindness. Despite decades of research, therapeutic options for the millions of patients suffering from these disorders remain severely limited, especially for treating earlier stages of pathogenesis when the opportunity to preserve the retinal structure and visual function is greatest. To address this urgent, unmet medical need, we employed a systems pharmacology platform for therapeutic development. Through integrative single-cell transcriptomics, proteomics, and phosphoproteomics, we identified universal molecular mechanisms across distinct models of age-related and inherited retinal degenerations, characterized by impaired physiological resilience to stress. Here, we report that selective, targeted pharmacological inhibition of cyclic nucleotide phosphodiesterases (PDEs), which serve as critical regulatory nodes that modulate intracellular second messenger signaling pathways, stabilized the transcriptome, proteome, and phosphoproteome through downstream activation of protective mechanisms coupled with synergistic inhibition of degenerative processes. This therapeutic intervention enhanced resilience to acute and chronic forms of stress in the degenerating retina, thus preserving tissue structure and function across various models of age-related and inherited retinal disease. Taken together, these findings exemplify a systems pharmacology approach to drug discovery and development, revealing a new class of therapeutics with potential clinical utility in the treatment or prevention of the most common causes of blindness.

rhodopsin | retina | eye | retinal degeneration | phosphodiesterase

At the mechanistic level, impaired physiological resilience to stress represents a common point of convergence across various age-related degenerative disorders of distinct etiologies (1). While somatic maintenance processes function with high efficiency in youth, such as in the effective repair and/or recycling of misfolded or aggregated protein adducts induced by stress, reduced efficiency of these processes in advanced age can result in aberrant accumulation of toxic aggregates, plaques, and other damage. This dysregulation can compromise cellular and organismal viability in the face of constant exposure to environmental stressors that eventually exceed the dynamic range of recovery by protective mechanisms (2). Age-related macular degeneration (AMD) is a leading cause of progressive, irreversible blindness in individuals over 50 y of age, with an estimated 11 million afflicted in the United States and approximately 196 million cases worldwide (3). Clinically, AMD susceptibility has been linked to cigarette smoking and obesity, both of which are known to induce cellular stress and inflammation, in part through epigenetic modifications (4, 5). Similarly, diabetic retinopathy is the leading cause of visual impairment and blindness in the diabetic population with an estimated 150 million cases worldwide, and disease pathogenesis involves microvascular complications secondary to chronic hyperglycemic stress, as well as inflammatory and oxidative stresses (6, 7). Lastly, in the most common inherited retinal disorder, retinitis pigmentosa, disease susceptibility and progression have been linked to increased proteomic stress, resulting from genetic mutations that accelerate the accumulation of misfolded or mislocalized protein aggregates (8, 9). Therefore, the pathogenesis of these age-related and inherited retinal diseases likely involves acute and/or chronic stress exposures that overwhelm the dynamic range of recovery by intrinsic somatic maintenance mechanisms. However, which signaling pathways modulate resilience to stress in these disease contexts is unclear.

Significance

In this study, we discovered small-molecule drugs that activate intrinsic biological mechanisms of stress resilience. Accordingly, we propose a new class of therapeutics, "stress resilience-enhancing drugs" (SREDs), for the treatment of acute and/or chronic stress-associated pathological conditions, focusing on age-related and inherited retinal diseases. Based on a systems pharmacology platform that leverages state-of-the-art disease modeling and characterization, our findings reveal important cell types and signaling pathways involved in modulating stress resilience and retinal degeneration. By exploiting integrative mechanisms of action in complex, multifactorial disorders, SREDs represent a promising strategy for clinicians to combat disease with superior efficacy in earlier stages of pathogenesis, thereby augmenting the arsenal of ophthalmic medications currently available in antiangiogenics, corticosteroids, and nonsteroidal anti-inflammatory drugs (NSAIDs).

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Cyclic nucleotide phosphodiesterases (PDEs) are a class of enzymes ubiquitously expressed throughout the human body. They play critical roles in a broad range of physiological processes, including regulation of the immune system (10), cardiovascular function (11), metabolism (12), reproduction (13), neurobiological processes of learning and memory (14), and vision (15). PDEs function enzymatically to hydrolyze the intracellular second messengers cAMP and/or cGMP, modulating various downstream cellular signaling pathways that control vital homeostatic processes (16). These include repair mechanisms that promote cell survival (i.e., somatic maintenance), as well as degenerative mechanisms that drive apoptosis and/or cell death (2). In human aging and disease, dysregulation of these signaling networks can lead to reduced efficiency of somatic maintenance and/or increased cell death, resulting in pathophysiological states of disease. Since PDEs represent key regulatory nodes that modulate second messenger signaling pathways, it is conceivable they could be pharmacologically targeted to upregulate somatic maintenance processes and improve cellular viability in disease states.

To date, only a limited number of PDE inhibitors have been approved by the United States Food and Drug Administration (FDA) for clinical use. Corresponding to a small subset in the wide range of physiological functions regulated by different PDE subtypes, clinical indications for these FDA-approved PDE inhibitors include cardiovascular disease (17), chronic obstructive pulmonary disorder (18), asthma (19), benign prostatic hyperplasia (20), and erectile dysfunction (21). Despite the relative scarcity of FDA-approved selective PDE inhibitors, the clinical utility of a PDE5-selective inhibitor (e.g., Viagra) in the treatment of erectile dysfunction exemplifies one of the most commercially successful ventures in the history of the pharmaceutical industry, generating consecutive annual revenues in excess of $1 billion for nearly two decades (22). Moreover, the most widely consumed drug in the world, caffeine, along with its metabolites, elicits certain neuroprotective effects via PDE inhibition, and caffeine intake has been associated with reduced risk of developing neurodegenerative disorders such as Parkinson disease (23). Indeed, PDEs represent an attractive class of drug targets with much untapped clinical potential, especially concerning neurodegenerative disorders for which no FDA-approved therapies exist. There are 11 different PDE subtypes (Fig. 1*A*), and each can be subdivided into different isoforms and splice variants (24). These unique PDEs exhibit differential cell type-specific expression, thus enabling the development of selective inhibitors to target cell type-specific functions and treat pathological conditions with greater efficacy and precision, while minimizing off-target effects (25).

Based on the premise that PDEs function as integrative signaling nodes that could be therapeutically targeted in the context of various retinopathies, we set out to investigate the effects of selective PDE-inhibitor therapy on the hallmark characteristics of age-related and inherited retinal diseases. We conducted in vitro studies utilizing cell culture models, as well as in vivo studies employing several murine models; namely, the photosensitive *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ double-knockout (dKO) mouse, which exhibits epigenetic and pathological hallmarks of human AMD (26, 27), the streptozotocin model of diabetic retinopathy (28, 29), and the *rd10* model of autosomal recessive retinitis pigmentosa (30, 31). The goal in each case was to delineate molecular mechanisms pertinent to therapeutic intervention and to determine whether stress resilience could be enhanced pharmacologically via selective PDE inhibition. Here, we report that selective targeting of PDEs, through stabilization of the transcriptome, proteome, and phosphoproteome, enhanced resilience to stress in the degenerating retina, facilitating preservation of tissue structure and function across various models of retinal disease.

Results

PDEs Involved in Regulating Retinal Homeostasis. Our initial focus was delineating molecular mechanisms governed by PDEs in the retina. First, we determined by single-cell RNA-sequencing (scRNA-seq) that all PDE subtypes and isoforms are expressed across the various cell types of the murine retina (Fig. 1*B*), indicating probable involvement of PDEs in regulating retinal homeostasis. Indeed, PDE6 isoforms exhibited high expression in rods and cones, consistent with the fundamental role of PDE6 in mediating visual phototransduction in photoreceptors via cGMP hydrolysis (24). Additionally, in the photosensitive dKO model of stress-inducible photoreceptor degeneration, we observed significant differential expression of most PDE isoforms in the retina 24 h after exposure to bright-light stress (Fig. 1*C*). Several PDEs became significantly upregulated with an average $\log_2$ fold-change (logFC) > 0.5 across many retinal cell types under stress, especially in photoreceptors, Müller glia, microglia, astrocytes, and horizontal cells. Indeed, increased PDE4D expression throughout the retina was confirmed at the protein level by immunohistochemistry (IHC) (*SI Appendix*, Fig. S1). Among the PDEs significantly upregulated under stress, PDE2, PDE4, and PDE11 can be inhibited selectively by the chemical compounds BAY 60-7550, rolipram, and BC11-38, respectively (10, 32). Thus, we reasoned that if the increased PDE activity in these cell types drives the pathogenesis of stress-induced retinal degeneration, then targeted pharmacological inhibition of these select PDEs would mitigate light damage in photosensitive dKO mice.

Pathways Regulated by Stress and PDEs in Human ARPE-19 Cells. The light-sensitive dKO model has the *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ genotype that phenotypically predisposes to accumulation of all-*trans*-retinal (atRAL) and associated cytotoxic adducts due to visual cycle deficiencies, and these toxic reactions are accelerated during exposure to bright-light stress (26, 33). To delineate the molecular mechanisms underlying atRAL-induced toxicity, we first employed an in vitro fluorescence assay of Ca^{2+}-associated cytotoxicity, using the human-derived ARPE-19 cell line. This approach is based on previous studies investigating atRAL exposure and apoptotic cell death, in the context of aberrant ionic gradients and intracellular Ca^{2+} elevation (34). In ARPE-19 cells exposed to 60 μM atRAL stress, we observed significantly increased intracellular Ca^{2+} by Fluo-3 AM staining relative to DMSO-treated controls, consistent with Ca^{2+}-associated cytotoxicity (*SI Appendix*, Fig. S2). In ARPE-19 cells treated with 10 μM BAY 60-7550, rolipram, or BC11-38 this atRAL-induced Ca^{2+} elevation was reduced by approximately 50%, suggesting that selective inhibition of PDE2, PDE4, or PDE11 confers protection against atRAL-induced cytotoxicity.

To obtain an unbiased, thorough understanding of the biological pathways implicated in atRAL-induced cytotoxicity and PDE-inhibitor–mediated cytoprotection, we performed a mass spectrometry-based quantitative proteomic analysis of ARPE-19 cells, using stable-isotope labeling of amino acids in cell culture. We identified differentially expressed proteins in ARPE-19 cells exposed to stress (60 μM atRAL or 5 mM H_2O_2) or treated with selective PDE inhibitors (10 μM BAY 60-7550, rolipram, or BC11-38), relative to vehicle-treated controls. From the lists of differentially expressed proteins, we filtered for those that were upregulated by both atRAL and oxidative stress and downregulated by all three PDE inhibitors; and conversely, those that were downregulated by both types of stress and upregulated by all three PDE inhibitors. This selection allowed us to focus on universal mechanisms of stress-induced cytotoxicity and PDE-inhibitor–mediated cytoprotection. The protein networks so identified were mapped

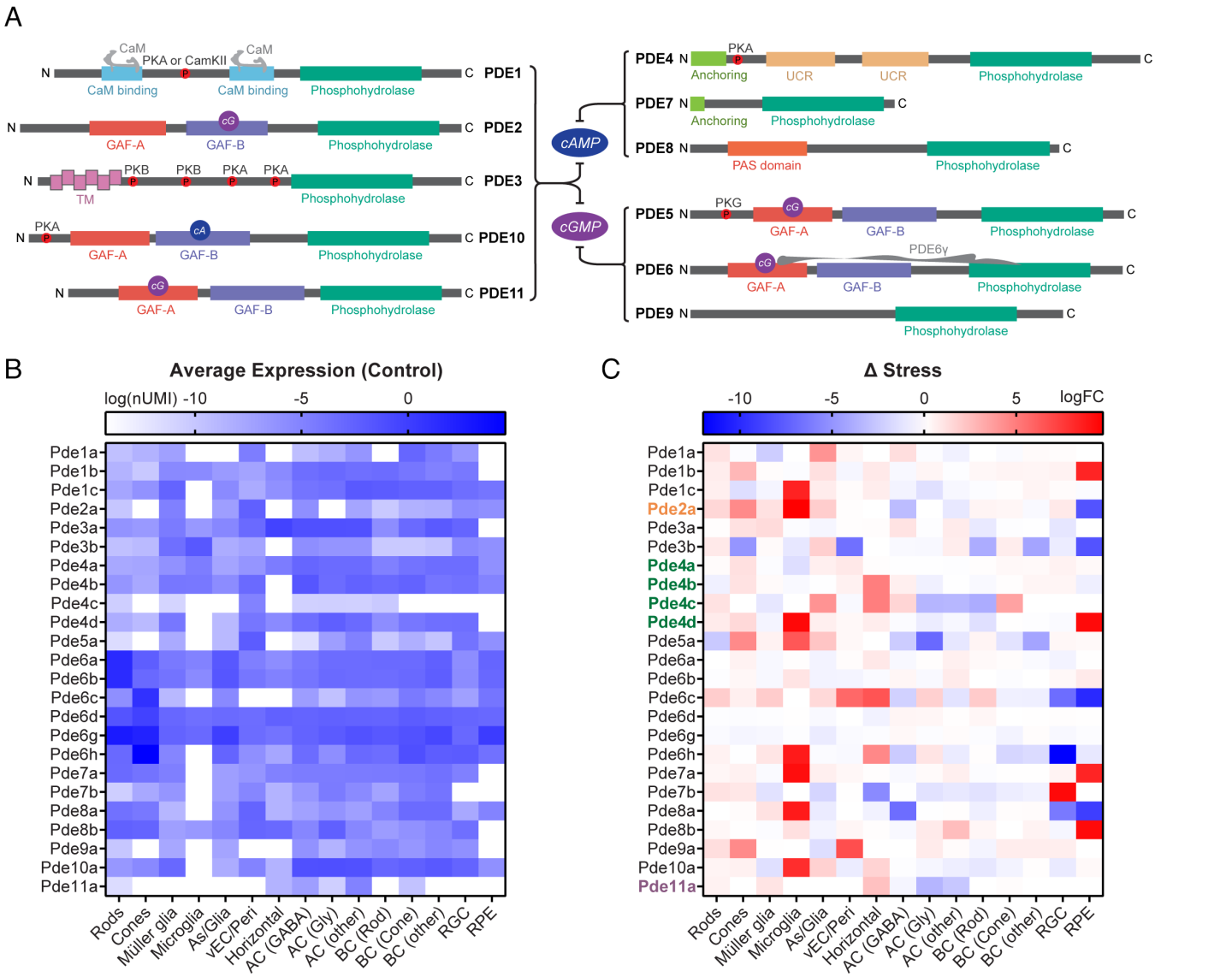

Fig. 1. PDE expression in murine retina. (*A*) Schematic representation showing domain organization in the eleven cyclic nucleotide PDE families and subtype-specific hydrolysis of cAMP and/or cGMP. The conserved phosphohydrolase catalytic domain is represented in green. Cam, Calmodulin; CamKII, calcium/calmodulin-dependent protein kinase II; cG/cA, cGMP/cAMP domain; PAS, Per-Arnt-Sim; PKG, protein kinase G; TM, transmembrane domain; UCR, upstream conserved region. (*B*) scRNA-seq heat map depicting average expression values of PDE isoforms by cell type in retinas of unstressed control *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ (dKO) mice, measured in normalized unique molecular identifier (nUMI) counts. AC, amacrine cell; As, astrocyte; BC, bipolar cell; RGC, retinal ganglion cell; RPE, retinal pigmented epithelium; vEC/Peri, vascular endothelial cell or pericyte. (*C*) scRNA-seq analysis reveals PDE isoforms become differentially expressed in dKO mice 1 d after exposure to bright-light stress, relative to unstressed controls. logFC, log fold change.

according to functional enrichments (*SI Appendix*, Fig. S3*A*), which revealed biological pathways of significance involving cellular response to stress, protein homeostasis, chromatin remodeling, immune regulation, post-translational modifications, and JAK-STAT signaling (*SI Appendix*, Fig. S3*B*). Individual proteins implicated in these pathways were differentially expressed under stress and with PDE-inhibitor treatment, and the degree of differential expression was quantified by logFC (*SI Appendix*, Fig. S3*C*).

Through the quantitative proteomic analysis of ARPE-19 cells, we identified mitochondrial apoptosis-inducing factor 1 (AIFM1) and endoplasmic reticulum calcium ATPase 2 (ATP2A2), both of which were upregulated under stress yet downregulated by PDE-inhibitor treatment, consistent with their previously described functions in driving apoptosis through Ca^{2+}-mediated signaling (35, 36). We also identified additional proteins implicated in various homeostatic processes, including stress-induced apoptosis, phagocytosis of apoptotic cells, signaling networks mediated by TNF or Ca^{2+}, histone modifications, DNA damage repair, autophagy, chaperone-mediated refolding, or the ubiquitin–proteasome system (*SI Appendix*, Table S1). Altogether, these in vitro studies support the conclusion that stress-induced cytotoxicity involves apoptosis driven by TNF- and Ca^{2+}-mediated signaling pathways, suggesting that PDE inhibition promotes resilience to stress in part through suppression of these apoptotic processes.

Selective PDE Inhibitors Mitigate Stress-Induced Retinal Degeneration. Having demonstrated in vitro attenuation of atRAL-induced cytotoxicity in human ARPE-19 cells by PDE-inhibitor treatment, we next investigated the effects of selective PDE-inhibitor therapy in vivo, using our photosensitive dKO model. These mice were administered BAY 60-7550, rolipram, or BC11-38 by intraperitoneal injection (2 mg/kg) 30 min prior to bright-light stress exposure, and their retinal phenotypes were characterized

1 wk later. Scanning laser ophthalmoscopy (SLO) and optical coherence tomography (OCT) were utilized to obtain images of the fundus and retinal cross sections, respectively (Fig. 2*A*). In vehicle-treated dKO mice exposed to stress, SLO imaging revealed characteristic autofluorescent puncta associated with phototoxicity and reactive inflammation, while OCT imaging revealed concomitant degeneration of the photoreceptor-containing outer nuclear layer (ONL), relative to WT and unstressed controls (Fig. 2*B*). Moreover, scotopic electroretinogram (ERG) recordings from these mice revealed significantly diminished average a-wave and b-wave amplitudes relative to unstressed controls, indicating impairment of outer and inner retinal function, respectively, concomitant with structural degeneration (Fig. 2*C*). However, in dKO mice treated with selective inhibitors of PDE2, PDE4, or PDE11 both retinal structure and function were protected from the degenerative effects induced by stress, thus demonstrating the efficacy of PDE-inhibitor therapy in vivo.

To confirm that the protective effects of selective PDE-inhibitor therapy are mediated by second messenger signaling pathways and associated somatic maintenance processes downstream, we first ruled out the possibility that off-target nonselective inhibition of PDE6 could have occurred, since inhibiting phototransduction during bright light exposure could also prevent light damage albeit through an independent mechanism. We performed a fluorescence enzymatic activity assay of PDE6-catalyzed cGMP hydrolysis using bovine ROS in vitro; with the nonselective PDE inhibitor vardenafil as a positive control, we observed an approximate two-fold reduction in PDE6 activity relative to the DMSO negative control at a vardenafil concentration of 1 μM (*SI Appendix*, Fig. S4 *A* and *B*). In contrast, the selective PDE inhibitors tested at the same drug concentration did not exhibit substantial impairment of PDE6 activity, suggesting that the observed protective effects of selective PDE inhibition do not require or involve any nonselective inhibition of PDE6 and/or phototransduction. To confirm this interpretation in vivo, we performed scotopic ERG analyses on unstressed dKO mice 30 min after intraperitoneal administration of 2 mg/kg PDE inhibitor (BAY 60-7550, rolipram, BC11-38, or vardenafil) or dimethyl sulfoxide (DMSO) vehicle. While vardenafil-treated mice exhibited a significant decrease in both a-wave and b-wave amplitudes, mice treated with selective inhibitors of PDE2, PDE4, or PDE11 displayed ERG amplitudes within the range of vehicle-treated controls (*SI Appendix*, Fig. S4 *C* and *D*). Taken together, these in vitro and in vivo analyses indicate that visual phototransduction is not substantially impaired by the selective PDE inhibitors, therefore supporting the potential clinical feasibility of this therapeutic approach for preserving vision in degenerative blinding diseases.

Molecular Mechanisms of Stress Resilience and PDE Inhibitor Therapy. After ruling out nonselective inhibition of phototransduction as a potential confounding factor, we performed scRNA-seq analyses on photosensitive dKO mice to identify molecular mechanisms responsible for the therapeutic effects of selective PDE inhibition, focusing on the PDE4- and PDE11-selective inhibitors that yielded the most significant improvement in photoreceptor viability. We first utilized Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction to generate a visual representation of transcriptomic differences between individual retinal cells (37). In a two-dimensional UMAP, the degree of difference between individual transcriptomic profiles is directly proportional to the distance between corresponding points, which segregate into distinct clusters that can be assigned according to cell type (Fig. 3*A*). Through a UMAP comparison highlighting retinal cells from unstressed versus stressed dKO mice, we identified certain clusters in which the effect of stress on transcriptomic changes was especially pronounced; namely, in the photoreceptors, Müller glia, astrocytes, and other glia (Fig. 3*B*). However, in the PDE-inhibitor–treated groups, these stress-induced transcriptomic shifts were largely attenuated (*SI Appendix*, Fig. S5), and their respective transcriptomic profiles generally resembled that of the unstressed controls. Among the most significant differentially expressed genes in the clusters of interest, we identified glutamine synthetase (*Glul*), which was reduced following acute stress exposure, especially in Müller glia, microglia, astrocytes, and retinal pigment epithelium (RPE)

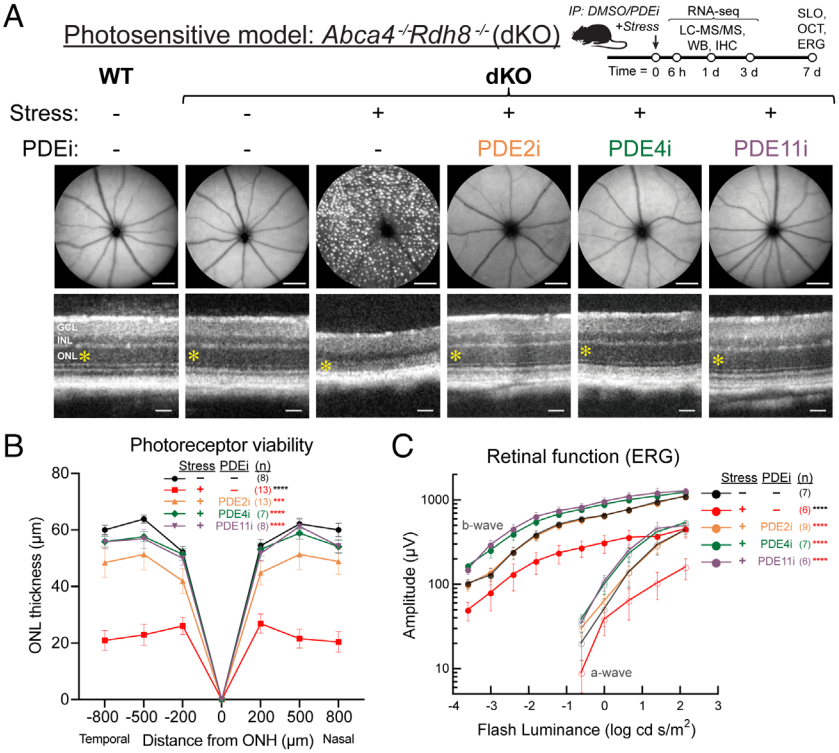

Fig. 2. Selective PDE inhibitors mitigate stress-induced retinal degeneration. (*A*) Representative SLO images (*Top* row) reveal autofluorescent puncta in the fundus of DMSO vehicle-treated dKO mice 7 d after exposure to bright-light stress, as compared to unstressed dKO and WT controls. SLO (Scale bars, 500 μm.) OCT images (*Bottom* row) from which thickness of the photoreceptor-containing outer nuclear layer (ONL, yellow asterisk) is measured. OCT (Scale bars, 50 μm.) GCL, ganglion cell layer; INL, inner nuclear layer. n = 3 per group. (*B*) Spider graph of ONL thickness as a function of distance from the optic nerve head (ONH). Combined SLO and OCT imaging analyses reveal dKO mice are protected from stress-induced retinal pathology by treatment with selective PDE inhibitors BAY 60-7550 (PDE2i), rolipram (PDE4i), or BC 11-38 (PDE11i). (*C*) Scotopic ERG recordings demonstrate retinal function impairment in stressed dKO mice (red) relative to unstressed controls (black). Treatment with PDE inhibitors in stressed dKO mice preserved photoreceptor function (a-wave; empty circles) and inner retinal function (b-wave; solid circles) to levels at or above those of unstressed, vehicle-treated controls. Repeated measures two-way ANOVA comparison with negative (black) or positive (red) control groups; ***P = 0.0002, ****P < 0.0001. Error bars represent SEM.

(Fig. 3*C*). Conversely, the PDE-inhibitor–treated mice exhibited upregulation of *Glul* to levels surpassing those of unstressed controls, suggesting that PDE-inhibitor therapy stimulates mechanisms of somatic maintenance to levels that support enhanced resilience to stress. Indeed, *Glul* has been implicated in the catalytic conversion of neurotoxic glutamate or ammonia to nontoxic glutamine, consistent with a neuroprotective role in somatic maintenance (38, 39).

In contrast, we also identified tumor necrosis factor receptor superfamily member 12A (*Tnfrsf12a*), a known inducer of apoptosis (40), which became significantly upregulated with stress, especially in cones, horizontal cells, Müller glia, microglia, astrocytes, and RPE (Fig. 3*D*). PDE-inhibitor therapy broadly mitigated stress-induced upregulation of *Tnfrsf12a* across most retinal cell types, and in some cases even suppressed expression to levels below that of unstressed controls, suggesting that the enhanced stress resilience phenotype enabled by selective PDE inhibition involves a synergistic combination of antiapoptotic effects and improved somatic maintenance. In further support of this interpretation, many of the additional genes we identified, which exhibited reduced expression in relevant cell types under stress but were upregulated by PDE-inhibitor therapy, are involved in somatic maintenance;

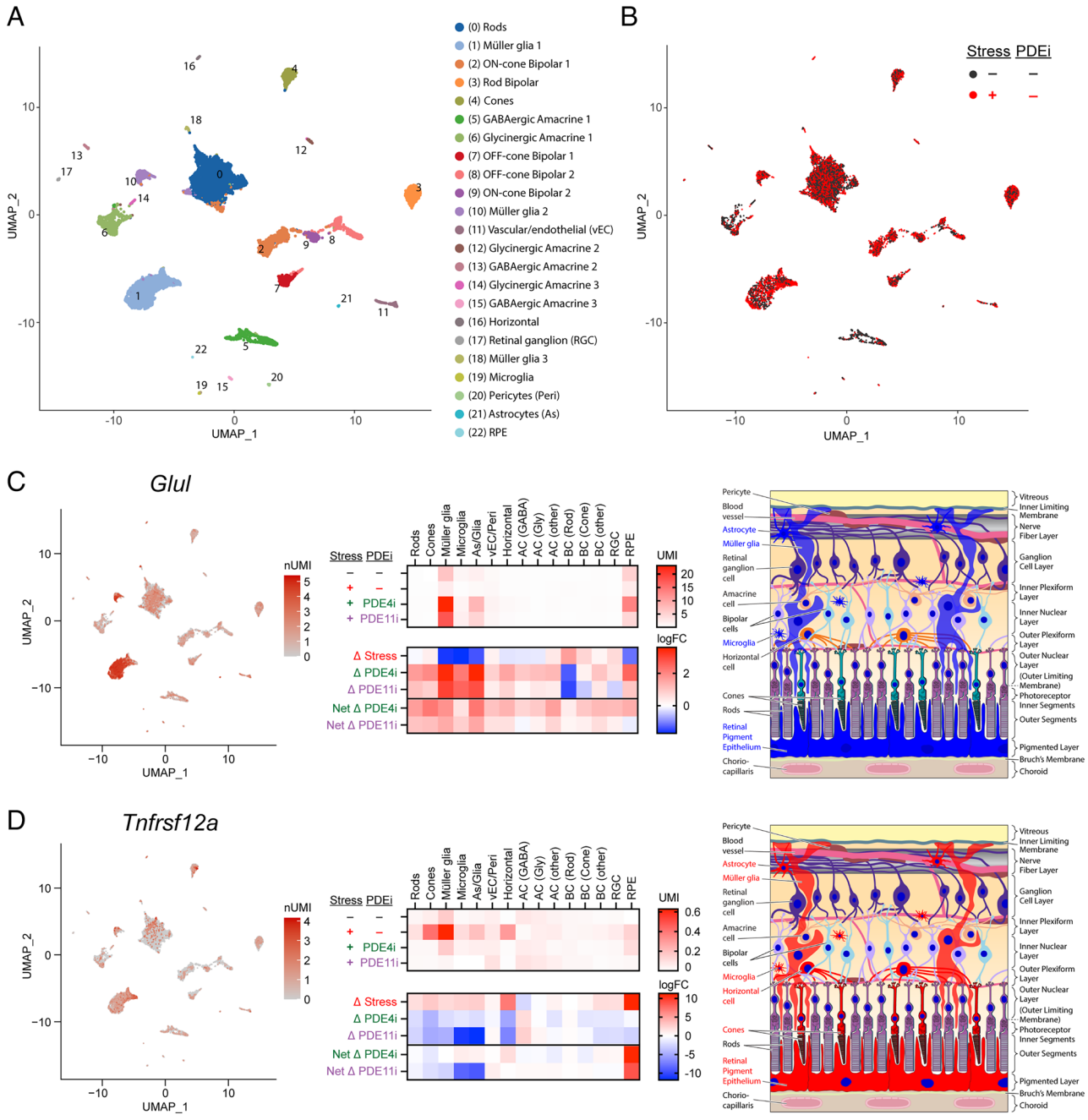

Fig. 3. scRNA-seq analysis of stress resilience mechanisms. (*A*) UMAP clustering of retinal cell types in dKO mice. (*B*) Cell types grouped by experimental condition, unstressed (black) or 1 d after bright-light stress (red). (*C*) Average *Glul* expression in retinal cell types, quantified by normalized unique molecular identifier (UMI) counts, and log fold change (logFC) reveals genes downregulated or upregulated 1 d after stress relative to unstressed controls (Δ Stress); in stressed mice treated with PDEi relative to vehicle-treated controls (Δ PDEi); and in stressed mice treated with PDEi relative to unstressed controls (Net Δ). Cell types exhibiting significant *Glul* downregulation (Δ Stress) with logFC < −1 are labeled and highlighted in blue in the cross-sectional retina diagram. (*D*) Average *Tnfrsf12a* expression in retinal cell types, quantified by UMI and logFC. Cell types exhibiting significant *Tnfrsf12a* upregulation (Δ Stress) with logFC > 1 are labeled and highlighted in red in the cross-sectional retina diagram.

and other genes, which became upregulated under stress but were attenuated by selective PDE inhibition, are implicated in driving apoptosis and/or cell death (*SI Appendix*, Table S2).

Given the biological pathways implicated at the level of gene transcription, we next sought to verify these processes in photosensitive dKO mice at the protein level, using combined label-free mass spectrometry-based quantitative proteomic and phosphoproteomic analyses. First, we identified differentially expressed proteins in retinas exposed to bright-light stress relative to unstressed controls. Next, we identified differentially expressed proteins in PDE-inhibitor-treated mice exposed to stress relative to vehicle-treated controls. From these lists of differentially expressed proteins, we filtered for those that were both upregulated by stress and downregulated by PDE-inhibitor therapy, and conversely for those that were both downregulated by stress and upregulated by PDE-inhibitor therapy. This selection process allowed us to focus on universal mechanisms of stress-induced retinal degeneration and PDE-inhibitor–mediated protection. The protein networks so identified were mapped according to functional enrichments, which revealed biological pathways of significance involving cellular response to stress, protein and/or mitochondrial homeostasis, chromatin remodeling, inflammatory response, post-translational modifications, apoptosis, and signaling by mTOR, JAK-STAT, or second messengers *SI Appendix*, Fig. S6 *A*–*C*) (41–44). Individual proteins within these functional pathways became differentially expressed under stress and with PDE-inhibitor treatment, and the degree of differential expression was quantified by logFC (Fig. 4*A*).

Through the quantitative proteomic analysis of dKO mice, we identified mitochondrial calcium uniporter protein and growth hormone-inducible transmembrane protein, both of which were downregulated by stress yet upregulated by PDE-inhibitor treatment, consistent with their previously described functions in mediating apoptosis through regulation of mitochondrial Ca^{2+} uptake in response to stress (45, 46). This analysis also revealed additional proteins of interest involved in promoting cell survival or apoptosis in response to stress, TNF or Ca^{2+}-mediated signaling, histone modifications, DNA damage repair, antioxidant defense, autophagy, chaperone-mediated refolding, or the ubiquitin–proteasome system (*SI Appendix*, Table S3). Notably, histone deacetylase 11 (HDAC11) and complement C3 (C3), both of which were upregulated by stress but attenuated by PDE-inhibitor therapy, have also been implicated in the pathogenesis of human AMD (4, 47), demonstrating commonality in the mechanisms driving retinal degeneration between the photosensitive dKO model and human subjects with AMD. Moreover, rod-derived cone viability factor (RdCVF), also known as nucleoredoxin-like protein 1 (NXNL1), a somatic maintenance protein that promotes cone photoreceptor survival (48, 49), was depleted by stress but partially restored with PDE-inhibitor therapy, and this finding was validated through quantitative Western blot analysis (Fig. 4*B*).

Lastly, a phosphoproteome-wide screen was conducted in parallel to delineate pertinent post-translational modifications in the context of phosphorylation-mediated signaling pathways associated with retinal degeneration or protection (Fig. 4*C*). As would be expected with PDE inhibition increasing intracellular cyclic nucleotide concentration and inducing PKA-dependent phosphorylation of downstream targets, we detected increased phosphorylation of cAMP-responsive element-binding protein 1 (CREB1) in PDE-inhibitor-treated dKO mice compared to vehicle-treated controls. This apparent protection from stress-induced CREB1 hypophosphorylation, in PDE-inhibitor–treated mice, was confirmed by quantitative Western blot analysis, both at the canonical serine 133 phospho-site (S133) and at serine 271 (S271)

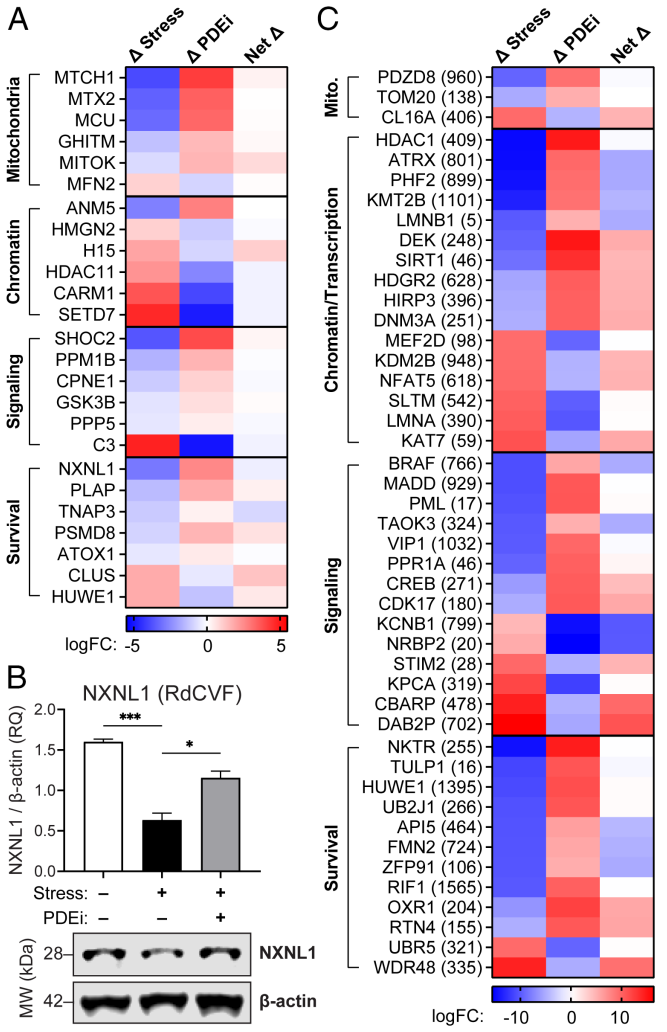

Fig. 4. Stress-induced proteomic and phosphoproteomic dysregulation is attenuated by PDE-inhibitor therapy. (*A*) Quantitative proteomic analysis reveals downregulated (blue) or upregulated (red) proteins, 1 d after bright-light stress exposure (n = 6) relative to unstressed (n = 6) controls (Δ Stress); in stressed dKO mice treated with 2 mg/kg BC11-38 (n = 3) relative to DMSO vehicle-treated controls (Δ PDEi); and in stressed mice treated with BC11-38 relative to unstressed vehicle-treated controls (Net Δ). (*B*) Quantitative Western blot analysis of NXNL1 (RdCVF) expression in unstressed and stressed mice treated with BC11-38 (PDEi) or vehicle. RQ, relative quantity. n = 3 per group, $*P < 0.05$, $***P < 0.001$. Error bars represent SEM. (*C*) Quantitative phosphoproteomic analysis reveals hypophosphorylated (blue) or hyperphosphorylated (red) proteins (phospho-sites). n = 3 to 6 per group. logFC, log fold change.

(*SI Appendix*, Fig. S6*D*). Therefore, these results exemplify how PDE-inhibitor therapy could attenuate stress-induced phosphoproteomic dysregulation and promote resilience to stress, in part through phosphorylation-mediated second messenger signaling pathways. Indeed, reduced phosphorylation and activity of CREB1 have generally been associated with neurodegenerative processes promoting apoptosis, whereas increased phosphorylation and activity of CREB1 have been associated with neuroprotective processes promoting somatic maintenance and survival (50–53).

Altogether, these transcriptomic, proteomic, and phosphoproteomic analyses support the hypothesis that selective PDE inhibition enhances resilience to stress in the retina, both by promoting somatic maintenance and by inhibiting apoptosis via cyclic nucleotide-mediated second messenger signaling pathways. To validate these findings and gain insights into the time course of pertinent protective and/or degenerative processes, we conducted a bulk RNA-seq analysis of whole dKO retinas collected at 6 h, 1 d, and

3 d after exposure to stress. We confirmed significant time-dependent decreases in expression of *Glul*, *Nxnl1*, *Xiap*, and *Tulp1*, consistent with stress-induced depletion of somatic maintenance processes (Fig. 5*A*). Conversely, we also confirmed time-dependent upregulation of *Tnfrsf12a* and *C3*, consistent with stress-induced apoptosis and complement activation, respectively (Fig. 5*B*). In further support of the anti-apoptotic effects conferred by PDE-inhibitor therapy, our bulk RNA-seq analysis revealed that both the intrinsic and extrinsic canonical apoptotic pathways, driven by *Trp53*, *Fas*, and *Tnfrsf1a*, are significantly upregulated at different time points after exposure to stress, relative to unstressed controls. To corroborate this transcriptional upregulation of apoptotic processes at the protein level, we utilized IHC to visualize TNFR1 expression in retinal cross-sections prepared from dKO mice. Peanut agglutinin (PNA) staining revealed photoreceptor degeneration, particularly in the outer segment (OS) layer in vehicle-treated dKO mice 1 d after exposure to stress, relative to unstressed controls (Fig. 5*C*); this OS degeneration was accompanied by widespread upregulation of TNFR1. Rolipram therapy attenuated TNFR1 upregulation while protecting photosensitive dKO mice from acute stress-induced photoreceptor degeneration.

Conserved Retinal Protection across Acute and Chronic Forms of Stress. We next questioned whether the therapeutic effect of selective PDE inhibition is limited to the photosensitive dKO model of acute stress, or if protection could be similarly conferred in models of chronic retinal degeneration. To investigate this possibility, we first employed the *rd10* mouse model of autosomal recessive retinitis pigmentosa, which exhibits progressive photoreceptor degeneration under standard lighting conditions (54, 55). Over the course of 2 wk, *rd10* mice were reared under standard lighting, on either a control (base) diet or a rolipram-infused rodent chow diet, and their retinal phenotypes were characterized at the end of the 2-wk period. Through OCT and SLO imaging, *rd10* mice on the rolipram diet exhibited protection from retinal degeneration, as well as attenuation of autofluorescent puncta associated with phototoxicity and reactive inflammation, compared to *rd10* mice on the control diet (*SI Appendix*, Fig. S7). In addition, IHC studies demonstrated reduced extrinsic apoptosis with PDE-inhibitor therapy; Fas signaling was largely attenuated by the rolipram diet (Fig. 6*A*), accompanied by significant improvement of photoreceptor viability (Fig. 6*B*). Consistent with retinal structure preservation in *rd10* mice on the rolipram diet, photopic ERG analyses revealed improvement in retinal function as well (Fig. 6*C*). Moreover, in another murine model of chronic retinal degeneration, the streptozotocin model of diabetic retinopathy, rolipram therapy alleviated pathological hallmarks in a dose-dependent manner, resulting in significant reduction of retinal oxidative stress and inflammation (Fig. 6 *D* and *E*), as well as protection from leukocyte-mediated endothelial cell death (Fig. 6*F*) and retinal capillary degeneration (Fig. 6*G*).

Taken together, these data indicate that the molecular mechanisms promoting stress resilience, which are enhanced by PDE-inhibitor therapy, may be broadly responsive across various retinopathies involving acute and/or chronic stressors. To evaluate the potential clinical relevance of these findings, we conducted IHC studies using human retina samples from donors who were diagnosed with either AMD or diabetic retinopathy. Compared to age-matched controls, AMD patients generally exhibited increased levels of heterochromatin (H3K9me3) throughout the retina (*SI Appendix*, Fig. S8*A*), indicating a global reduction in chromatin accessibility. Conversely, patients with diabetic retinopathy exhibited reduced GLUL expression (*SI Appendix*, Fig. S8*B*), particularly in the outer plexiform and inner nuclear layers. Respectively, these findings are consistent with stress-induced upregulation of HDAC11 and associated epigenetic hallmarks of AMD (4, 26), as well as glutamate-associated neurotoxicity in the diseased retina (38).

Discussion

Recent clinical trials aimed at developing treatments for AMD have primarily focused on singular disease pathways. These include visual cycle modulators (56), complement inhibitors (57), and growth receptor inhibitors (58), none of which have achieved desirable results or received FDA approval. The most prevalent form of AMD (dry), in earlier stages of pathogenesis, represents approximately 80 to 90% of the total number of cases, yet no treatments have been approved for dry AMD to date (3). Likewise, for retinitis pigmentosa and diabetic retinopathy, safe and efficacious therapies are urgently needed. To address this therapeutic deficiency, we employed a systems pharmacology approach that identified pertinent cell types and signaling pathways for therapeutic targeting. Pharmacological inhibition of select PDEs enhanced resilience to acute and chronic forms of stress, exemplifying a prototypical therapeutic platform that exploits integrative mechanisms of action for the treatment of complex, multifactorial disorders. Based on this model, the retina functions as a complex, heterogenous tissue comprised of multiple cell types, which signal via intercellular communication to modulate intrinsic stress resilience and homeostasis of the entire system. Therefore, it is conceivable that both stress-resilient and degenerative retinal phenotypes involve complex signaling networks, which are modulated either directly by a primary defect in a particular cell type, or indirectly by other retinal cells downstream of the primary defect. Accordingly, for complex diseases such as AMD, in which numerous intracellular and intercellular pathways are perturbed, the systems pharmacology platform enables global normalization of cellular and tissue homeostasis, representing a multitargeted therapeutic strategy with superior efficacy and reduced side effects in comparison to traditional monotherapy (59–61).

In this study, we applied the principles of systems pharmacology to identify universal molecular mechanisms of stress resilience and degeneration across acute and chronic models of retinopathy. Combined transcriptomic, proteomic, and phosphoproteomic analyses led to the identification of pertinent cell types and signaling pathways, many of which have indeed been implicated in human AMD, retinitis pigmentosa, and/or diabetic retinopathy. For instance, in the dKO model that recapitulates hallmarks of AMD, unbiased screening by scRNA-seq initially identified stress-induced upregulation of PDE transcripts, particularly in microglia and RPE, as well as in Müller glia and astrocytes; this effect could potentially be attributed, in part, to phagocytosis of other retinal cell types expressing the PDE transcripts. However, by experimental design, we selected 1 d after stress as the time point for scRNA-seq analyses based on prior studies that had comprehensively characterized the time course for stress-induced retinal degeneration in the dKO model (26, 62). At 1 d after stress, the effect of phagocytosis or cell loss is negligible, as the structure of the retina remains largely intact, with cell loss beginning around 5 d after stress; thus, our scRNA-seq analyses should reflect cell-specific transcriptomic changes directly induced by stress. These findings implicate Glul expression, particularly in Müller glia, microglia, astrocytes, and RPE, corroborating prior studies on the neuroprotective role of Glul (38, 39). Notably, stress-induced depletion of Glul may also be relevant in the clinical context of diabetic retinopathy, as it would lead to neurotoxic glutamate accumulation, which has been positively associated with proliferative disease (63). Likewise, unbiased proteome-wide

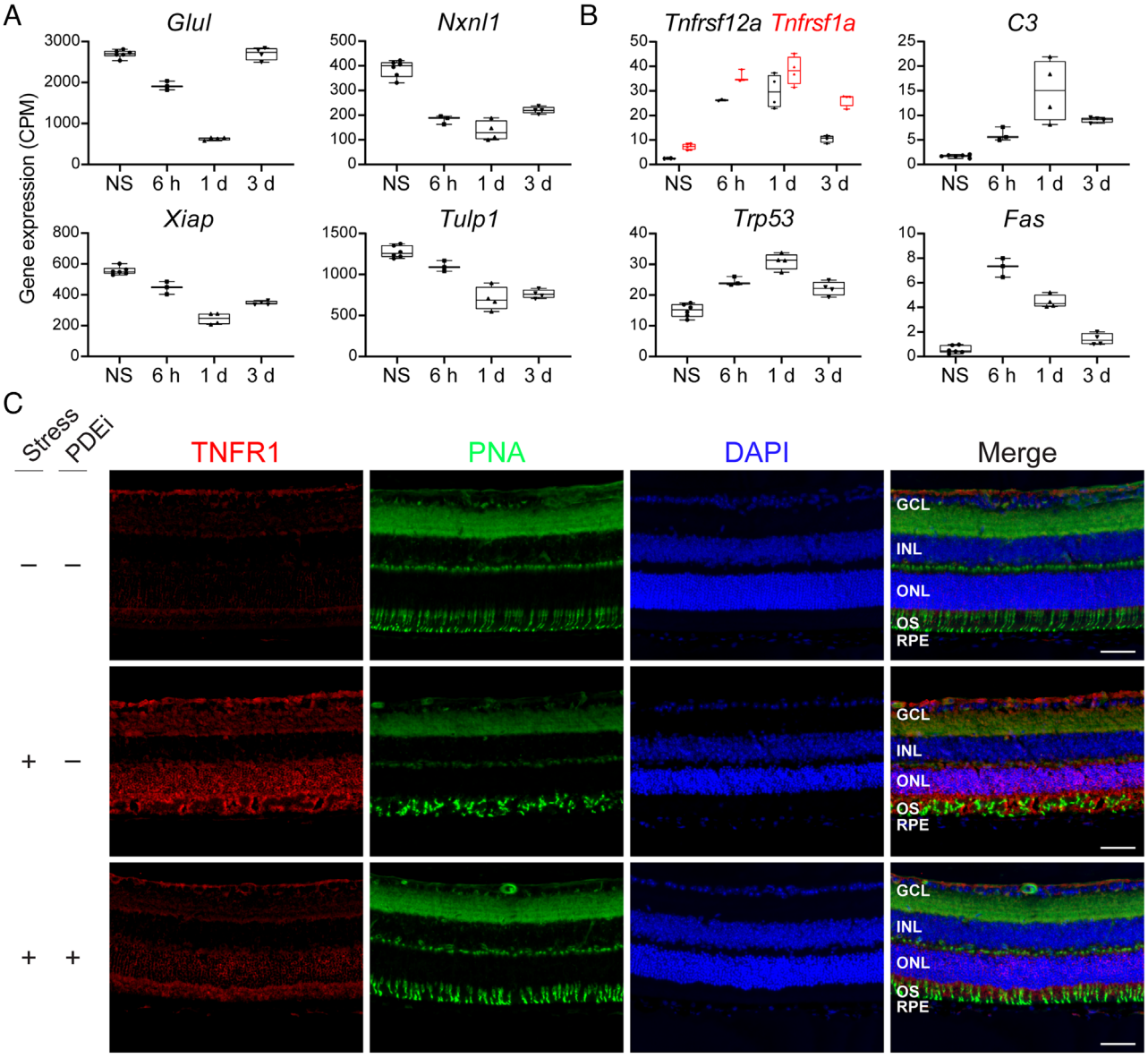

Fig. 5. Reduced somatic maintenance and increased apoptosis in stressed dKO retina. (*A* and *B*) Bulk RNA-seq analysis of retinas collected from dKO mice 6 h (n = 3), 1 d (n = 4), and 3 d (n = 4) after exposure to bright-light stress reveals significant differentially expressed genes 1 d after stress (adjusted *P* value < 0.05), confirming (*A*) decreased somatic maintenance (*Glul, Nxnl1, Xiap, Tulp1*) and (*B*) increased intrinsic (*Trp53*) and extrinsic (*Tnfrsf/Fas*) apoptosis, relative to unstressed (NS) controls (n = 6). Boxes and whiskers represent interquartile and minimum-maximum ranges, respectively. CPM, counts per million. (*C*) IHC analysis of retinal cross-sections demonstrates TNFR1 upregulation at the protein level with concomitant degeneration of cone photoreceptors (PNA) in dKO mice 1 d after exposure to stress, relative to unstressed controls. In dKO mice treated with rolipram (PDEi), TNFR1-mediated apoptosis and cone degeneration are mitigated 1 d after exposure to stress, relative to vehicle-treated controls. Representative sections shown are from the same dorsal region of the retina in each mouse. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segment layer; RPE, retinal pigment epithelium. (Scale bars, 50 μm.)

screening revealed that expression of RdCVF was depleted in degenerating retina, consistent with its previously characterized role in promoting photoreceptor survival (49, 64); in the context of retinitis pigmentosa, restoration of RdCVF expression by gene therapy has been shown to alleviate cone cell death and improve retinal function (48, 65). Conversely, HDAC11, C3, and TNF were upregulated in degenerating retina, in line with clinical findings from AMD patients (4, 47, 66).

Previously, we had demonstrated that the photosensitive dKO model recapitulates epigenetic hallmarks of human AMD, exhibiting a global reduction in chromatin accessibility through decreased euchromatin (i.e., H3K27ac) and increased heterochromatin (i.e., H3K9me3) following stress-induced retinal degeneration. Based on this premise, we devised a pharmacological intervention to mitigate disease by epigenetic reprogramming and demonstrated its efficacy in dKO mice (26). This approach has since been validated by others, given the recent finding that hallmarks of aging and age-related diseases involve loss of epigenetic information over time, which may be reversible through manipulations of the epigenome (67). Building upon our initially proposed strategy of direct pharmacological modulation (i.e., selective inhibition of histone deacetylases or methyltransferases), we propose herein an intervention that suppresses HDAC11 secondary to selective PDE inhibition; both these approaches yield a similar result in the context of attenuating stress-induced epigenetic dysregulation.

In summary, the processes mediating stress resilience and degeneration are integrally regulated by cyclic nucleotide signaling and can be therapeutically targeted. Selective PDE-inhibitor therapy enhanced resilience to stress through suppression of degenerative pathways (e.g., TNF, C3, HDAC11, Trp53, Fas), in parallel with

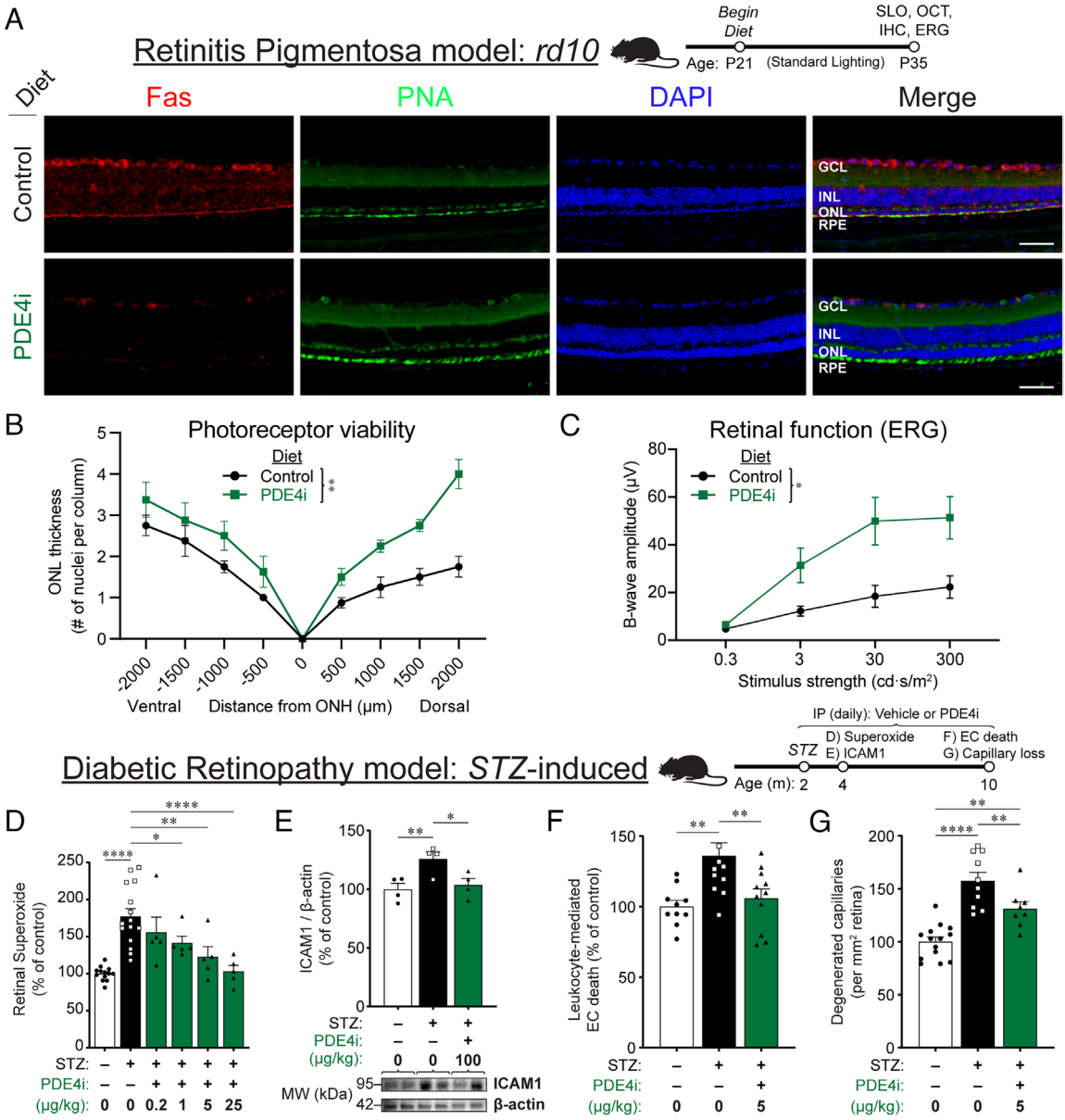

Fig. 6. Chronic retinal degeneration is attenuated by long-term PDE-inhibitor therapy across distinct disease models. (*A*) Representative IHC images from *rd10* mice at 2 mm dorsal to the optic nerve head (ONH) show increased Fas signaling after two wk on the control diet, attenuated in mice on the rolipram (PDE4i) diet. DAPI labels nuclei, and peanut agglutinin (PNA) labels cone photoreceptors. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. (Scale bars, 50 μm.) (*B*) Spider graph of ONL thickness as a function of distance from ONH in *rd10* mice. n = 4 per group, repeated measures two-way ANOVA ($F_{(1, 6)} = 17.04$, $^{**}P = 0.0062$). (*C*) Photopic ERGs from *rd10* mice demonstrate improved retinal function on the rolipram diet relative to control. n = 4 per group, two-way ANOVA ($F_{(1, 6)} = 7.896$, $^{*}P = 0.0308$). (*D–G*) Mice having streptozotocin (STZ)-induced diabetes developed hallmarks of diabetic retinopathy, as well as molecular abnormalities implicated in disease pathogenesis: (*D*) retinal superoxide, (*E*) ICAM1-dependent inflammation, (*F*) leukocytemediated cytotoxicity in retinal endothelial cells (EC), and (*G*) degeneration of retinal capillaries; all were inhibited by rolipram, administered daily by intraperitoneal (IP) injection in DMSO at the indicated doses. Data are expressed as a percentage of the value of non-diabetic controls. n ≥ 5 per group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. Error bars represent SEM.

synergistic upregulation of somatic maintenance and protein homeostasis mechanisms (e.g., Glul, RdCVF, Xiap, Tulp1, pCREB-S133/S271) (Fig. 7). Given that selective PDE inhibition suppressed molecular mechanisms that drive a global reduction in chromatin accessibility (e.g., HDAC11) while promoting clearance of neurotoxic substrates (i.e., GLUL upregulation), it is conceivable that such a therapeutic strategy could be similarly effective in clinical cases of AMD and diabetic retinopathy, respectively. Altogether, the results from our combined in vitro and in vivo studies demonstrate proof-of-concept for the systems pharmacology platform, culminating in the discovery of a therapeutic strategy that enhances conserved mechanisms of stress resilience and could potentially be applied to the most common causes of blindness, irrespective of specific disease etiology or underlying genetic mutations.

Through comprehensive transcriptomic and proteomic profiling, we gained insights into the pathological processes driving retinal degeneration and the protective mechanisms of somatic maintenance stimulated by PDE-inhibitor therapy. However, we may have missed some important elements. Membrane proteins could potentially have been underrepresented in sample preparations due to their low relative abundance and hydrophobicity, which would decrease the probability of detection by liquid chromatography coupled with mass spectrometry (LC-MS/MS). Acknowledging this possibility, we measured the relative

abundance of membrane proteins identified through our combined proteomic analyses of ARPE-19 cells; 1,082 membrane proteins were detected out of 2,046 total proteins, i.e., ~53%. Similarly, our combined proteomic analysis of dKO retina identified 1,567 membrane proteins out of 3,350 total proteins, i.e., ~47%. Hence, while certain membrane-associated proteins may have escaped detection, we successfully identified many relevant proteins and pathways involved in modulating retinal degeneration and protection. Nevertheless, recent biochemical advances enabling improved membrane protein enrichment and detection likely will yield increased sensitivity in future applications (68), facilitating the identification of low-abundance proteins of potential relevance to the degenerative or protective processes discovered herein.

Based on previous studies that had demonstrated the efficacy of GPCR-targeted Metoprolol-Bromocriptine-Tamsulosin (MBT) combination therapy (69), extending the systems pharmacology framework to the context of PDE-inhibitor therapy requires consideration of the complex intracellular cyclic nucleotide signaling mechanisms between the GPCR- and PDE-targeted interventions. Whereas PDE inhibition increases intracellular cyclic nucleotide concentrations, MBT therapy reduces second messenger availability; thus, the two therapeutic approaches might be expected to yield opposing outcomes if every cell in the retina were equivalent. However, a systems pharmacology paradigm supports several potential scenarios under which this apparent contradiction may be resolved. First, the retina is a complex tissue comprised of multiple cell types, so drug targets could be differentially expressed across the distinct cell types. Accordingly, there may be no substantial contradictory action between the two treatments on intracellular cyclic nucleotide concentrations in given cell type. For example, if the retinal cells that express PDE11 do not express the GPCRs targeted by MBT therapy, and vice versa, then either increased cyclic nucleotide concentration in the PDE11-expressing cell types or decreased cyclic nucleotide concentration in the GPCR-expressing cell types could produce a similar result, with intercellular communication between the distinct retinal cell types converging on a common physiological response at the tissue level. Secondly, a recent discovery revealed that GPCRs likely signal via subcellular microenvironments with highly localized concentrations of cAMP, known as receptor-associated independent cAMP nanodomains (RAINs), corresponding to thousands of self-sufficient, independent signaling units (70). Thus, the distribution of RAINs could also account for the apparent contradiction, as any single cell could contain RAINs modulated by MBT therapy which are distinct and separate from the cyclic nucleotide microenvironments modulated by selective PDE-inhibitor therapy. Moreover, as the systems pharmacology paradigm includes the phenomenon of biased agonism (71), it becomes less challenging to rationalize how integration of differential effects on cyclic nucleotide concentrations in different cell types or microenvironments within the retina could culminate in a common stress-resilient retinal phenotype. Future studies leveraging recent advances in chemogenetic technologies will provide important mechanistic insights to delineate cell-specific responses and cyclic nucleotide-mediated signaling pathways in each cell type pertinent to retinal degeneration and protection (72).

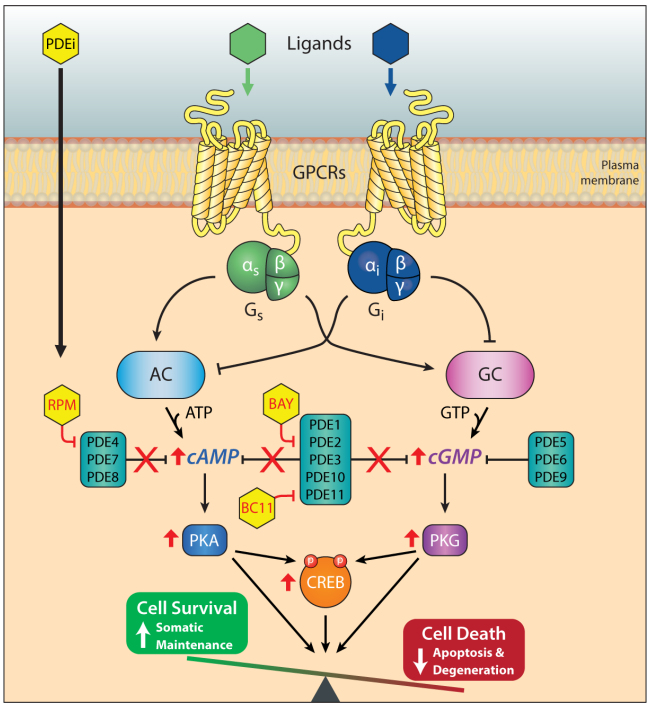

Fig. 7. Molecular mechanisms of stress resilience enhanced by PDE-inhibitor (PDEi) therapy. Treatment with selective inhibitors of PDE2 (BAY 60-7550; BAY), PDE4 (rolipram; RPM), and PDE11 (BC11-38; BC11) decreases cyclic nucleotide hydrolysis, resulting in increased intracellular concentrations of cAMP and/or cGMP and activation of the PKA/PKG-CREB signaling axis (PDEi effects highlighted in red), which confers a stress-resilient phenotype by stimulating downstream somatic maintenance processes (e.g., Glul, RdCVF, Xiap, Tulp1, pCREB-S133/S271) that promote cell survival while inhibiting degenerative or apoptotic processes (e.g., Tnfrsf, C3, HDAC11, Trp53, Fas, etc.) that drive cell death.

Given that the billions of cells that make up the brain and retina and the trillions of cells comprising the rest of the human body are all derived from a common eukaryotic ancestor, it is certainly possible, if not probable, that at least some vital somatic maintenance and pathological processes are common to various degenerative diseases of distinct etiologies, as well as across humans, mammals, and murine disease models. While it is important to acknowledge certain limitations, the intrinsic advantages of employing highly tractable animal models that recapitulate hallmarks of human retinopathies cannot be overstated. For instance, although it may be fundamentally impossible to replicate the chronic, multifactorial pathogenesis of human AMD in the mouse with no cone-enriched macula, it is nevertheless reasonable to employ the dKO model as an acute, stress-inducible surrogate that effectively recapitulates epigenetic hallmarks such as reduced global chromatin accessibility; as well as other major pathological features of AMD, including photoreceptor degeneration, lipofuscin accumulation, drusen deposition, and late-onset choroidal neovascularization (26). The fundamental goal in using murine models for this study was to identify universal molecular mechanisms of degeneration and protection, thereby revealing new drug targets and pharmacological interventions, such as the selective PDE inhibitors described herein, with potential clinical utility to treat both age-related and inherited retinal degenerations causing blindness.

Despite the seemingly disparate pathological mechanisms underlying the vast array of age-related diseases, a common underlying thread across every disease manifesting in advanced age is the loss of resilience to stress (1). This theory of human aging has been confirmed independently across distinct academic disciplines, from mathematics and statistics to physiology and medicine (73–75). It is, therefore, conceivable that chronic degenerative disorders, such as those associated with aging, could be treated with drugs intentionally designed to enhance physiological resilience to stress. Here, we propose establishing a new class of therapeutics specifically for this intention, under the category of "stress resilience-enhancing drugs" (SREDs). Indeed, our data support the potential use of selective PDE inhibitors as prototypical SREDs in the context of treating age-related and inherited retinal dysfunctions. Since this therapeutic paradigm

would likely involve a long-term prophylactic course of treatment, additional investigations will be required to evaluate the clinical feasibility of such an approach. Safety and toxicology studies will determine side-effect profiles of the aforementioned selective PDE inhibitors as chronically administered systemic therapies, providing insights on the relative advantages and disadvantages of oral versus direct ophthalmic routes of administration. Indeed, Phase I and II clinical trials conducted on rolipram, which had been evaluated for the treatment of major depressive disorder (MDD), demonstrated an acceptable safety and tolerability profile (76–78). Moreover, rolipram-mediated attenuation of stress-induced inflammation and complement activation (79), coupled with neuroprotection through improved protein homeostasis (80), has also been corroborated previously. However, clinical development and commercialization were ultimately discontinued in favor of a similarly efficacious drug for MDD, imipramine, which was not associated with nausea or emesis as had occasionally been reported by patients on rolipram. Since such adverse events were reported in a small subset of patients, lesser in severity and frequency than universally accepted safety standards, rolipram could be considered safe and tolerable as an oral medication for our proposed clinical indications; especially given the absence of efficacious alternatives with comparable safety and tolerability profiles for the treatment of dry AMD. Alternatively, a sustained-release preparation of rolipram could be developed for intravitreal delivery, thereby avoiding adverse systemic effects associated with the oral route of administration. In parallel, pharmacokinetic and pharmacodynamic studies will determine whether the prototypical SREDs described herein require additional chemical modifications or optimization for human use through conventional medicinal chemistry approaches. Ultimately, it is our expectation that SREDs will someday serve as a standard of care for human aging, effectively providing patients the means to diminish suffering from debilitating ailments for which there currently exist no viable therapeutic options, thereby extending human lifespan and healthspan irrespective of disease etiology.

Materials and Methods

ARPE-19 cells were utilized for in vitro studies, and in vivo studies were conducted using the photosensitive *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mouse that exhibits hallmarks of human AMD, the streptozotocin mouse model of diabetic retinopathy, and the *rd10* mouse model of retinitis pigmentosa. Retinal phenotypes were characterized using OCT, SLO, IHC, and ERG. Integrative single-cell transcriptomics (scRNA-seq), proteomics, and phosphoproteomics (LC-MS/MS) were also performed. Statistical significance was determined by the two-tailed Student's *t* test, one- or two-way (ANOVA), or as otherwise indicated; P values < 0.05 were considered statistically significant. Additional details are available in the *SI Appendix*.

Data, Materials, and Software Availability. RNAseq data have been deposited in the NCBI GEO data repository (GSE208760) (81). Proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (PXD035841) (82).

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