



Cone Phosphodiesterase-6 γ ' Subunit Augments Cone PDE6 Holoenzyme Assembly and Stability in a Mouse Model Lacking Both Rod and Cone PDE6 Catalytic Subunits

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Rod and cone phosphodiesterase 6 (PDE6) are key effector enzymes of the vertebrate phototransduction pathway. Rod PDE6 consists of two catalytic subunits PDE6 α and PDE6 β and two identical inhibitory PDE6 γ subunits, while cone PDE6 is composed of two identical PDE6 α' catalytic subunits and two identical cone-specific PDE6 γ' inhibitory subunits. Despite their prominent function in regulating cGMP levels and therefore rod and cone light response properties, it is not known how each subunit contributes to the functional differences between rods and cones. In this study, we generated an *rd10/cpfl1* mouse model lacking rod PDE6 β and cone PDE6 α' subunits. Both rod and cone photoreceptor cells are degenerated with age and all PDE6 subunits degrade in *rd10/cpfl1* mice. We expressed cone PDE6 α' in both rods and cones of *rd10/cpfl1* mice by adeno-associated virus (AAV)-mediated delivery driven by the ubiquitous, constitutive small chicken β -actin promoter. We show that expression of PDE6 α' rescues rod function in *rd10/cpfl1* mice, and the restoration of rod light sensitivity is attained through restoration of endogenous rod PDE6 γ and formation of a functional PDE6 $\alpha'\gamma$ complex. However, improved photopic cone responses were achieved only after supplementation of both cone PDE6 α' and PDE6 γ' subunits but not by PDE6 α' treatment alone. We observed a two fold increase of PDE6 α' levels in the eyes injected with both PDE6 α' plus PDE6 γ' relative to eyes receiving PDE6 α' alone. Despite the presence of both PDE6 γ' and PDE6 γ , the majority of PDE6 α' formed functional complexes with PDE6 γ' , suggesting that PDE6 α' has a higher association affinity for PDE6 γ' than for PDE6 γ . These results suggest that the presence of PDE6 γ' augments cone PDE6 assembly and enhances its stability. Our finding has important implication for gene therapy of PDE6 α' -associated achromatopsia.

Keywords: phosphodiesterase 6 (PDE6), *rd10*, *Cpfl1*, adeno-associated viral vector (AAV), achromatopsia, gene therapy, rod, cone

INTRODUCTION

Cyclic nucleotide phosphodiesterases 6 (PDE6) belongs to the highly conserved mammalian PDEs composed of 11 different families that modulate cellular levels of the second messengers, cAMP and cGMP, by controlling their rates of degradation (Beavo, 1995; Francis et al., 2001). PDE6 is the key regulator of cytoplasmic cGMP concentration in rod and cone photoreceptor cells. Because of its primary role in controlling cGMP, regulation of PDE6 is essential for the speed, sensitivity, recovery and adaptation of visual signals (Burns and Arshavsky, 2005; Fu and Yau, 2007). Rod PDE6 is composed of two catalytic subunits α and β encoded by the *PDE6A*, *PDE6B* genes, and two inhibitory subunits γ encoded by *PDE6G*. In contrast, cone PDE6 is composed of two identical catalytic subunits of α' encoded by *PDE6C* and two cone-specific inhibitory subunits γ' encoded by *PDE6H* (Gillespie and Beavo, 1988; Hamilton and Hurley, 1990; Li et al., 1990).

Despite the important role it plays in photoreceptor function, the structure and function relationship of PDE6 is not well understood due to failure of its functional expression in various *in vitro* systems (Qin et al., 1992; Piriev et al., 1993, 2003; Qin and Baehr, 1994). Electron microscopic analysis of purified rod PDE6 catalytic dimers showed that the catalytic domains are located at the C-terminus, and are highly conserved among all known members of PDE family (Beavo, 1995). The N-terminal domains of PDE6 catalytic subunits consist of two regulatory cGMP binding GAF motifs (GAF A and GAF B) termed for their presence in cGMP-regulated PDE, adenylyl cyclases and the *E. coli* protein Fh1A (Aravind and Ponting, 1997). It is known that there is a synergistic effect between PDE6 γ and cGMP binding to the catalytic subunits because: (1) PDE6 γ binding to the catalytic subunits enhances the affinity of cGMP for the noncatalytic sites; and (2) when cGMP binds to the GAF domains, the affinity of PDE6 γ for the catalytic subunits increases (Cote et al., 1994; Yamazaki et al., 2002). The region of PDE6 γ responsible for this effect has been mapped to the central polycationic domain of PDE6 γ known to have high affinity for the catalytic dimer (Artemyev and Hamm, 1992; Mou and Cote, 2001). Functional mapping of PDE6 γ with the catalytic subunits showed that the C-terminus of PDE6 γ is a key inhibitory domain. It interacts directly with the active site of PDE6 and blocks access of substrate to the catalytic pocket and therefore controls cGMP hydrolysis (Granovsky et al., 1997; Barren et al., 2009; Zhang and Artemyev, 2010). The middle region of PDE6 γ stabilizes the overall binding affinity of PDE6 γ with the catalytic subunits (Zhang et al., 2012).

One unique feature of rod PDE6 among the PDE family is that its catalytic subunits are composed of heterodimer $\alpha\beta$ subunits. Evidence suggests that PDE6 γ may form structurally and functionally distinctive interactions with the α - and β -subunits, and this asymmetric binding of PDE6 γ to PDE6 $\alpha\beta$ may represent an important regulatory mechanism in rod phototransduction (Guo et al., 2005, 2006). The molecular mechanism underlying the distinctive photoresponses of rods vs. cones is poorly understood and is one of the fundamental questions remaining in photoreceptor biology. The potential roles of rod and cone PDE6 in phototransduction is of particular

interest because PDE6's functions in rods and cones as effector enzymes in controlling cGMP levels in the outer segments is key to fully understanding of rod and cone photoresponses. We have shown previously that rods of *rd10* mice expressing cone PDE6 α' delivered by an adeno-associated virus (AAV) vector are more sensitive to light than wild type rods, most likely due to the slower shutoff of their light responses (Deng et al., 2013). A similar study employing a transgenic approach showed that exchange of cone PDE6 α' for rod PDE6 $\alpha\beta$ partially mimics the features of light adaptation (Majumder et al., 2015). Here, we study the association affinities among different subunits of PDE6 following AAV-mediated delivery in *rd10/cpfl1* mice. The *rd10/cpfl1* mice carry mutations in both rod PDE6 β and cone PDE6 α' , and they also lack rod PDE6 $\alpha\gamma$ and cone PDE6 γ' . We find that cone PDE6 α' has an intrinsically higher affinity for its partner PDE6 γ' than for PDE6 γ , and the presence of PDE6 γ' augments cone PDE6 holoenzyme assembly and enhances its stability.

MATERIALS AND METHODS

Animals

Rd10, *cpfl1*, and wild-type C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). *Rd10* and *cpfl1* mice were crossed to generate homozygous *rd10/cpfl1* mutant mice. Genotyping of *rd10* and *cpfl1* were performed according to previously published methods (Chang et al., 2007; Kolandaivelu et al., 2011). The mice were maintained in the University of Florida Health Science Center Animal Care Services Facilities in a continuously dark room, except for husbandry at ~400 lux illuminance. Physiological experiments were performed under dim red illumination using a Kodak number 1 Safelight filter (transmittance > 560 nm). All experiments were approved by the local Institutional Animal Care and Use Committees at the University of Florida and West Virginia University and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and NIH regulations.

Construction and Packaging of AAV Vectors

The murine PDE6 γ' cDNA was synthesized by GenScript (Piscataway, NJ, USA) and subcloned under the ubiquitous, constitutive small chicken β -actin promoter (smCBA; Haire et al., 2006) in self-complementary AAV vectors to create sc-smCBA-mPDE6 γ' . Construction of smCBA-mPDE6 β and smCBA-mPDE6 α' were described previously (Deng et al., 2013). All constructs were packaged in AAV serotype 8-Y733F by transfection of H293 cells according to previously published methods (Zolotukhin et al., 1999).

Subretinal Injections

All vectors were adjusted to the same titer of 5×10^{12} vector genomes/ml before injection. One microliter of vector was injected subretinally into the left eyes of *rd10/cpfl1* pups at postnatal 14 (P14) while the right eyes served as untreated controls. For co-injection, smCBA-mPDE6 α'

and sc-smCBA-mPDE6 γ ' vectors were mixed at a 2:1 ratio and 1 μ l injected. Subretinal injections were performed as previously described (Pang et al., 2006, 2008). Briefly, eyes were dilated with 1% atropine (Akorn, Inc. Lake Forest, IL, USA) and 2.5% phenylephrine hydrochloride solution (Paragon Biotek, Portland, OR, USA). An aperture within the pupil area was made through the cornea with a 30 gauge disposable needle. A 33-gauge blunt needle mounted on a 5- μ l Hamilton syringe (Hamilton Co., Reno, NV, USA) was then introduced through the corneal opening, avoiding the lens and reaching the subretinal space. Injections were visualized by fluorescein-positive subretinal bleb. 1% atropine eye drops and neomycin/polymyxin B/dexamethasone ophthalmic ointment (Bausch and Lomb Inc. Tampa, FL, USA) were given after injection.

ERG Analysis

At 5 weeks post-injection, dark-adapted and light-adapted electroretinograms (ERGs) were recorded separately using a UTAS Visual Diagnostic System equipped with Big Shot Ganzfeld (LKC Technologies, Gaithersburg, MD, USA) according to protocols previously describe (Deng et al., 2013). Scotopic rod recordings were performed with three increasing light intensities at -1.6 , -0.6 , and 0.4 log cds/m² after overnight dark adaptation. Ten responses were recorded and averaged at each light intensity. Photopic cone recording were taken after mice were adapted to a white background light of 30 cds/m² for 10 min. Recordings were performed with four flash intensities at 0.1, 0.7, 1.0 and 1.4 log cds/m² in the presence of 30 cds/m² background light. Fifty responses were recorded and averaged at each intensity. Scotopic and photopic b-wave amplitudes from untreated, treated *rd10/cpf1* and wild-type controls at each intensity were averaged and used to generate a standard deviation.

Immunohistochemistry and Morphology

Three days after ERG recordings, mice were sacrificed and eyes were enucleated for immunohistochemical analysis. Eyes were fixed in 4% paraformaldehyde at room temperature for 3 h. Cornea, lens and vitreous were removed from eyes without disturbing the retina. The remaining eyecup was rinsed with PBS and then cryoprotected by immersion in 30% sucrose in PBS for 4 h. Eyecups were then embedded in cryostat compound (Tissue TEK OCT, Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen at -80°C . Embedded eyecups were sectioned at 12 μ m thickness, rinsed in PBS, and blocked in 3% BSA, 0.3% Triton X-100 in PBS for 1 h at room temperature. Primary antibodies were: PDE6 α ' (3184P, A polyclonal antibody raised in rabbit using purified His-tagged mouse PDE6 α ' protein (amino acids 1–115) as the immunogen, gift of Dr. Visvanathan Ramamurthy, West Virginia University, Morgantown, WV, USA, 3184P denotes the rabbit number used for raising cone PDE6 α ' antibody; Kirschman et al., 2010; Kolandaivelu et al., 2011), rhodopsin, blue-cone opsin and red/green-cone opsin (Millipore Bioscience Research Reagents). All primary antibodies were diluted 1:1000 in 1% BSA in PBS and incubated with sections overnight at 4°C . The sections were then washed

three times with PBS, incubated with IgG secondary antibody tagged with Alexa-594 (Invitrogen) at 1:500 dilution and Lectin peanut agglutinin (PNA) conjugated to an Alexa Fluor 488 (Invitrogen) at 1:200 dilution in PBS at room temperature for 1 h and washed with PBS. Sections were mounted with Vectashield Mounting Medium for Fluorescence (H-1000, Vector lab, In. Burlingame, CA, USA) and coverslipped. Sections were analyzed with a Zeiss CD25 microscope fitted with Axiovision Rel. 4.6 software.

For morphology analysis, eyecups were prepared as described above, then paraffin-embedded and sectioned at 4 μ m through the optic nerve, followed by H&E staining.

Immunoprecipitation (IP)

PDE6 complexes and total protein density were analyzed by immunoprecipitation (IP) with ROS-1 monoclonal antibody and immunoblotting. The frozen eyecups (3 each) were homogenized in 400 μ l of IP buffer containing protease and phosphatase inhibitors and 10 mM iodoacetamide (10 mM Tris-HCl, pH 7.5, 100 mM KCl, 20 mM NaCl, 1 mM MgCl₂) using a pellet pestle in a 1.5 ml Eppendorf tube on ice (5 s \times 4). To solubilize the proteins, Triton X-100 was added to a final concentration of 1% and samples were incubated at 4°C for 30 min. Supernatants were collected by centrifuging at 10,000 \times g for 5 min at 4°C and incubated with 1.5 μ g ROS-1 monoclonal antibody coupled protein A/G beads for 3 h. Unbound proteins were removed and beads were washed in wash buffer (1X PBS containing 0.1% Triton X-100). PDE6 subunits bound to ROS-1 were eluted from the beads by adding 1X SDS/PAGE sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.005% bromophenol blue, 5% 2-mercaptoethanol) and boiling for 5 min. Eluted proteins were separated by 4%–20% SDS-polyacrylamide gel (Bio-Rad) and immunoblotting was performed as described earlier with catalytic and inhibitory subunits specific rod or cone PDE6 antibodies (Kolandaivelu et al., 2011; Deng et al., 2013). RetGC1 antibody was obtained from Dr. David Garbers (Deceased, University of Southwestern Medical School, Dallas, TX, USA). Membrane scanning and protein densities were measured with an Odyssey Infrared Imaging System (LI-COR Biosciences). Images are representative of at least three independent experiments.

Statistical Analysis

Scotopic a- and b-wave and photopic b-wave amplitudes at indicated flash intensities were compared by one-way ANOVA with the *post hoc* Bonferroni test to compare means at individual flash intensities. Protein quantifications were analyzed by one-way ANOVA with Bonferroni *post hoc* test to compare means of each treatment. Data were expressed as mean \pm SEM.

RESULTS

Histological Analysis of *rd10/cpf1* Mouse Retinas

We first compared rod photoreceptor cell degeneration in *rd10/cpf1* mice to *rd10* mice reared in the dark. Histological

examination of hematoxylin and eosin (H&E) stained retinal sections revealed progressive outer nuclear layer (ONL) thinning in *rd10/cpfl1* retinas mirroring the retinal degeneration seen in *rd10* mice (Figure 1A). Dark-reared *rd10/cpfl1* mice showed similar ONL as in the wild-type mice 2 weeks of age, however, outer and inner segments appeared to be shorter. Retinal degeneration was rapid with only half of ONL remaining at 4 weeks. By 8 weeks, only 2–3 layers of ONL were present. We established that the rate of rod photoreceptor cell loss in *rd10/cpfl1* mouse is very similar to that of *rd10* mice, as previously reported (Chang et al., 2007).

We then characterized the rate of cone photoreceptor cell degeneration by comparing the cone opsin immunohistochemistry pattern in 2, 4 and 8 week-old *rd10/cpfl1* and *cpfl1* mice using a mixture of red/green- and blue-opsin antibodies. One previous study showed that although *cpfl1* retinas display grossly normal morphology and layering, there is vacuolization of a small subset of cells in the photoreceptor layer indicating cone loss as early as 3 weeks, with continued cone degeneration and complete cone loss by 10 weeks of age (Chang et al., 2009). *Rd10/cpfl1* mice show a cone opsin staining pattern similar to *cpfl1* at 2 weeks of age, followed by rapid cone photoreceptor cell degeneration with a majority of cones absent by 4 weeks of age. By 8 weeks, cone opsin staining was undetectable (Figure 1B). Cones of *rd10/cpfl1* mice degenerated slightly faster than those in *cpfl1* mice most likely due to concomitant rod degeneration. Furthermore, *rd10/cpfl1* mice exhibited both flat scotopic and photopic ERG responses at 3 weeks of age (data not shown).

Rod and Cone Function in PDE6 α' and PDE6 α' +PDE6 γ' -treated *rd10/cpfl1* Retinas

We first characterized the expression of cone PDE6 α' in *rd10/cpfl1* mice treated with AAV8 Y733F-smCBA-PDE6 α' . The vector was subretinally delivered into one eye of postnatal 14 (P14) *rd10/cpfl1* mice, while the contralateral eyes remained untreated and served as controls. PDE6 α' expression was analyzed by immunostaining at 5 weeks post-injection. In C57BL/6 wild-type eyes, PDE6 α' is expressed specifically in cones (Figure 2). Untreated *rd10/cpfl1* eyes showed no PDE6 α' expression, whereas injected eyes showed robust PDE6 α' expression in both rods and cones.

We next characterized the rod function in PDE6 α' - and PDE6 α' +PDE6 γ' -treated *rd10/cpfl1* eyes by full-field ERG analysis. Expression of PDE6 α' or PDE6 α' +PDE6 γ' driven by smCBA promoter led to significant restoration of scotopic ERG responses at 5 weeks post-injection (Figures 3A–C). Both average b-wave and a-wave amplitudes in treated eyes are significantly higher than their undetectable ERG responses in contralateral untreated eyes at three light intensities tested. The average rod-mediated b-wave amplitude at a flash intensity of $-1.6 \log \text{ cds m}^{-2}$ was $166 \pm 14 \mu\text{V}$ (mean \pm SEM) in PDE6 α' -treated eyes, significantly higher than untreated controls ($n = 6$, $P < 0.001$, $F = 253.0$), and approximately 50% of those in the wild-type controls. The average rod-mediated a-wave amplitude

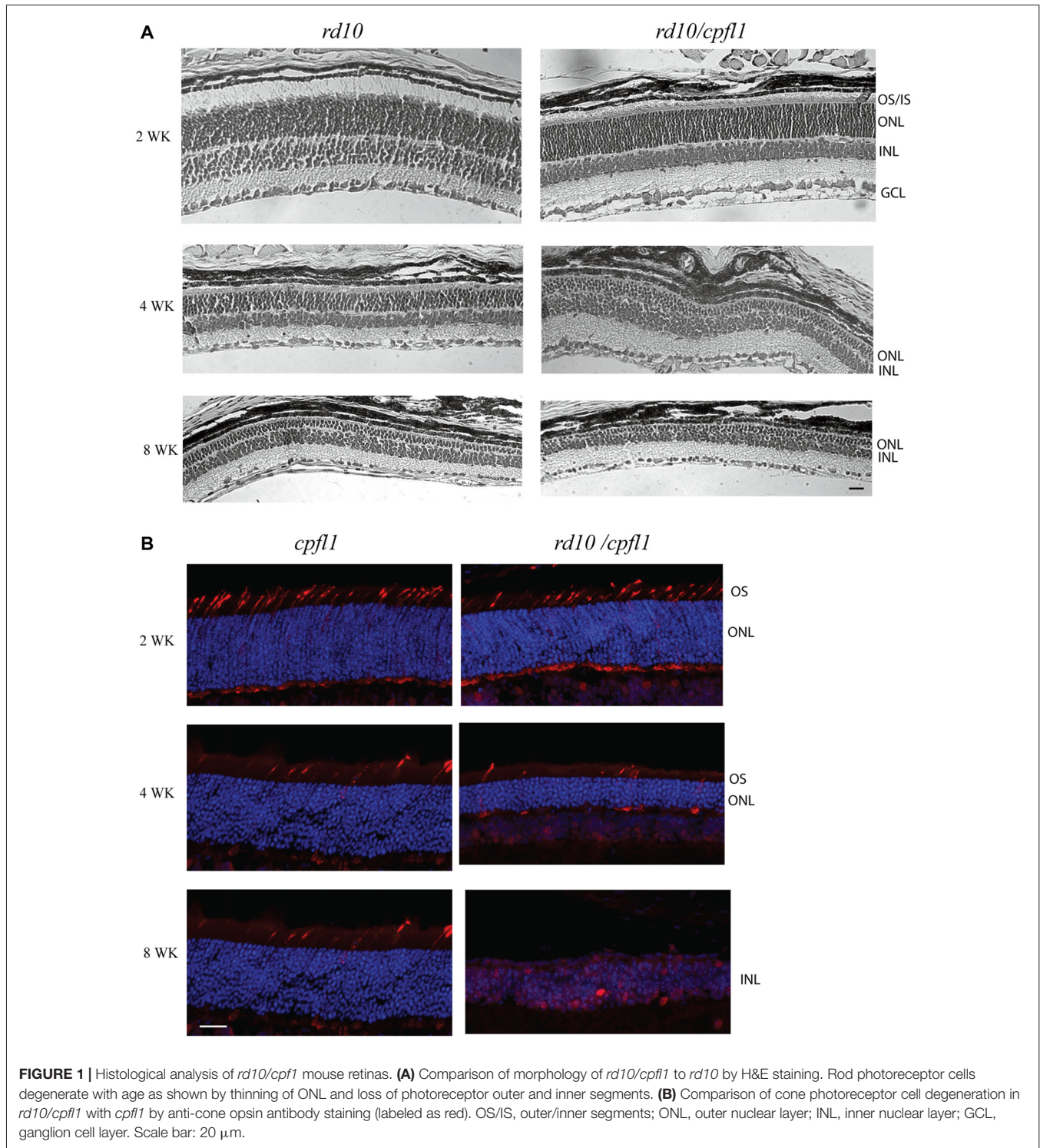
at a flash intensity of $-1.6 \log \text{ cds m}^{-2}$ was $21 \pm 4.9 \mu\text{V}$ in PDE6 α' -treated eyes, significantly higher than untreated controls ($n = 6$, $P < 0.01$, $F = 28.34$). Measured rod ERG responses were similar in eyes receiving PDE6 α' alone or PDE6 α' and PDE6 γ' mixture ($n = 6$, $P > 0.05$).

Rescue of rod photoreceptor cells in PDE6 α' -treated eyes was also confirmed by examination of retinal morphology and immunostaining for rhodopsin. Hematoxylin and eosin staining (H&E) showed partial retinal structure preservation in treated eyes with 7–8 layers of photoreceptor nuclei retained in the ONL, while only one layer remained in contralateral untreated eyes. Retinal outer segments were also preserved in treated eyes (Supplementary Figure S1A). Consistent with this result, immunostaining with a rhodopsin antibody showed much stronger rhodopsin expression in treated eyes compared to almost undetectable staining in untreated eyes (Supplementary Figure S1B).

In contrast to robust rod rescue in *rd10/cpfl1* mice by PDE6 α' , rescue of cone function was significantly muted in PDE6 α' -treated eyes (Figures 3D,E). To test the hypothesis that the presence of PDE6 γ' might assist proper folding of cone PDE6 holoenzyme and improve cone ERG rescue, PDE6 γ' driven by a smCBA promoter packaged in a fast-acting self-complimentary AAV vector was co-injected with PDE6 α' . Improved photopic ERG responses were obtained with an average cone b-wave amplitude of $38 \pm 1.5 \mu\text{V}$ ($n = 6$) at $1.4 \log \text{ cds m}^{-2}$, which was significantly higher than amplitudes from eyes treated with PDE6 α' alone ($12 \pm 1.9 \mu\text{V}$, $P < 0.001$, $F = 580.4$). Immunostaining with M/S-opsin antibodies and PNA in co-injected eyes showed cone morphology rescue (Supplementary Figure S1C). Mild cone morphology rescue was also observed in PDE6 α' -treated eyes (data not shown), however, these cones mostly existed because of slower degeneration due to rescue of rods. Our results suggest that the presence of PDE6 γ' from an earlier onset expression vector may help the proper folding, assembly, and stabilization of PDE6 α' more efficiently. The stability of the PDE6 $\alpha'\gamma'$ complex could be vital for enhancing the survival and function of cones.

Presence of PDE6 γ' Augments Cone PDE6 Assembly and Stability

Rd10/cpfl1 mice carry mutations in both rod PDE6 β and cone PDE6 α' . Next we investigated the levels of PDE6 α' , PDE6 γ' and PDE6 γ subunits by Western blot analysis from retinal extracts of C57BL/6 wild-type control, uninjected *rd10/cpfl1*, and *rd10/cpfl1* injected with PDE6 β , PDE6 α' , or PDE6 α' +PDE6 γ' . We took advantage of the fact that cone specific PDE6 γ' has a slightly higher molecular weight than rod PDE6 γ (Gillespie and Beavo, 1988) and used the same antibody to distinguish them. Western blot analysis showed that untreated *rd10/cpfl1* eyes lacked all three rod PDE6 subunits (Figure 4A; Supplementary Figure S2), and PDE6 α' treatment restored expression of rod PDE6 γ subunits, similar to eyes treated with rod PDE6 β (Figure 4A). However, cone PDE6 γ' expression was not detected in PDE6 α' -treated eyes. In contrast, *rd10/cpfl1* eyes co-injected with PDE6 α' +PDE6 γ' showed significantly higher levels of



expression of both PDE6 α ' and PDE6 γ ' (**Figure 4A**). Qualitative analysis of the amount of PDE6 α ', PDE6 γ and PDE6 γ ' from these samples showed that PDE6 α ' levels were approximately double in co-injected eyes compared to eyes receiving only PDE6 α ' (**Figure 4B**, top panel, $N = 3$, $P < 0.005$). The levels of PDE6 γ in PDE β - and PDE6 α '-treated eyes were similar ($N = 3$, $P > 0.05$)

and were about half of that in the wild-type control ($N = 3$, $P < 0.001$). However, the levels of PDE6 γ were reduced ($N = 3$, $P < 0.05$) while the amount of PDE6 γ ' was about twice as much as PDE6 γ in co-injected eyes (**Figure 4B**, bottom panel). PDE6 γ ' is delivered in half of the vector genomes of PDE6 α ' (vector genome ratio 1:2). PDE6 γ ' expression is expected to occur in both

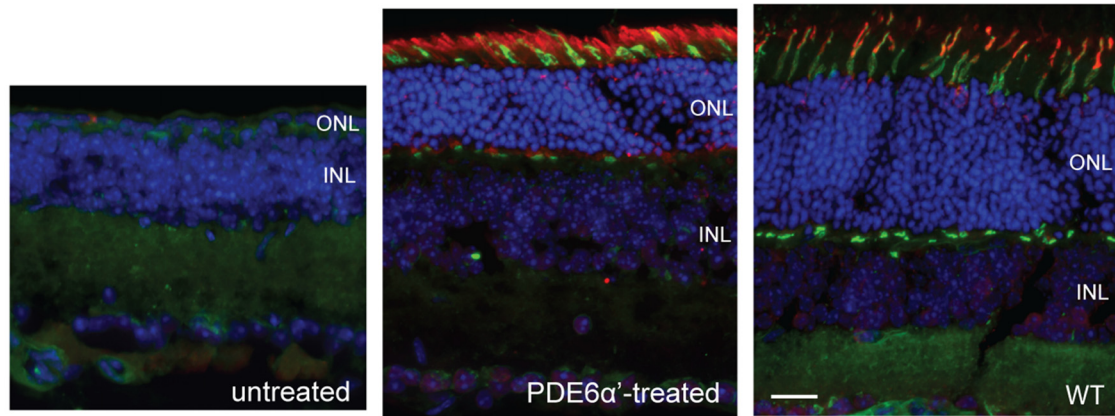


FIGURE 2 | Detection of phosphodiesterase 6 (PDE6) α' expression by immunofluorescence in *rd10/cpfl1* retinas following delivery of adeno-associated virus (AAV)-smCBA-PDE6 α' . No PDE6 α' expression is detected in untreated eyes (left panel), while PDE6 α' (labeled as red) was expressed in both rods and cones in treated eyes (middle panel). PDE6 α' is expressed exclusively in cones in wild type mice (right panel). Cones were labeled by peanut agglutinin (PNA) as green. Note the much thicker ONL in the treated retina compared to that in the untreated control. Scale bar, 20 μ m.

rods and cones following AAV vector delivery because we are using the nonspecific, ubiquitous smCBA promoter. However, we do not have a PDE6 γ' -specific antibody to demonstrate this by immunohistochemistry. The higher PDE6 α' levels in co-injected eyes is mostly likely due to the presence of PDE6 γ' .

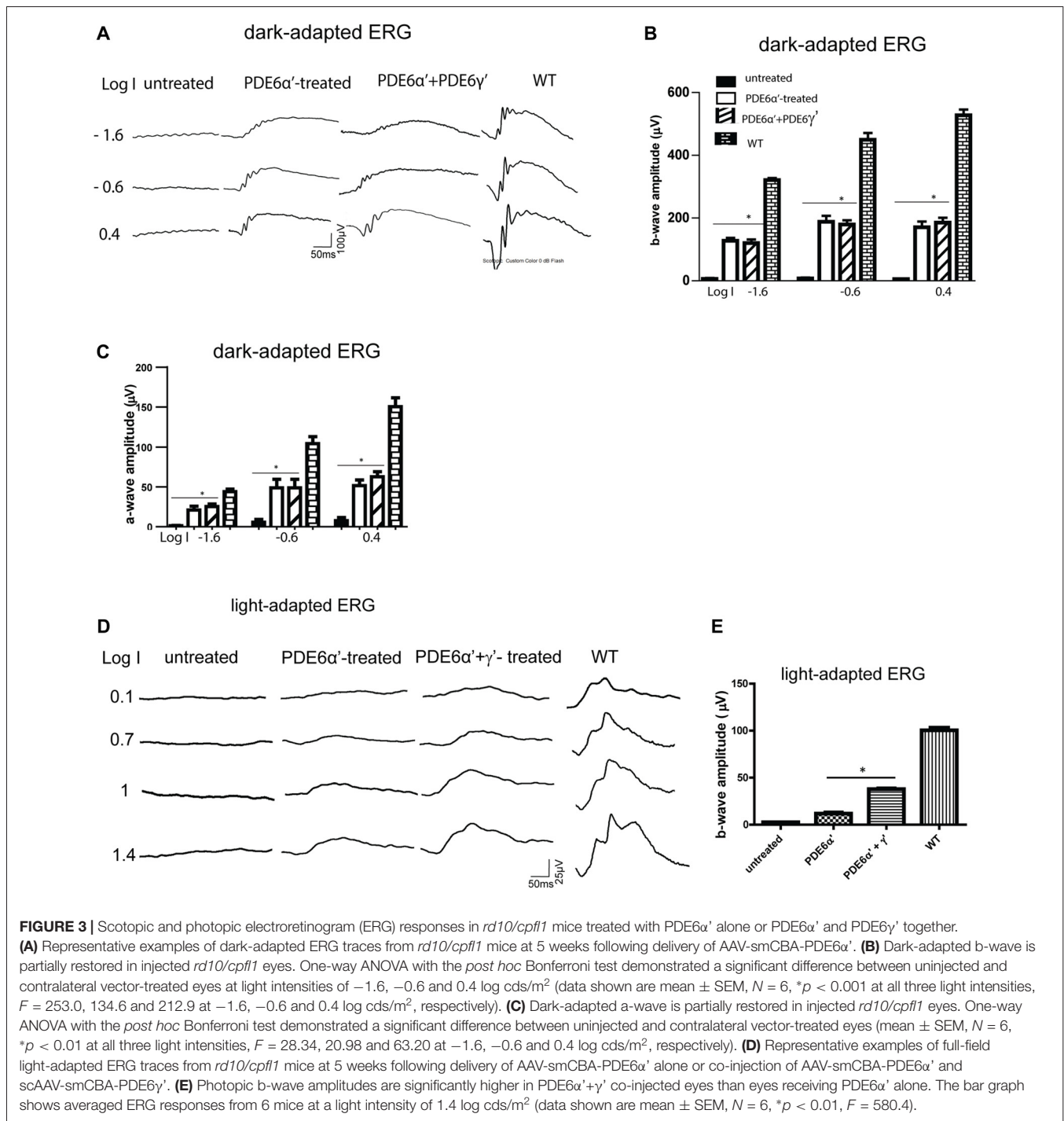
Finally, we investigated the assembly status of PDE6 from the above samples by IP using ROS-I, a mouse monoclonal antibody that exclusively recognizes assembled and functional PDE6 complexes in both rods and cones (Kolandaivelu et al., 2009, 2011). We have shown previously that restoration of light sensitivity by cone PDE6 α' in rods of *rd10* mice was attributed to functional complex formation between cone PDE6 α' and rod PDE6 γ (Deng et al., 2013). Here we confirm that rescue of rod function by PDE6 α' in *rd10/cpfl1* mice is also due to stabilization of endogenous PDE6 γ and the resultant functional complex formation of PDE6 $\alpha'\gamma$ (Figure 4C, Supplementary Figure S2). Interestingly, in PDE6 α' +PDE6 γ' co-injected eyes, the majority of PDE6 α' coupled with PDE6 γ' , with only a small amount of PDE6 α' associated with rod PDE6 γ (Figure 4C), although PDE6 γ was restored due to presence of PDE6 α' . These results suggest that the reduced level of PDE6 γ in co-injected eyes might be the result of degradation due to its reduced coupling to PDE6 α' . Overall, these results clearly demonstrate that cone PDE6 γ' delivered with PDE6 α' specifically enhances cone PDE6 assembly and its stability, while assembly of endogenous rod PDE6 γ with PDE6 α' is severely reduced in presence of PDE6 γ' .

DISCUSSION

We studied the association among PDE6 subunits *in vivo* using *rd10/cpfl1* mice. The *rd10/cpfl1* mice have no detectable rod and cone ERG function, and carry mutations in both rod PDE6 β and cone PDE6 α' . Interestingly, these mice also lack rod PDE6 α and cone PDE6 γ' . The loss of both rod and cone PDE6 in these

mice lead to early onset and rapid rod and cone photoreceptor cell degeneration. We show here that AAV vector-mediated expression of cone PDE6 α' driven by a constitutive promoter can rescue rod-mediated light responses and this functional rescue is achieved through stabilization of endogenous rod PDE6 γ and complex formation of PDE6 $\alpha'\gamma$. However, improved photopic ERG responses were attained only in eyes co-injected with vectors expressing both PDE6 α' and PDE6 γ' , but not in the eyes injected with the PDE6 α' vector alone. The levels of PDE6 α' are significantly higher in co-injected eyes and the majority of PDE6 α' complexed with PDE6 γ' , although PDE6 γ is also detected. These results demonstrate that PDE6 α' has higher intrinsic affinity for PDE6 γ' and its presence facilitates cone holoenzyme assembly and augmented its stability.

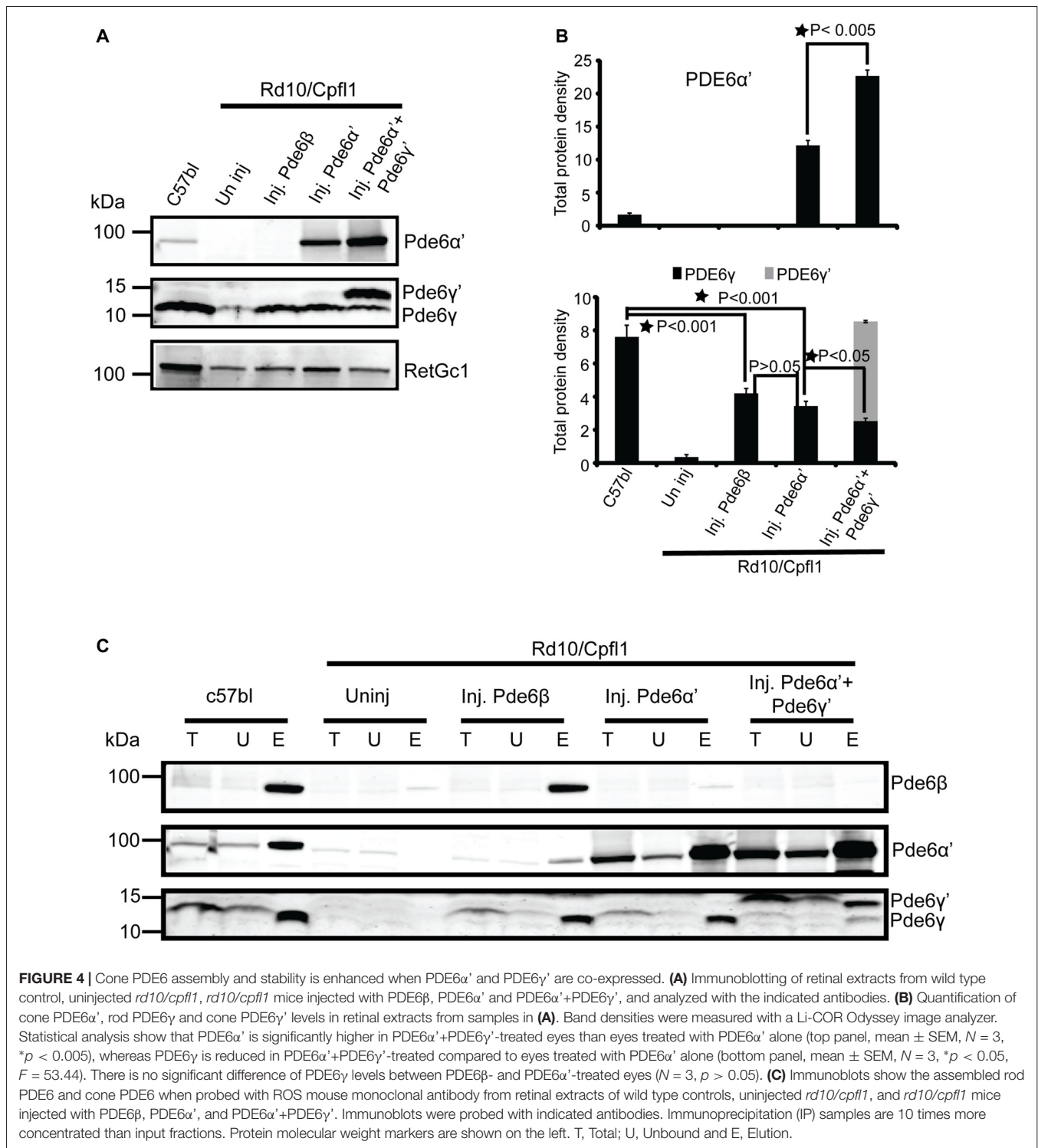
One of the biggest challenges hampering PDE6 research is failure of functional expression of this enzyme in various *in vitro* systems. It was recently shown that AIPL1 is an obligate chaperone required for heterologous expression of active PDE6 α' in cultured cells, and that the presence of PDE6 γ dramatically increases the proportion of correctly folded, functional PDE6 produced in the presence of AIPL1 (Gopalakrishna et al., 2016). Our results showing that the presence of PDE6 γ' helps to stabilize cone PDE6 holoenzyme is consistent with this observation. In our experiment, we also find that PDE6 γ' increases the stability of PDE6 α' and facilitates cone PDE6 assembly demonstrated by the significant elevated amount of PDE6 α' as well as assembled and functional cone PDE6. We also found that levels of PDE6 γ is reduced in co-injected eyes compared to eyes received PDE6 α' alone. We speculate that this reduction is the result of degradation due to its reduced coupling to PDE6 α' when PDE6 γ' is present. This is supported by previous studies suggesting that stability of PDE6 complex is dependent on the association of its interacting partners. First, in *rd10* mice carrying a mutation of PDE6 β , PDE6 α and PDE6 γ were not detected (Deng et al., 2013). Second, AIPL1 interacts with PDE6 α and the interaction is essential for PDE6 assembly. In the absence



of AIPL1, all PDE6 subunits are degraded regardless of their normal synthesis (Kolandaivelu et al., 2009). Therefore, our data support the same conclusion that PDE6 γ' potentiates the proper folding of PDE6 α' and plays a critical role in the maturation of cone PDE6 holoenzyme.

An interesting question raised by this study is why PDE6 γ' enhances cone PDE6 assembly and stability compared with rod PDE6 γ ? A previous study demonstrated that the rod PDE6 γ naturally exists in mouse cones and can couple with cone PDE6 α'

to protect cone function and stability (Brennenstuhl et al., 2015). Our data show that the PDE6 α' can couple with PDE6 γ when PDE6 γ' is absent. However, when PDE6 γ' is present along with the endogenous PDE6 γ , PDE6 α' has a higher association affinity for its natural partner. Such a higher affinity might play a role in the faster light response kinetics of cone photoreceptor cells. Although the overall three-dimensional structure of the rod PDE6 holoenzyme was defined by using negative-stain electron microscopy, the quaternary structure of PDE6 $\alpha\beta$ as well as the



interface between PDE6 $\alpha\beta$ and their inhibitory subunits PDE6 γ remains elusive (Kameni Tcheudji et al., 2001; Goc et al., 2010). In addition, the heterogeneity of the rod PDE6 α and β catalytic subunits in terms of their interactions with PDE6 γ is poorly understood. There is evidence that PDE6 γ may form distinctive structural and functional interactions with the PDE6 α and PDE6 β

subunits (Guo et al., 2005). Despite the fact that rod and cone PDE6 subunits share similar domain organizations, the detailed structural relationships between PDE6 α' and γ' is unknown and cone PDE6 may have a different mode of regulation and therefore display different enzymatic kinetics. Single cell recordings from rods expressing PDE6 $\alpha'\gamma$ and PDE6 $\alpha'\gamma'$ would give detailed

information regarding light signaling properties between the two types of rods and might reveal if PDE6 γ' plays a role in the faster light response of cones. The ideal model to perform this experiment would be a knock-in mouse line with endogenous PDE6 γ replaced with cone PDE6 γ' . In these mice, PDE6 γ' is expressed at the same level as PDE6 γ in wild type mice, therefore detailed light response kinetics can be compared. Single cell recordings were not performed here due to the stochastic nature of AAV vector expression.

In our previous study, we showed that rod visual performance measured by optomotor responses was significantly improved in *rd10* mice treated with PDE6 α' but remained subpar compared to wild-type controls (Deng et al., 2013). We would expect similar improvement of scotopic visual acuity in *rd10/cpfl1* mice treated with PDE6 α' alone or a PDE6 $\alpha'\gamma'$ mixture. We also note that although improved photopic ERG responses were obtained with co-injection of vectors expressing both PDE6 α' and PDE6 γ' , there is a possibility that these photopic recordings were partially derived from rods expressing cone PDE6 $\alpha'\gamma'$. These rods might be desensitized and could not be saturated by standard scotopic conditions, although our photopic ERG recordings were performed after longer than standard light exposure time for saturating normal rods. One approach to address this issue would be to express PDE6 γ' under a cone-specific promoter instead of a constitutive promoter such as CBA. However, our best cone-preferred promoter still confers some level of expression in the mouse rods (Dyka et al., 2014).

In summary, we show that cone PDE6 α' has an intrinsically higher affinity for its partner PDE6 γ' than for rod PDE6 γ

and that the presence of PDE6 γ' helps to stabilize cone PDE6 holo-enzyme and augment its assembly. This finding might be important for designing future gene therapy studies for treating patients with PDE6 α' -associated achromatopsia.

AUTHOR CONTRIBUTIONS

W-TD and SK designed and performed the experiments, and wrote the manuscript. JL, PZ and VC conducted experiments. AD contributed to the data analysis. VR and WH contributed to study supervision. W-TD, SK, AD, VR and WH contributed to the critical revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnmol.2018.00233/full#supplementary-material>

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Conflict of Interest Statement: WH and the University of Florida have a financial interest in the use of AAV therapies, and WH owns equity in a company (AGTC Inc.) that might, in the future, commercialize some aspects of this work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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