Phosphodiesterase 6β Expression In Developing Mouse Retina

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The *rd1* mouse is a well-studied model of retinitis pigmentosa (RP), an inherited retinal degenerative disease affecting approximately 1 in 4000 people. It is characterized by a mutation in the *Pde6b* gene that codes for Phosphodiesterase 6β (PDE6β), a downstream effector of phototransduction. *Pde6b* gene expression occurs embryonically in mouse retina, whereas other proteins involved in phototransduction are expressed around postnatal day 5 (P5). The primary aim of this study is to investigate the temporal and spatial expression pattern of PDE6β protein during photoreceptor development. Using Western blots with wild type and *rd1* mouse retinas from P2 – P21 we demonstrated that PDE6β protein is expressed in wild type retinas by P2 and is not detected in *rd1* retinas. The earliest detection of PDE6β in wild type retinas by immunohistochemistry was at P6, where it was confined to the apical region of the photoreceptor layer. The expression of PDE6β protein prior to differentiation of photoreceptor cells and prior to expression of other phototransduction proteins is consistent with the hypothesis that PDE6β may play a role during photoreceptor development distinct from its role in phototransduction. Our lab previously showed that Prenylated Rab Acceptor 1 (PRA1), a vesicular trafficking protein, is downregulated in the developing *rd1* retina, although its function in the retina is unknown. The second aim of this study was to explore the relationship between PRA1 and PDE6β. We used immunohistochemistry to determine whether the two proteins are co-localized during the postnatal differentiation period. However, no co-localization between PDE6β and PRA1 was detected. The function of PRA1 in developing retina remains to be elucidated.

Abbreviations: cGMP – cyclic guanosine monophosphate; CNG – cyclic nucleotide-gated; Crx – cone-rod homeobox protein; E – embryonic day; ER – endoplasmic reticulum; GFP – green fluorescent protein; IS – inner segment; NDS – normal donkey serum; Nrl – neural retina leucine zipper protein; OCT – Optimal Cutting Temperature; OS – outer segment; P – postnatal day; PBS – phosphate buffered saline; PDE6 – phosphodiesterase 6; PKG – protein kinase G; PRA1 – Prenylated Rab Acceptor 1; RP – Retinitis pigmentosa; Rho – rhodopsin; RPC – retinal progenitor cells

Keywords: photoreceptor; retinal degeneration; PDE6β; cGMP; PRA1; development; mouse; trafficking

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Introduction

Retinitis pigmentosa (RP), a disease characterized by rod photoreceptor cell degeneration, is the leading cause of inherited blindness (Hartong et al., 2006). Over a hundred different gene mutations have been linked to RP, resulting in variation in age of onset and severity of the disease (Daiger et al., 2007). The *rd1* mouse is an animal model of an early-onset form of the disease with a mutation in the *Pde6b* gene (Farber et al., 1994). The role of *Pde6b* in phototransduction is well characterized in mature retinas. However, *rd1* rod photoreceptors degenerate during retinal development. Here we consider the hypothesis that *Pde6b* could have a function during photoreceptor development distinct from its role in phototransduction.

Normal Retinal Development in Mice

The genesis of rodent photoreceptors proceeds over a long period. Rod precursors exit the cell cycle between embryonic day 18 (E18) and P5 (Young, 1985; Rapaport et al., 2004). However, terminal differentiation of photoreceptors is not complete until P21. After final mitosis, rod precursor cells begin to express rod-specific genes, such as those required for phototransduction of light. Subsequent morphogenesis involves axonal outgrowth, synapse formation and differentiation of the apical structures unique to photoreceptors, such as inner segment (IS) compartments that are distal to the cell body and contain most of the cytoplasmic organelles, and outer segments (OSs), which are ciliary organelles that extend from ISs and incorporate phototransduction proteins on membrane disks. OS differentiation and disk formation can first be seen by electron microscopy around P6-8 (Sanyal and Bal, 1973).

The phototransduction process requires expression of signaling molecules that are trafficked through the IS to OS disks. It begins with light photons being absorbed by rhodopsin, which then activates transducin, a heterotrimeric G-protein. Transducin, in turn, activates phosphodiesterase 6 (PDE6) (Baylor, 1996). Activated PDE6 hydrolyzes cyclic guanosine monophosphate (cGMP) to 5'GMP. cGMP is a second messenger that regulates cyclic nucleotide-gated (CNG) channels on the plasma membrane of OSs. CNG channel activation results in Na^+ and Ca^{2+} entry leading to photoreceptor synaptic signaling.

rd1 Retinal Development and Degeneration

The *rd1* mutation occurs in *Pde6b*, the gene that codes the rod-specific β subunit of phosphodiesterase 6 (PDE6β) (Farber et al., 1994). PDE6β forms a heterotetramer with PDE6α and two PDE6γ subunits (Beavo, 1995). The mutated PDE6β cannot catalyze the conversion of cGMP to 5'GMP, and, as a result, cGMP accumulates in photoreceptors. High levels of cGMP cause CNG channels to remain open, allowing calcium and sodium cation accumulation in the cell, and preventing phototransduction (Farber et al., 1994).

Rod photoreceptors degenerate rapidly in the *rd1* retina beginning around P10, during photoreceptor morphogenesis. Rods have completely degenerated by P21, when retinal development is complete in the wild type (wt) retina (LaVail and Sidman, 1974).

Bibb et al. (2001) showed that *Pde6b* is expressed at E12, very early in normal retinal development. Genes for the other components of the PDE6 holoenzyme, *Pde6a* and *Pde6g*, are first expressed at P1. Other proteins in the phototransduction pathway, such as rhodopsin, are expressed even later, beginning around P5. *Nrl*, a transcription factor necessary for rod cell fate determination, is also expressed at P1, later than *Pde6b* (Bibb et al., 2001). cGMP levels are two-fold greater in *rd1* whole retinas compared to wt by P6 (Lolley and Farber, 1976). Although cell death follows increased intracellular cGMP, the specific pathway is not well understood (Dickison et al., 2012).

An Alternate Role of PDE6β

Synthesis and post-translational modification of PDE6 subunits take place in ISs where the ER and Golgi body are located, whereas PDE6 functions on membrane discs located in OSs in mature photoreceptors. Therefore, PDE6β requires transport to reach its final phototransduction location in OSs.

Previously, our lab found that the vesicular trafficking-related protein PRA1 is significantly and consistently downregulated in *rd1* retinas during early photoreceptor development, from P2 to P8 (Dickison et al., 2012). PRA1 binds prenylated Rab GTPases and plays an important role in vesicular trafficking but its function in the retina is unknown.

While the molecular function of PDE6β in phototransduction in the mature retina has been well characterized, its developmental expression pattern in mouse retina in either temporal or spatial aspect has not been investigated. The relationship between PDE6β and the trafficking protein PRA1 is also unexplored. Knowledge of the PDE6β expression pattern and its possible relationship with PRA1 expression would help us better understand the early molecular events that precede *rd1* photoreceptor degeneration and evaluate the possibility of a role for PDE6β in

regulating early rod photoreceptor development. Here we characterize the abundance and localization of PDE6β and its spatial relationship to PRA1 during mouse retinal development. We determined that PDE6β protein expression is detectable by P2, very early in photoreceptor cell differentiation. However, no co-localization between PDE6β and PRA1 was detected. Based on these results, we discuss whether early expression could reflect an alternative function for PDE6β during mouse photoreceptor development.

Material and Methods

Animals and Reagents

Animals used in these studies were bred in-house. They include wt and *rd1* mice, both on a C57Bl/6 background, and Nrl-GFP transgenic mice, generously provided by Anand Swaroop (Akimoto et al., 2006), that were back-crossed to either wt or *rd1* mice for nine generations to produce Nrl-GFP wt or Nrl-GFP *rd1* mice on the same C57Bl/6 background. The Nrl promoter specifically drives the expression of GFP only in rod photoreceptors (Akimoto et al., 2006). All experiments on mice followed the National Institutes of Health Guidelines on Laboratory Animal Welfare and protocols approved by the Saint Louis University Institutional Animal Care and Use Committee. All reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Western Blot Assay

In order to measure protein expression, 14 samples for Western blot assays were harvested, one from each of two mouse strains, wt and *rd1*, at each of seven different ages: P2, P4, P6, P8, P10, P12, and P21. For each sample, 5-10 mice from a single litter were euthanized, both retinas were dissected from each animal and the retinal tissue was then homogenized by sonication in cold Radio Immune Precipitation Assay buffer (10mM Tris-Cl pH 8.0, 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate and 0.1% SDS). The resulting samples were assayed to determine the protein concentration. An equal amount of total protein from each sample was loaded onto SDS polyacrylamide gradient gel. Protein samples then underwent SDS-PAGE at a voltage of 120V for 1 hr and were transferred to nitrocellulose membrane at 36V for overnight.

The nitrocellulose membrane was blocked with 5% nonfat dry milk in TBST for 2 hrs; the membrane was incubated with goat anti-PDE6β polyclonal antibody (1:150, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4^{o}C and incubated with HRP-conjugated anti-goat IgG secondary antibody (Santa Cruz, Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:4000) for 1 hr. After incubation with ECL solution (Sigma-Aldrich, St. Louis, MO; CPS160), the PDE6β protein on the membrane was visualized with a GE LAS4000 imager. To visualize β-actin, the same nitrocellulose membrane was incubated in 0.2M NaOH solution for 15 minutes to strip bound antibodies and then blocked in 5% milk again, followed by incubating with primary antibody against β-actin (Abcam, Cambridge, MA; Ab 8226, mouse monoclonal, dilution 1:500). The assay was repeated three times.

Cryostat Tissue Preparation

To collect tissue samples for immunohistochemistry, two to three mice were euthanized from each of two mouse strains (Nrl-GFP wt and Nrl-GFP *rd1*) and each of seven ages (P2, P4, P6, P8, P10, P12, and P21). For double label immunohistochemical studies (see below), two to three mice were euthanized from each of two mouse strains (wt and *rd1*) and each of two ages (P4 and P6). After animals were euthanized, both eyecups were dissected in cold phosphate buffered saline (PBS) and fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer. Eyecups were rinsed in 0.1M phosphate buffer three times on ice, followed by incubation in 30% sucrose at 4°C overnight for cryoprotection. Eyecups were then incubated in a 1:1 mixture of 30% sucrose and Optimal Cutting Temperature compound (OCT, EMS, Hatfield, PA) for 1 hr, followed by incubation in 100% OCT compound for another hour. Eyecups were then flash frozen in OCT compound. Twelve micron tissue sections were cut on a Leica CM 1850 cryostat (Leica, Buffalo Grove, IL) and three sections were placed on

each gelatin-subbed slide. Slides were stored for up to two weeks in a -20°C freezer with desiccant until used for immunohistochemistry.

Immunohistochemistry

In order to determine protein localization, slides from at least two different retinas for each mouse strain and each age were incubated in 4% paraformaldehyde for 1 minute. Slides then were placed into Coplin jars containing PBS and rinsed three times for 15 minutes each. Subsequently, sections were blocked with 2% normal donkey serum (NDS, Jackson ImmunoResearch Laboratories, West Grove, PA) and 0.3% Triton X-100 in PBS for 20 minutes, followed by incubating with goat anti-PDE6β polyclonal antibody (1:150, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS with 0.3% Triton X-100 and 2% NDS at 4°C overnight. For controls, the same steps were followed, except no primary antibody was used.

On the next day, slides were washed in PBS for three 10-minute periods, followed by blocking for 20 minutes and staining with rabbit anti-goat Cy3 secondary antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:500 in blocking solution for 1 hr in the dark. Next, slides were washed for three 20 minutes periods, mounted with coverslips on Vectashield media (Vector Laboratories, Burlingame, CA), and sealed with nail polish.

Double Label Immunohistochemistry

Double label experiments were performed using slides from P6 and P8 samples from both wt and *rd1* eyecups. The same procedure for immunohistochemistry described above was followed with two modifications: first, rabbit anti-PRA1 antibody (1:50, Abgent, San Diego, CA) was added to the primary antibody incubation step concurrently with anti-PDE6β and, secondly, donkey anti-rabbit Alexa 488 antibody (1:400, Molecular Probes, Eugene, OR) was mixed with donkey anti-goat Cy3 antibody (1:500, Sigma-Aldrich, St. Louis, MO) for the secondary antibodies.

Confocal Microscopy

Fluorescence photomicrographs of sections were taken using a Zeiss LSM 510 Meta laser confocal microscope under a 63x/1.4 oil objective. Only areas near the optic nerve were used for imaging. The sections were scanned by 488 nm or 543 nm laser for green fluorescent protein (GFP)/Alexa 488 or Cy3 fluorescence signal, respectively. The generated images were false-colored to show GFP/Alexa 488 signal as green and Cy3 signal as red. Only one slice in the middle of image stacks was selected for analysis. No post-adjustment for brightness or contrast was made to the images.

Results

PDE6β Was Detected in Wild Type Mouse Retina by P2

To determine the temporal expression pattern of PDE6β during photoreceptor cell differentiation, P2-P21 retinas from *rd1* and wt mice were compared in a Western Blot assay (Figure 1). No PDE6β was detected at any age in *rd1* retina, since the antibody only recognizes a sequence on the C-terminal that is completely lost due to the early stop codon generated in the *rd1 Pde6b* mutant gene. In wt retinas, PDE6β was detected at P2, the earliest age examined, with a distinct but weak signal. The expression level of PDE6β increased steadily from P2 to P21, prior to and during formation of rod photoreceptor OSs.

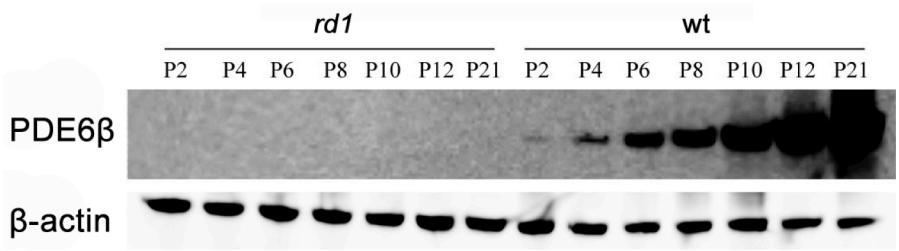

Figure 1. PDE6β protein expression during *rd1* and wt mouse retina development. The protein expression of PDE6β in wt retina was detected as early as P2 by Western Blot analysis. No PDE6β expression was detected in the *rd1* retina at any age. β-actin was used as a loading control. Representative image shown.

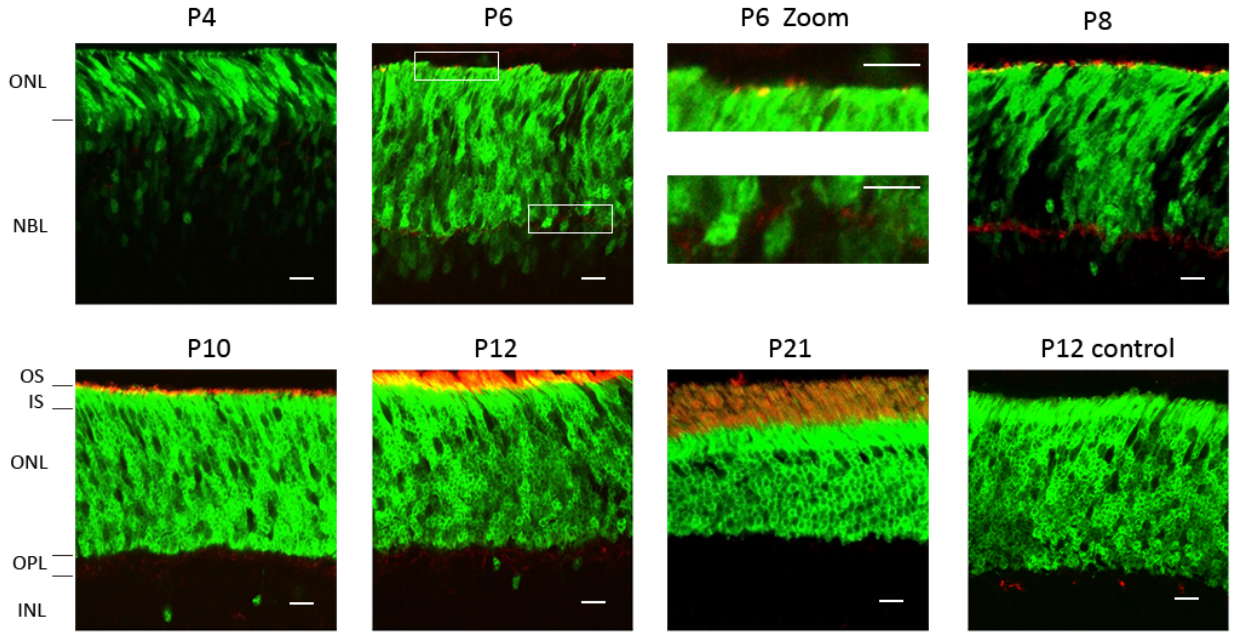

Figure 2. Spatial pattern of PDE6β expression in developing Nrl-GFP wt retinas. PDE6β (red) could be detected by confocal microscopy from P6. Localization was confined to the growing outer segment. Nonspecific staining was also detected in the outer plexiform layer, suggested by the no-primary-antibody control (only P12 shown here). GFP labeled rods are stained green to provide orientation. P: post-natal day; ONL: outer nuclear layer; NBL: neuroblast layer; OS: outer segment; IS: inner segment; OPL: outer plexiform layer; INL: inner nuclear layer. Scale bars = 10 μm in all images; zoom = 2.25X magnification.

PDE6β Immunoreactivity Was Detected by P6

Retinal sections from Nrl-GFP transgenic mice were immunostained for PDE6β (Figure 2). At P4, no signal from PDE6β was detected. At P6, PDE6β started to accumulate at the apical surface of differentiating rod photoreceptors. From P6 to P21, with the formation and growth of rod OSs, PDE6β steadily increased in expression and was localized exclusively to the OS. Minor non-specific staining was seen in the outer plexiform layer in the central retina region for both control and experimental sections.

PDE6β Did Not Co-Localize with PRA1 Protein

PRA1 plays a role in vesicular trafficking and its expression is downregulated in *Pde6b* mutant retinas (Dickison et al., 2012), suggestive of a functional link between these proteins. Therefore, we investigated whether PDE6β co-localizes with PRA1 during early development of wt mouse retina. The PRA1 protein was seen in both photoreceptors and inner retinal cells (Figure 3). PDE6β protein did not show co-localization with PRA1 at either P6 or P8, the ages examined. Note that PRA1 is most enriched in photoreceptor ISs whereas PDE6β staining was limited to the OS region.

Discussion

PDE6β Is Expressed Early in Photoreceptor Differentiation

In our study, we confirm that PDE6β protein is detectable at P2 by Western Blot, but on a low level. Since the PDE6β gene is expressed approximately two weeks before rhodopsin and other phototransduction genes (Bibb et al., 2001) and since we have shown here that the PDE6β protein is also expressed very early, it is reasonable to consider whether, in addition to its well-characterized function in phototransduction, PDE6β plays a role in early development of photoreceptors. PDE6α and PDE6γ are expressed later than PDE6β, but approximately four days earlier than rhodopsin

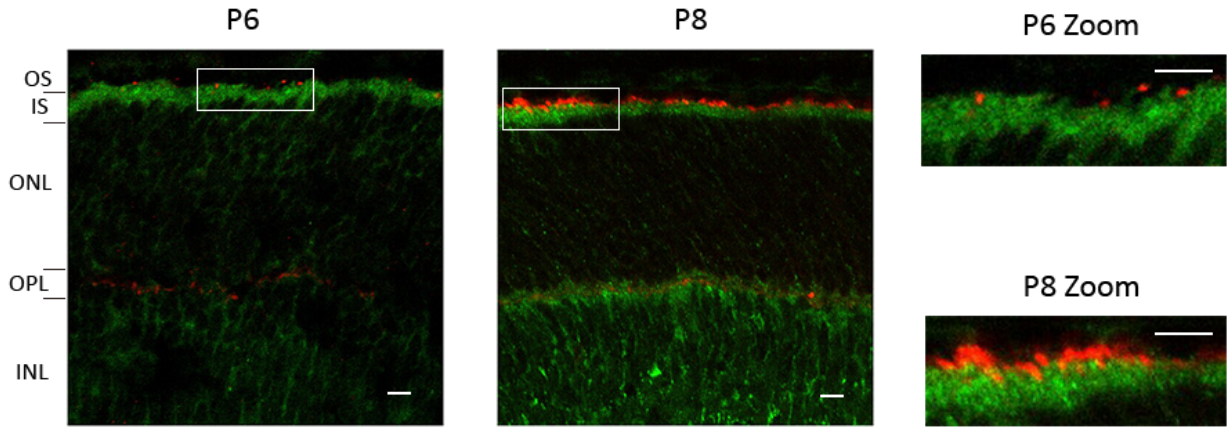

Figure 3. Localization of PRA1 and PDE6β proteins. No co-localization could be seen at either P6, which is the earliest age when PDE6β becomes detectable, or P8. Green: PRA1; Red: PDE6β; P: post-natal day; OS: outer segment; IS: inner segment; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer. Scale bars = 10 μm in all images; zoom = 2.5X magnification.

(Bibb et al., 2001), suggesting that the holoenzyme could assemble and act by regulating levels of cGMP and its downstream receptors, such as protein kinase G (PKG) during early rod differentiation. Although embryonic expression of PDE6β protein remains to be confirmed, PDE6β could regulate development prior to PDE6α or -γ expression via possible novel interactions, such as forming a homodimer, as is the case for all other members of the PDE family, or forming a heterodimer with other family members.

During mid- to late-stage photoreceptor differentiation, cGMP may participate in the regulation of photoreceptor development by regulating CNG channel activity and, thus, Ca^{2+} levels in the cell. We have previously shown that CNG channel expression increases acutely at P6 from a very low level at the margin of detection (Zhang et al., 2014). This suggests CNG channels are possibly not involved in the very early, i.e., prior to P4, regulation of photoreceptor development. Future studies are needed to determine whether the initially-expressed PDE6 may regulate PKG activity.

Spatial Expression Pattern of PDE6β Is Consistent with Morphological Development of Photoreceptors

Outer segments, where the PDE6 heterotetramer is functionally located, begin to differentiate as early as P5 as characterized by swelling of the region apical to the connecting cilium (Sanyal and Bal, 1973). At P6, the swelling continues and starts to contain variously shaped and sized vesicles, which are later fused into membrane discs. Flat vesicles (immature disc membranes) are partially stacked at P8, but are not as yet arranged regularly as observed in adult photoreceptors (Sanyal and Bal, 1973). OSs elongate at a rapid and almost linear rate from P11 to 17, reaching adult length by approximately P21 (LaVail, 1973).

Although PDE6β protein was not detected by immunostaining at P4, prior to OS differentiation, our data clearly show that PDE6β accumulates at the apical region of photoreceptors at P6, consistent with transport through cilia. From P8 to P21, with the generation and maturation of membrane discs, PDE6β continues to be trafficked to photoreceptor OSs, where the phototransduction machinery is assembled.

As stated earlier, our data show PDE6β was detected by Western Blot from P2, while by immunostaining only from P6. This discrepancy may be due to the PDE6β antibody being less sensitive in immunostaining than in Western Blot. Alternatively, the expressed PDE6β protein could be diffusely distributed within photoreceptors at P4, making it below the level of detection by immunostaining. A third possibility is that the PDE6β epitope is masked prior to P6, particularly if it forms an atypical dimer or is bound to trafficking proteins necessary for transport from endoplasmic

reticulum (ER) to the apical membrane. This hypothesis is supported by our observation that even at later ages, no PDE6β signal was detected in photoreceptor ISs, where PDE6β is synthesized and post-translationally modified.

PRA1 Does Not Co-Localize with PDE6β

PRA1 is a 21 kDa protein that serves in cargo selection, vesicle budding and in docking and fusion of vesicles to target membranes (Martincic et al., 1997; Bucci et al., 1999). Two proteins, AIPL1 and PDE6δ, have been reported to play a role in PDE6β trafficking, but the precise mechanism is not known (Ramamurthy et al., 2004; Zhang et al., 2007; Kolandaivelu et al., 2009). Since PRA1 is downregulated in the *rd1* retina that lacks PDE6β, it may also function in PDE6β trafficking. If this is the case, we would predict that PRA1 and PDE6β co-localize in developing mouse photoreceptors.

In our study, PRA1 labeling was diffuse and punctate throughout the retina and particularly in photoreceptor IS and synaptic layers, consistent with its known function in vesicular trafficking. However, no co-localization was seen at either P6, the earliest age PDE6β was detected, or P8, when OSs begin to elongate. This may suggest the transport of PDE6β from IS to OS is via vesicles not directly bound by PRA1. Alternatively, as discussed above, the PDE6β epitope could be masked during trafficking or below the level of detection, technical concerns that could be circumvented in future experiments using fluorescence resonance energy transfer. Consistent with this possibility, AIPL1 has been shown to directly interact with PDE6α and -β by immunoblotting and to be necessary for proper folding and trafficking of the assembled PDE6 heterotetramer (Kolandaivelu et al., 2009). However, AIPL1 also appears localized to the IS and synaptic layers (Ramamurthy et al., 2003) where it cannot overlap with PDE6α or -β in the OS.

In summary, our results demonstrate early onset of PDE6β expression, before other phototransduction proteins, which is consistent with the hypothesis that PDE6β may have a function during photoreceptor development distinct from its role in phototransduction. The observed spatial expression pattern of PDE6β is consistent with development of photoreceptor OSs and assembly of the phototransduction machinery. Localization of PDE6β prior to initiation of OS differentiation could not be detected, possibly due to epitope masking during post-translational modification and trafficking. Further work is required to determine why the vesicle-trafficking protein PRA1 is downregulated in the *rd1* mutant retina. Future studies should also focus on identifying and confirming the downstream targets of PDE6β, for instance PKG, that may participate in modulating photoreceptor development. Identifying potential targets of PDE6β could elucidate cellular processes disrupted in developing *rd1* mouse photoreceptors. Further investigations into how molecular functions are disrupted would enhance our overall understanding of early onset RP.

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