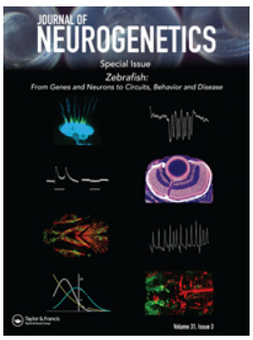

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Neurotoxicity of cGMP in the vertebrate retina: from the initial research on *rd* mutant mice to zebrafish genetic approaches

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ABSTRACT

Zebrafish are an excellent animal model for research on vertebrate development and human diseases. Sophisticated genetic tools including large-scale mutagenesis methodology make zebrafish useful for studying neuronal degenerative diseases. Here, we review zebrafish models of inherited ophthalmic diseases, focusing on cGMP metabolism in photoreceptors. cGMP is the second messenger of phototransduction, and abnormal cGMP levels are associated with photoreceptor death. cGMP concentration represents a balance between cGMP phosphodiesterase 6 (PDE6) and guanylate cyclase (GC) activities in photoreceptors. Various zebrafish cGMP metabolism mutants were used to clarify molecular mechanisms by which dysfunctions in this pathway trigger photoreceptor degeneration. Here, we review the history of research on the retinal degeneration (*rd*) mutant mouse, which carries a genetic mutation of PDE6b, and we also highlight recent research in photoreceptor degeneration using zebrafish models. Several recent discoveries that provide insight into cGMP toxicity in photoreceptors are discussed.

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Zebrafish; photoreceptor degeneration; genetic mutants; retina; Aipl1; Pde6c

Introduction

In vertebrates, the neural retina consists of six major classes of neurons: retinal ganglion cells, which form the innermost layer of the optic cup, three types of interneurons, namely amacrine cells, bipolar cells, and horizontal cells, which form the intermediate nuclear layer, and two types of photoreceptor neurons, namely rods and cones, which mediate phototransduction under dim and normal light conditions, respectively (Figure 1(A)). In the last several decades, the neural retina has been used as an excellent platform to study neuronal differentiation in the central nervous system of vertebrates. In addition, the neural retina provides a good model for studying neuronal degeneration and regeneration. Here, we focus on photoreceptor degeneration caused by genetic mutations of a central component of phototransduction molecule, cGMP phosphodiesterase 6 (PDE6) by reviewing previous mouse and human studies and zebrafish research, and identify its underlying mechanism, cGMP toxicity, which causes photoreceptor cell death.

Photoreceptor structure and the phototransduction pathway

Cone and rod photoreceptors mediate phototransduction in the retina under dim and normal light conditions, respectively. Because zebrafish are diurnal, they have retinas rich in cones as well as rods, making them a convenient model to study cone pathologies (Fadool & Dowling, 2008). Humans have red-, green-, and blue-sensitive cones, while zebrafish have four types, which express red, green, blue, and UV opsins, respectively, and which employ a planar pattern called the row mosaic (Raymond, Barthel, Rounsifer, Sullivan, & Knight, 1993).

Photoreceptors have highly specialized functional structures, formed by an outer segment (OS), an inner segment containing a mitochondria-rich ellipsoid, a nucleus, and a synaptic terminal (Figure 1(B)). The OS consists of photoreceptive membranes that form multiple stacked membrane disks, like piles of coins. These accommodate a large number of phototransduction molecules, such as rhodopsin and opsins (Goldberg, Moritz, & Williams, 2016), indicating that phototransduction is initiated in the OS. The transport of phototransduction molecules and membrane components from the inner segment to the OS is mediated through a specialized primary cilium called the connecting cilium (Wang & Deretic, 2014).

The molecular network for the vertebrate phototransduction cascade is shown in Figure 2. In vertebrate photoreceptors, cyclic nucleotide-gated (CNG) channels determine membrane potential by promoting Na^{+}/Ca^{2+} ion flow into or out of cells. Under dark conditions, a high level of cGMP maintains CNG channels in the open state, maintaining a steady influx of Na^{+}, and Ca^{2+}. Once the photoreceptors are exposed to light, opsins or rhodopsin activates a G-protein, transducin, which subsequently activates PDE6. Activated

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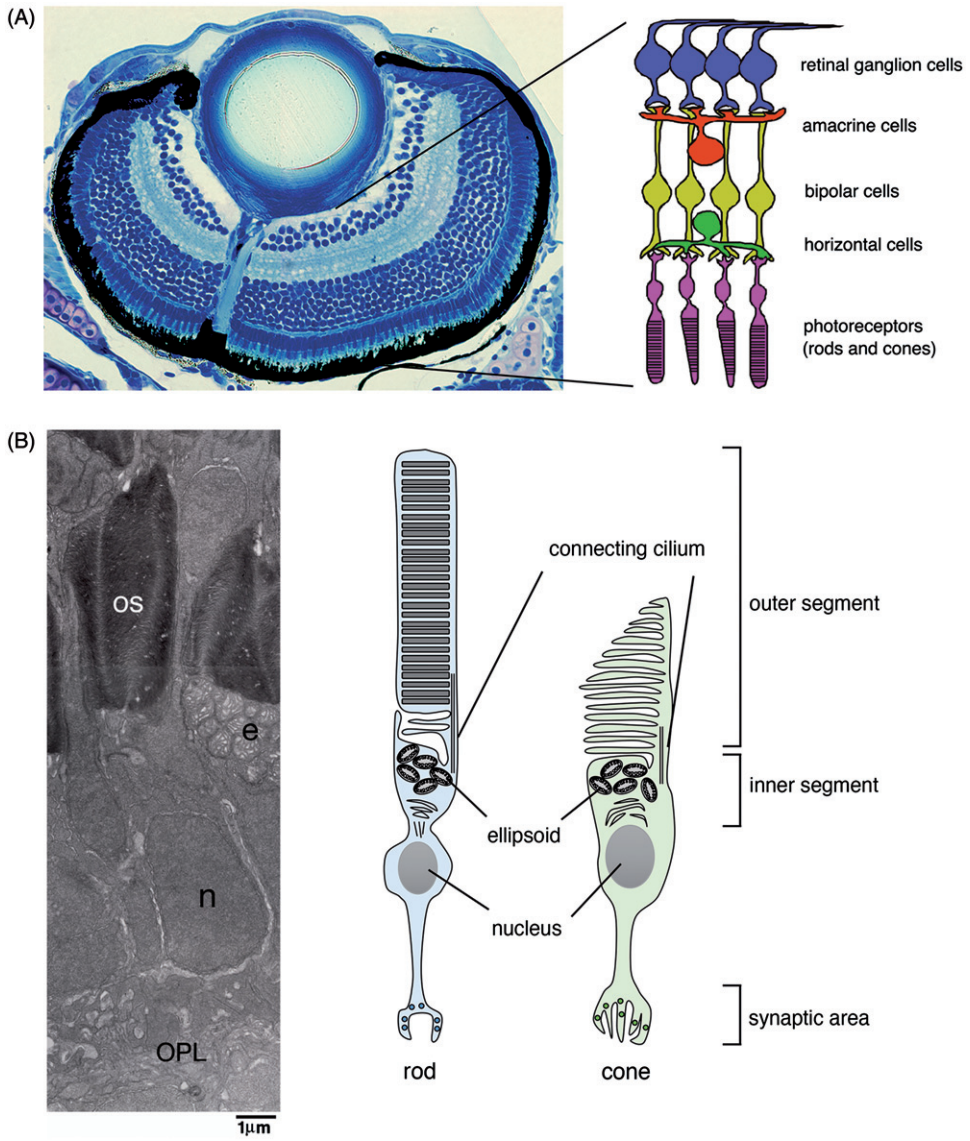

Figure 1. Zebrafish retina. (A) Section of a 7 dpf zebrafish retina (left) and a schematic drawing of the retinal neural circuit, which comprises six major classes of retinal neurons (right). (B) Electron microscopic (EM) image (left) and schematic drawing (right) of rod and cone photoreceptors. Photoreceptors have specialized structures: the outer segment (OS), the inner segment, and a synaptic terminus. The inner segment contains the mitochondria-rich ellipsoid. The connecting cilium extends from the inner segment to the OS. The OS consists of multiple, stacked, photoreceptive membrane disks and accommodates a large number of phototransduction molecules, including opsins, transducins, and Pde6.

PDE6 hydrolyses cGMP. The reduced intracellular concentration of cGMP causes cGMP to dissociate from the CNG channel, which closes the channel. This subsequently reduces the ion influx that hyperpolarizes photoreceptors. Guanylate cyclase-activating proteins (GCAPs) function as Ca^{2+} sensors. When Ca^{2+} ions bind to GCAPs, GCAPs inhibit guanylate cyclase (GC) activity. On the other hand, when Ca^{2+} ions dissociate from GCAPs, this activates GC activity. After light stimuli close the CNG channel, resulting in decreased Ca^{2+} influx, GCAPs activate GC to recover cGMP levels. Thus, in the phototransduction process, PDE6 and GC are functionally linked by intracellular Ca^{2+} levels and regulate the cGMP concentration in photoreceptors.

The phototransduction pathway is shared by both rod and cone photoreceptors; however, phototransduction components differ in rods and cones. For example, rods possess the visual pigment, rhodopsin, while opsins are present in cones. Furthermore, rod and cone transducin, PDE6, GC, GCAP and CNG channels differ in their subunit compositions (Table 1) (Larhammar, Nordstrom, & Larsson, 2009). In rods, PDE6 is a hetero-tetramer consisting of two closely related α and β subunits and two identical γ subunits, while cone PDE6 consists of two homodimers of α′ and γ′ subunits (Lagman, Franzen, Eggert, Larhammar, & Abalo, 2016). PDE6 α, β, α′, γ, and γ′ subunits are also designated as PDE6a, PDE6b, PDE6c, PDE6g, and PDE6h, respectively.

Phototransduction molecules are transported from the ER and Golgi bodies to the OS. Rhodopsin constitutes the majority of OS-resident proteins and belongs to the G-protein-coupled receptor family, which has seven transmembrane domains. The last four amino acids comprising the VXPX motif at the C-terminus of rhodopsin are important for trafficking to the OS and are targeted by a small GTPase, Arf4, which interacts with Rab11 and Rab8 to sort rhodopsin

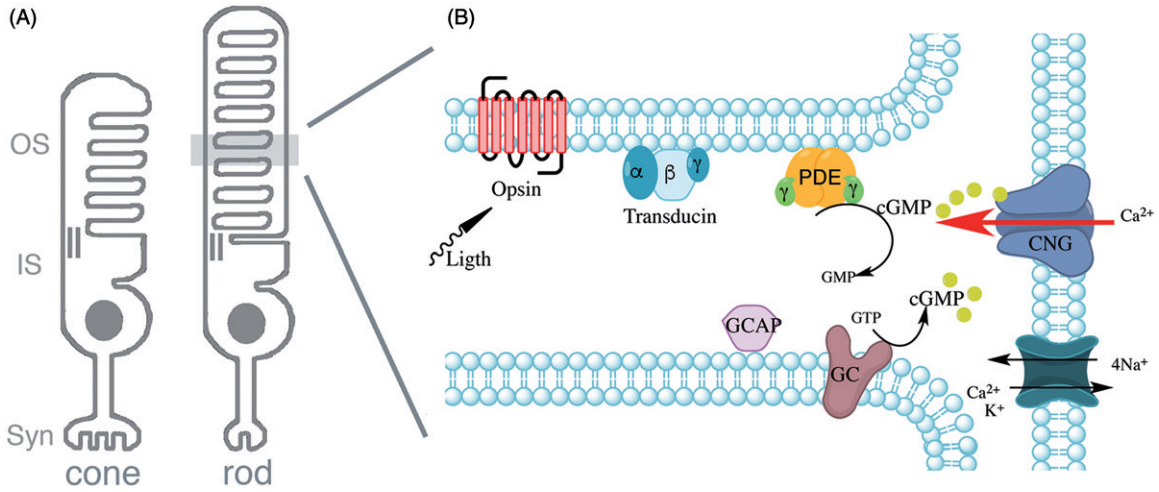

Figure 2. Photoreceptors and the phototransduction pathway. (A) Diagram of cone and rod photoreceptors showing the different compartments, the outer (OS) and inner segment (IS), the nucleus, and the synapsis (Syn). (B) Schematic representation of the phototransduction pathway. Photon absorption and activation of opsin lead to dissociation of transducin α subunit from β/γ subunits, which subsequently activates PDE6. Activated PDE6 then catalyzes cGMP hydrolysis. Reduction of cGMP closes CNG channels on the plasma membrane and reduces intracellular Ca^{2+} levels. Then, hyperpolarization of the photoreceptor ceases neurotransmitter release. Reduced [Ca^{2+}] activates GCAP, which stimulates GC to synthesize cGMP, opening CNG channels again.

Table 1. Nomenclature of rod- and cone-specific subunits of phototransduction molecules in human and zebrafish. In zebrafish, some of the subunits have extra paralogs caused by gene duplication in teleost fish.

			rod		cone	
			human	zebrafish	human	zebrafish
rhodopsin			RHO			
L-opsin					opn1lw (red)	opn1lw1/2 (red)
					opn1mw (green)	
						opn1mw1-4 (green)
S-opsin					opn1sw (blue)	opn1sw (UV)
						opnsw2 (blue)
transducin		α subunit	GNAT1		GNAT2	
		β subunit	GNB1	GNB1a, 1b	GNB3	GNB3a, 3b
		γ subunit	GNGT1		GNGT2	GNGT2a, 2b
PDE6	catalytic subunit	α subunit	PDE6a			
		β subunit	PDE6b			
		α' subunit			PDE6c	
	inhibitory subunit	γ subunit	PDE6g	PDE6ga, 6gb		
		γ' subunit			PDE6h	PDE6ha, 6hb
CNG channel		α subunit	CNGA1	CNGA1a, 1b	CNGA3	CNGA3a, 3b
		β subunit	CNGB1	CNGB1a, 1b	CNGB3	CNGB3.1, 3.2
GC		GUCY2D	retGC1		retGC1	zGC3
		GUCY2F	retGC2	zGC1		
				zGC2		
GCAP		GUCA1A	GCAP1	GCAP1	GCAP1	GCAP5
		GUCA1B	GCAP2	GCAP2	GCAP2	GCAP7
						GCAP3
						GCAP4
APIL1			AIPL1	AIPL1a	AIPL1	AIPL1b

into transport vesicles budding from the trans-Golgi network and delivering them to the OS (Pearring, Salinas, Baker, & Arshavsky, 2013). Many other proteins transported to the OS are associated with membranes via lipid modifications. Transducin α and γ subunits, GCAPs, and opsin kinase GRK1 are lipidated by farnesylation, acylation, and myristoylation (Pearring *et al.*, 2013). Rod PDE6 is farnesylated and geranylgeranylated on its α and β subunits (Anant *et al.*, 1992; Qin, Pittler, & Baehr, 1992), respectively, while cone PDE6 is likely geranylgeranylated on both of its α′ subunits (Wensel, 2008). Following prenylation of the C-terminal cysteine of PDE6, which ends in a CAAX domain, the last three amino acids are removed by the endoprotease RAS-converting enzyme 1 (RCE1) (Christiansen, Kolandaivelu,

Bergo, & Ramamurthy, 2011) and subsequently prenylcysteine is methylated by isoprenylcysteine carboxyl methyltransferase (IMCT) (Christiansen *et al.*, 2016). These two modifications are critical for trafficking functional PDE6 to the OS. Photoreceptor-specific GCs, retGC1 and retGC2 (also designated as GUCY2D and GUCY2F), are trans-membrane proteins. Double knockdown of retGC1 and retGC2 impairs protein levels of GCAP1, transducins, PDE6, and GRK1, as well as their delivery to the OS, suggesting that these lipidated proteins are transported to the OS via GC-bearing transport vesicles (Baehr *et al.*, 2007). These findings indicate that intracellular protein modification and transport tightly regulate functional integrity of phototransduction molecules.

Retinal degeneration diseases in humans

Retinal degeneration is a devastating condition leading to blindness. Here, we will focus on the inherited retinal degenerative diseases, such as retinitis pigmentosa, cone-rod dystrophy, achromatopsia, and Leber congenital amaurosis (LCA) among others. Retinitis pigmentosa is characterized by night blindness and loss of mid-peripheral visual field in its early stages. Later, as the disease progresses, loss of far peripheral visual field and central vision occur, suggesting rod-cone dystrophy in which primarily rods degenerate, followed by the second loss of cones (Hartong, Berson, & Dryja, 2006). Cone-rod dystrophy is a distinct type of retinal disease causing initial cone degeneration followed by the loss of rods (Hamel, 2007). Achromatopsia is a retinopathy, which presents loss of cone functions, leading to color blindness, poor visual acuity, photophobia, and nystagmus. Characteristically, it appears at birth or infancy of patients (Aboshiha, Dubis, Carroll, Hardcastle, & Michaelides, 2016). LCA is a severe disease with congenital loss of rods and cones at early stages (den Hollander, Roepman, Koenekoop, & Cremers, 2008). Over 238 genes are known to participate in these retinal diseases (RetNet, http://www.sph.uth.tmc.edu/RetNet). These genes are roughly classified into several distinct functional groups, which are classified depending upon whether they impact phototransduction (Rattner, Sun, & Nathans, 1999), retinoid metabolism (Saari, 2012), intracellular transport through the connecting cilium (Rachel, Li, & Swaroop, 2012), or establishment of the OS (Goldberg *et al.*, 2016). However, because of the large number of genes associated with retinal degeneration, it is difficult to develop an efficient treatment. So far, only partial positive results have been obtained through treatment with neuroprotective compounds, gene therapy, cell transplantation, or artificial devices (Cuenca *et al.*, 2014).

cGMP metabolism in photoreceptor degeneration

As mentioned above, genetic mutations of a variety of genes are associated with photoreceptor degeneration in humans. Hereafter, we focus on cGMP metabolism in photoreceptor degeneration. A good starting point is an early finding in a spontaneous mouse mutant, retinal degeneration (*rd*) (Keeler, 1924). The *rd* mutation induced photoreceptor degeneration in accordance with Mendelian inheritance. In 1974, Farber and Lolley reported that cGMP concentration is elevated in degenerating photoreceptors of the *rd* mutant mouse (Farber & Lolley, 1974). Following this study, it was reported that high concentrations of cGMP are linked to photoreceptor degeneration in frog (Farber & Lolley, 1977) and dog retinas (Aquirre, Farber, Lolley, Fletcher, & Chader, 1978). These reports raised the possibility that abnormal accumulations of cGMP cause photoreceptor degeneration in the vertebrate retina.

Indeed, cGMP is the key messenger in phototransduction in vertebrate photoreceptors, and the *rd* mutant gene encodes a rod-specific subunit of PDE6, PDE6b (Bowes *et al.*, 1990). Soon after, genetic mutations in rod-specific subunits of PDE6, PDE6a, and PDE6b were identified in human patients suffering from retinitis pigmentosa (Huang *et al.*, 1995; McLaughlin, Sandberg, Berson, & Dryja, 1993), further suggesting that cGMP metabolic dysfunctions are important for understanding human genetic retinal diseases. In parallel, a genetic mutation was found in rod transducin α-subunit (also called GNAT1) in human patients with the Nougaret form of congenital stationary night blindness (Dryja, Hahn, Reboul, & Arnaud, 1996). Mutations in the cone transducin α-subunit (also designated GNAT2) were identified in human patients with Achromatopsia, which shows total lack of cone functions (Kohl *et al.*, 2002). *Gnat1* mutant mice show that rods fail to show the response to flash light by single-cell recording, however display a very mild retinal degeneration (Calvert *et al.*, 2000). *gnat2* mutant zebrafish show no detectable level of cone α transducin and relatively normal retinal morphology (Brockerhoff *et al.*, 2003). These data suggest that a blockade of phototransduction upstream of PDE6 in both rods and cones does not induce photoreceptor degeneration to the level of the *rd* mutant mice. These observations also suggest that cGMP metabolic dysfunction is more directly linked to photoreceptor degeneration.

cGMP levels are maintained by a balance between PDE6 and GC activities. In humans and mice, there are two GCs, retGC-1 and retGC-2. retGC1 is expressed in both rods and cones, whereas retGC2 is expressed only in rods (Table 1) (Yang, Foster, Garbers, & Fulle, 1995). Gain-of-function mutations of retGC-1 are likely to increase cGMP concentration and to cause autosomal dominant cone-rod dystrophy (Tucker *et al.*, 1999; Wilkie *et al.*, 2000). Furthermore, a genetic mutation, Y99C, in GCAP1 disrupts the 3rd EF hand motif, thereby preventing Ca^{2+} binding to GCAP1. Thus, GCAP1 is released from Ca^{2+}-mediated inhibition and permanently activates retGC1 (Payne *et al.*, 1998), resulting in autosomal dominant cone dystrophy (Sokal *et al.*, 1998). These observations also support the idea that elevated cGMP levels trigger photoreceptor degeneration in humans and mice. Conversely, loss-of-function mutations of retGC-1 are expected to decrease cGMP production and reported to induce LCA in human (Perrault *et al.*, 1996). It was also reported that cGMP levels are significantly reduced in chick *GC1* mutant (Semple-Rowland, Lee, Van Hooser, Palczewski, & Baehr, 1998), suggesting that lower cGMP levels link to

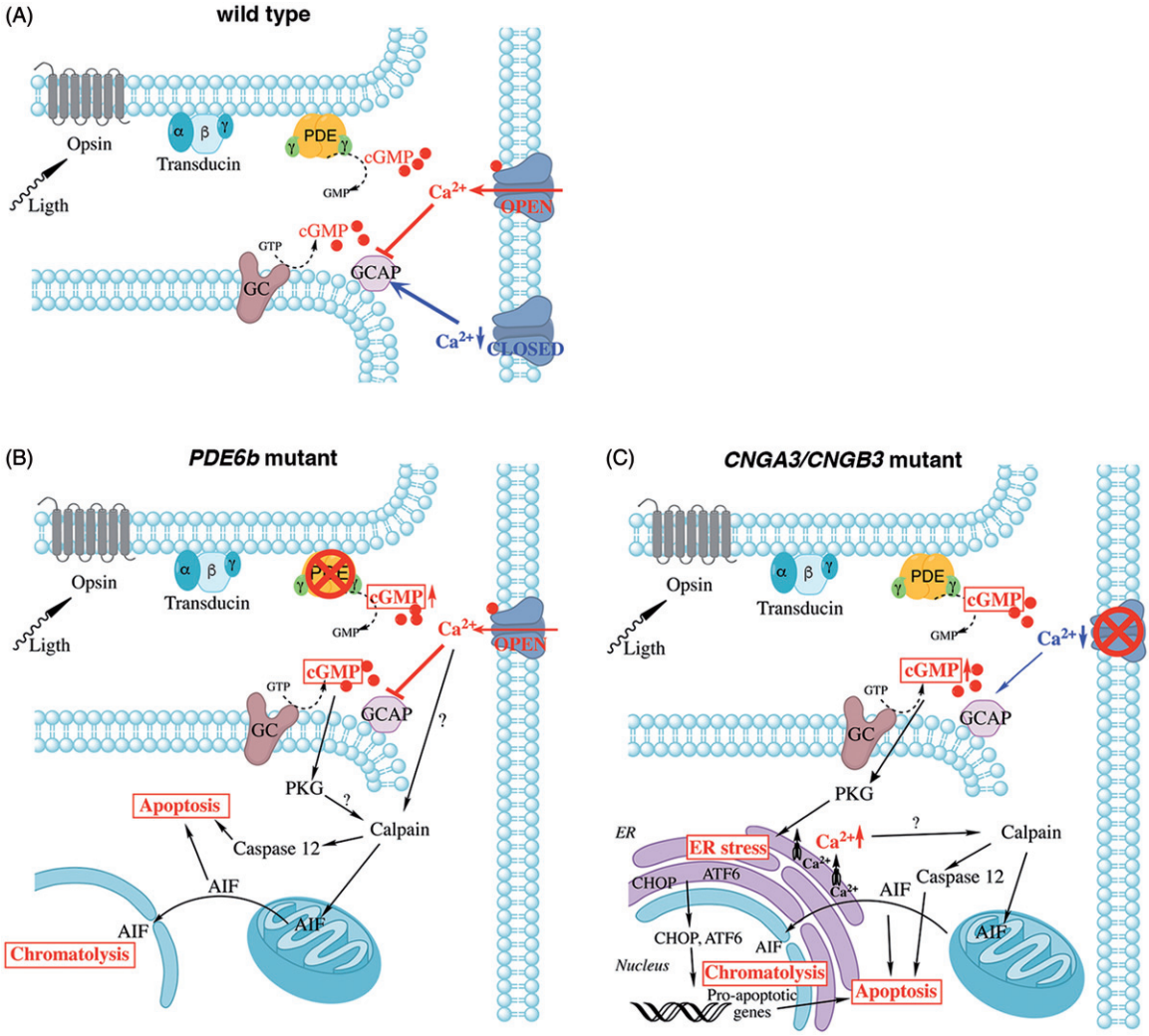

Figure 3. Possible cone cell death pathways in response to cGMP accumulation. (A) Diagram of the phototransduction pathway in wild-type photoreceptors. Intracellular cGMP levels are regulated by a balance between PDE6 and GC activity. Ca^{2+} influx through CNG channels inhibits GCAPs, so the closure of CNG channels decreases intracellular Ca^{2+} levels, which subsequently activate GCAP and then increase GC activity, resulting in cGMP production. (B) Apoptotic pathways in *Pde6b* mutant mice. The absence of PDE6b activity induces the accumulation of cGMP. High levels of cGMP chronically open CNG channels, which subsequently enhance a series of Ca^{2+}-dependent cellular processes, including the activation of calpain. Calpain activates AIF to translocate from mitochondria to nuclei, in which AIF promotes chromatin degradation. Calpain also activates caspase 12 to promote apoptosis. (C) The apoptotic pathway of *Cnga3/Cngb3* mutant mice. The absence of cone CNG channels decreases Ca^{2+} influx, which may cause persistent activation of GCAPs and retGC1, resulting in elevated cGMP. Highly elevated cGMP activates PKG and enhances ER stress response regulators, including ATF6 and CHOP. Activation of calpain, AIF, and caspase 12 is also observed.

photoreceptor degeneration. As we mentioned before, double knockdown of retGC-1 and retGC-2 markedly decreases the level of PDE6 in mice (Baehr *et al.*, 2007), indicating some interdependence between PDE6 and GC activity. These findings show that dysfunction on cGMP metabolism results in abnormal cGMP levels, which are toxic to photoreceptors.

Functional CNG channels in photoreceptors are hetero-tetramers consisting of α subunits (also designated as CNGA) and β subunit (also designated as CNGB) (Larhammar *et al.*, 2009). Rods and cones use distinct subunits. Rods have CNGA1 and CNGB1, whereas cone channels consist of CNGA3 and CNGB3 (Table 1). The hetero-tetramer channel consists of three CNGA1 and one CNGB1 in rods (Zheng, Trudeau, & Zagotta, 2002), and two copies of CNGA3 and CNGB3 in cones (Peng, Rich, & Varnum, 2004). Mutations of CNGA3 and CNGB3 were reported in patients with achromatopsia (Kohl *et al.*, 1998; Kohl *et al.*, 2000). In *Cnga3* mutant mice, accumulation of cGMP causes photoreceptor degeneration (Xu *et al.*, 2013), supporting the correlation between high levels of cGMP and photoreceptor cell death.

Mechanisms underlying photoreceptor cell death triggered by cGMP accumulation

During the past twenty years, many studies have elucidated the molecular mechanism underlying photoreceptor degeneration caused by defects in PDE6 function (Figure 3(A–B)). In the *rd* mouse, mutations of the *Pde6b* gene are likely to cause accumulation of cGMP in rods. Consistently, rods first degenerate, followed by cones. Since PDE6b is expressed exclusively in rods, cone degeneration is triggered by loss of rods, probably through the bystander effect

(Frasson *et al.*, 1999; Narayan, Wood, Chidlow, & Casson, 2016). Thus, two questions arise. First, what molecular mechanism mediates rod death in the *rd* mouse? Second, what is the molecular mechanism that induces cone death in response to rod loss in the *rd* mouse? The first question also raises a series of other questions. If cone *pde6* gene, *pde6c,* is mutated, do these mutant cones also accumulate cGMP and undergo degeneration? In this case, does loss of *pde6c* mutant cones affect survival of genetically healthy rods?

Early studies on the *rd* mutant mouse focused on the first question, the molecular mechanism underlying rod cell death. In *rd* mice, rods undergo a common mode of cell death, apoptosis (Chang, Hao, & Wong, 1993; Portera-Cailliau, Sung, Nathans, & Adler, 1994). However, overexpression of anti-apoptotic factors Bcl2 or BclXL showed conflicting results. Joseph and Li reported that overexpression of Bcl2 or BclXL did not inhibit photoreceptor degeneration in *rd* mice (Joseph & Li, 1996); however, Chen *et al.* found that overexpression of Bcl2 rescued photoreceptor cell death in *rd* mice (Chen *et al.*, 1996). Furthermore, the involvement of caspases is controversial. Activation of caspases and release of cytochrome C were reported in the *rd* mouse photoreceptors (Jomary, Neal, & Jones, 2001), whereas caspase activation was not observed in *rd* mice (Doonan, Donovan, & Cotter, 2003). Yoshizawa *et al.* reported that caspase inhibition mildly rescued photoreceptor degeneration in *rd* mice (Yoshizawa *et al.*, 2002), whereas Zeiss *et al.* showed that photoreceptor degeneration proceeded even in *rd caspase3*$^{-/-}$ double mutant mice (Zeiss, Neal, & Johnson, 2004). Although photoreceptor death is associated with apoptotic traits such as DNA fragmentation, it is likely that the caspase-independent pathway mediates rod degeneration in *rd* mutant mice.

Accumulation of intracellular cGMP is expected to maintain high levels of Ca^{2+} via CNG channels. It was reported that application of a Ca ion channel blocker rescues photoreceptor degeneration and visual functions in *rd* mice (Frasson *et al.*, 1999), although there were conflicting reports (Pawlyk, Li, Scimeca, Sandberg, & Berson, 2002; Pearce-Kelling *et al.*, 2001). Knockdown of rod-specific subunits of CNG channels, CNGA1 and CNGB1, strongly rescues rod photoreceptor degeneration in *rd* mice (Paquet-Durand *et al.*, 2011; Tosi *et al.*, 2011), suggesting that deleterious Ca^{2+} influx through CNG channels causes rod cell death in the absence of PDE6b. Consistently, it was reported that calcium-activated proteases, calpains, are highly activated in degenerating *rd* mutant photoreceptors (Paquet-Durand *et al.*, 2006). One calpain substrate is apoptosis-inducing factor (AIF). AIF was discovered as the first protein that mediates caspase-independent apoptosis (Susin *et al.*, 1999). AIF is a flavoprotein located in the mitochondrial intermembrane space and is involved in energy and redox metabolism (Hangen, Blomgren, Benit, Kroemer, & Modjtahedi, 2010; Modjtahedi, Giordanetto, Madeo, & Kroemer, 2006). Under stress conditions, AIF is cleaved and translocates to the nucleus, leading to chromatinolysis in a caspase-independent manner. AIF release from mitochondria is stimulated by not only calpain-mediated cleavage, but also by reactive oxygen species (ROS) (Churbanova & Sevrioukova, 2008) and poly(ADP-ribose) (PAR) polymer (PARP) (Yu *et al.*, 2002; Yu *et al.*, 2006), which is produced by PAR polymerase 1, a family of proteins involved in DNA repair and genome instability, suggesting that oxidative stress and subsequent DNA damage modify calpain-mediated cleavage of AIF. Retinas of *rd* mice exhibit increased activity of calpains and PARP, which mediate mitochondrial AIF release (Paquet-Durand *et al.*, 2007; Sanges, Comitato, Tammaro, & Marigo, 2006). Blockade of the calpain or PARP pathway inhibits nuclear translocation of AIF and rod cell death in *rd* mice (Murakami, Ikeda, *et al.*, 2012; Sahaboglu *et al.*, 2010; Sanges *et al.*, 2006). These data suggest that AIF and its regulatory mechanisms induce rod photoreceptor cell death in the absence of PDE6b. In addition to the Ca^{2+}/calpain/AIF pathway, cGMP-dependent protein kinases (PKG) are activated and regulate photoreceptor cell death in *rd* mice (Paquet-Durand, Hauck, van Veen, Ueffing, & Ekstrom, 2009). Very recently, it was also reported that microglia eliminate photoreceptors in *rd* mice (Zhao *et al.*, 2015), suggesting that microglial phagocytosis contributes to rod cell elimination in the absence of PDE6b.

Zebrafish forward genetics generate visual mutants

Zebrafish are widely used as a vertebrate model in developmental genetics, because of their high fertilization rate, large numbers of eggs per female, rapid embryonic development (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995), and an established chromosome linkage map (Geisler *et al.*, 1999; Knapik *et al.*, 1998; Postlethwait *et al.*, 1994). Moreover, zebrafish embryos are transparent, so a series of excellent fate-map studies (Kimmel, Warga, & Schilling, 1990; Woo & Fraser, 1995) and high-resolution time-lapse live imaging techniques have been applied to early stage of development (Olivier *et al.*, 2010) and also combined with an anatomical atlas documenting gene expression in the nervous system (Ronneberger *et al.*, 2012).

The pioneering work of Streisinger showed the utility of this genetic model to study neuronal development using mutant strains (Grunwald & Streisinger, 1992; Streisinger, Walker, Dower, Knauber, & Singer, 1981). Soon after, several laboratories carried out large-scale, forward-genetic screening with the mutagenic agent, ethyl nitrosourea (ENU) (Driever *et al.*, 1996; Haffter *et al.*, 1996), or viral insertion to identify recessive mutants in zebrafish (Amsterdam *et al.*, 1999). Because zebrafish depend heavily upon vision, they are especially useful for research on the visual system (Fadool & Dowling, 2008). Different behavioral tests have been established to evaluate visual performance, such as the optomotor response (OMR) (Neuhauss *et al.*, 1999) and the optokinetic response (OKR) (Brockerhoff *et al.*, 1995). Visual impairment can be evaluated as soon as 5 days post-fertilization (dpf), when cone photoreceptors start to function (Rinner, Rick, & Neuhauss, 2005). Several types of screening focusing on the visual system were performed. Mutants were identified based upon OKR, OMR, or electroretinogram (ERG)

responses (Brockerhoff *et al.*, 1995; Muto *et al.*, 2005; Neuhauss *et al.*, 1999; Nishiwaki *et al.*, 2008) and retinal morphology (Gross *et al.*, 2005; Malicki *et al.*, 1996; Masai *et al.*, 2003). This mutant collection is an essential resource for various studies of retinal developmental biology and functions.

Zebrafish cone-specific subunit of PDE6, PDE6c, mutants

As mentioned above, genetic mutations of rod-specific subunits of PDE6, PDE6a and PDE6b, cause retinitis pigmentosa in humans (Huang *et al.*, 1995; McLaughlin *et al.*, 1993). Furthermore, the *rd* mutant gene encodes PDE6b (Bowes *et al.*, 1990). In both cases, rods initially degenerate, followed by a progressive degeneration of cones. Because cGMP concentration is elevated in degenerating photoreceptors of *rd* mutant mice (Farber & Lolley, 1974), these findings suggest that chronically high levels of cGMP trigger rod photoreceptor degeneration in mice and humans. One interesting question is whether a similar situation occurs in cones. Does knockdown of cone-specific subunits of PDE6 cause cone degeneration? Until 2009, no genetic mutation of cone-specific subunit of PDE6, α' subunit (also designated as PDE6c), had been reported in mice or humans. Another interesting issue is the interdependence of rod and cone maintenance. Since PDE6b is a rod-specific subunit, cone degeneration in *rd* mouse mutants is likely to depend on the initial loss of rods, suggesting a 'bystander effect', in which healthy neurons undergo cell death when they are neighbor to dead or dying neurons (Frasson *et al.*, 1999). Several possibilities were proposed (Narayan *et al.*, 2016): (1) degenerating rods may release toxic materials to cones, (2) rods may secrete factors that support cone survival, (3) rod loss may affect delivery of nutrients to cones via blood vessels and glia, or (4) rod loss may decrease oxygen consumption, leading to increase of reactive oxygen species, which cause cone damage by oxidative stress. However, it was not clear whether rods show a similar bystander effect in response to cone loss. Interestingly, knockdown of retGC1 in mice shows cone-specific dystrophy, but rods do not degenerate despite a reduced response in rod-mediated ERG (Yang *et al.*, 1999), suggesting that the interdependence between rod and cone maintenance is not mutual. Thus, the interdependence of rod and cone maintenance is interesting to understand homeostasis of retinal functions and also important from a clinical point of view.

A cue to understand rod survival in the cone-degenerating environment came from zebrafish mutagenesis. A nonsense allele of zebrafish *pde6c* mutants was isolated by Brockerhoff's lab (Stearns, Evangelista, Fadool, & Brockerhoff, 2007). In this allele, loss of Pde6c activity induces rapid cone degeneration at embryonic stages. Some rods also undergo degeneration, although rod degeneration is limited to areas with a low rod density. Concurrently, we also isolated a hypomorphic mutant allele of *pde6c,* named *eclipse* (*els*) (Nishiwaki *et al.*, 2008). The *els* mutant has a missense mutation in the *pde6c* gene, causing an amino acid substitution in a conserved regulatory domain called GAF-A. This allele causes cell death in cones and probably rods at an early embryonic stage (Nishiwaki *et al.*, 2008). In later developmental stages, cones undergo slow, but progressive degeneration, and are completely eliminated by 6 months post-fertilization (mpf), but rods are retained to form the outer nuclear layer (Figure 4(A)), suggesting that cones are eventually eliminated in the absence of cone Pde6. It was reported that transplanted wild-type cones persist in zebrafish null *pde6c* mutant retinas (Lewis, Williams, Lawrence, Wong, & Brockerhoff, 2010). These findings indicate that as in the case of rod PDE6, cone PDE6 is required for cone maintenance in a cell-autonomous manner.

Zebrafish *pde6c* mutants provide an interesting insight on the interdependence between rod and cone maintenance. In both null and hypomorphic alleles of zebrafish *pde6c* mutants, some rods seem to undergo cell death at embryonic stages from 6 to 9 dpf (Nishiwaki *et al.*, 2008; Stearns *et al.*, 2007). Since the maturation of rods proceeds until 3 weeks post-fertilization (wpf) in zebrafish (Branchek & Bremiller, 1984), it is likely that cone cell death influences the survival of maturing rods. Interestingly, we observed a slow but normal OKR under dim light conditions in zebrafish *els* mutant larvae at 3 wpf, suggesting rod-mediated scotopic vision (Nishiwaki *et al.*, 2008). In *els* mutants, almost all cone cells are eliminated, but rods are retained with normal subcellular morphology at 6 mpf (Figure 4(A)) (Nishiwaki *et al.*, 2008). Thus, although rods are deformed and some of them are likely to undergo cell death in embryonic stages, the *els* mutant larvae maintain scotopic vision. From these observations, loss of cones does not compromise scotopic vision and rod maintenance in adult zebrafish. After zebrafish *pde6c* mutants were reported, human *PDE6C* mutations were reported and linked to autosomal recessive achromatopsia (Chang *et al.*, 2009; Thiadens *et al.*, 2009). These findings suggest that, like zebrafish *pde6c* mutants, cone-specific degeneration occurs in human *PDE6C* patients.

However, it remains unknown why the bystander effect of rods in the cone-degenerating environment is weak. In *rd10* mutant mice, a missense mutation occurs in exon 13 of the *Pde6b* gene (Chang *et al.*, 2002). Rod photoreceptor death is characterized by apoptotic features, but receptor-interacting protein (RIP) kinase mediates necrotic cone cell death in *rd10* mutant mice (Murakami, Matsumoto, *et al.*, 2012), suggesting that programmed necrosis, called necroptosis, plays a critical role in non-cell-autonomous cone cell death in the absence of rod PDE6. These data suggest that bystander effects of cones in the absence of rod PDE6 are linked to necroptosis. A weak bystander effect of rods in the cone-degenerating environment may be explained if rods do not have molecular machinery to receive death signals for necroptosis or if the cone-degenerating environment does not release signals for necroptosis. Second, in zebrafish *els* mutants, rod numbers recover from embryonic to adult stages, and this recovery depends on retinal regeneration triggered by Müller cells (Nishiwaki *et al.*, 2008). If rod regeneration is active enough to mask non-cell-autonomous rod degeneration in zebrafish *pde6c* mutants, it is expected that rods would gradually recover during development. However, as Müller cell-mediated neuronal regeneration is

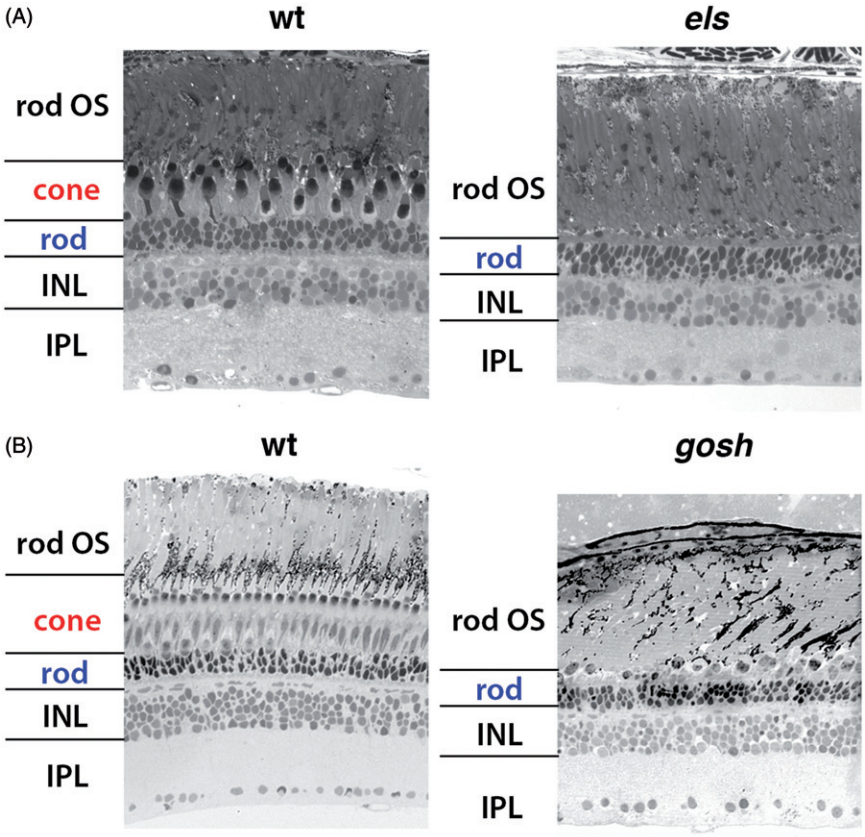

Figure 4. Retinal phenotypes of zebrafish *pde6c* and *aipl1b* mutants. (A) Semi-thin sections of zebrafish wild-type and *pde6c* (*els*) mutant retinas at 6 mpf. Cone photoreceptors are exclusively eliminated, but rods are retained, suggesting cone-specific cell death. Adapted, with permission, from Nishiwaki *et al.* (2008). (B) Semi-thin sections of zebrafish wild-type and *aipl1b* (*gosh*) mutant retinas at 3 mpf. The *gosh* mutant shows cone-specific cell death very similar to that of *els* mutants. Adapted, with permission, from Iribarne *et al.* (2017).

active in fish but not in mammals (Wan & Goldman, 2016; Wilken & Reh, 2016), it is hard to imagine that a similar regeneration mechanism supports rod functions in human achromatopsia patients carrying a *PDE6c* mutation. It is interesting to investigate whether rod cell number and functions are maintained throughout development in human achromatopsia.

Zebrafish aryl hydrocarbon receptor-interacting protein-like 1 (*Aipl1*) mutant

As previously mentioned, PDE6 is synthesized and anchored to the ER membrane by prenylation of a C-terminal cysteine located in the CAAX motif. Further modification of the CAAX motif is required for the stability of PDE6 subunits and their transport to the OS (Christiansen *et al.*, 2011; Christiansen *et al.*, 2016). In addition to these modifications, the maturation and functional integrity of PDE6 depends on aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1). *AIPL1* mutation was originally identified as the cause of human LCA4 (Jacobson *et al.*, 2011; Sohocki *et al.*, 2000). Retinal phenotypes in mice with an *Aipl1* mutation were studied as an animal model for human LCA4. *Aipl1* null mutant mice show rapid degeneration of both rods and cones in early post-natal stages (Dyer *et al.*, 2004; Liu *et al.*, 2004; Ramamurthy, Niemi, Reh, & Hurley, 2004). Analyses of *Aipl1* mutant mice revealed that AIPL1 is required for maintenance of rod-specific subunits of PDE6, PDE6a, and PDE6b. A transcription factor, neural retinal leucine zipper (NRL), promotes rod differentiation by suppressing cone specification (Mears *et al.*, 2001). In *Aipl1;Nrl* double mutant mice, almost all retinal photoreceptors are specified as cones, but cones fail to mediate phototransduction and undergo degeneration through a loss of PDE6c (Kolandaivelu, Singh, & Ramamurthy, 2014). Furthermore, *Aipl1* mutant mice expressing human AIPL1 under the control of rod-specific *Nrl* promoter showed defects in cone phototransduction and maintenance (Kirschman *et al.*, 2010). Even though both cases employed artificially cone-dominated environments, these data exclude the possibility that cone loss of *Aipl1* mutant mice results entirely from the loss of rods. Instead, the authors suggested that AIPL1 is cell autonomously required for cone function and survival.

Biochemical analyses revealed that in the absence of AIPL1, rod and cone PDE6 subunits are synthesized normally, but are degraded through the ubiquitin–proteasome system (Kolandaivelu, Huang, Hurley, & Ramamurthy, 2009; Kolandaivelu *et al.*, 2014). Thus, AIPL1 is not implicated in synthesis of PDE6, but helps to achieve proper functioning, by assisting correct folding, prenylation, and assembly of PDE6 subunits (Kolandaivelu *et al.*, 2009). Recently, it was proposed that AIPL1 also interacts with the inhibitory subunit, PDE6g, after PDE6 assembly (Yadav, Majumder, Gakhar, & Artemyev, 2015). Thus, AIPL1 is an important

regulator for rod and cone PDE6 function in mammalian photoreceptors.

Very recently, we reported that the zebrafish *gold rush* (*gosh*) mutant harbors a mutation in *aipl1* (Iribarne *et al.*, 2017). The zebrafish *gosh* mutant was originally identified as an OMR-defective mutant (Muto *et al.*, 2005). We examined retinal phenotypes of the *gosh* mutant and found that they are very similar to those of the zebrafish hypomorphic *pde6c* mutant, *els*. The *gosh* mutant does not show OKR, and displays abnormal photoreceptor morphology, such that some photoreceptors undergo cell death during the embryonic stage. At 3 mpf, cones are eliminated, but rods are retained in the outer nuclear layer in the *gosh* mutant retina (Figure 4(B)). However, a complementation test between the *gosh* and *els* mutations indicated normal photoreceptor morphology and OKR in trans-heterozygous embryos, suggesting that the *gosh* mutant gene is not *pde6*c. We cloned the *gosh* mutant gene and found that it encodes Aipl1. There are two *aipl1* homologous genes, namely *aipl1a* and *aipl1b*, annotated on the zebrafish genomic database. *aipl1a* is expressed in rods and UV cones, whereas *aipl1b* is expressed in all four types of cones. The *gosh* gene encodes Aipl1b, a cone-specific Aipl1. We found that cone Pde6c is markedly reduced in the *gosh* mutant, and that the *gosh* mutation genetically interacts with the zebrafish *pde6c* mutation, *els*. These data suggest that Aipl1 is required for Pde6c stability and function in zebrafish. The requirement of AIPL1 for PDE6 is conserved across vertebrate species from fish to humans.

The zebrafish cone *gc (gc3)* mutant, *zatoichi*

Mammalian photoreceptors express two GCs. retGC1 is expressed in rods and cones, whereas retGC2 is expressed only in rods. In zebrafish, there are three retGCs, gc1–3. *gc3* is expressed in all cones, whereas *gc1–2* are expressed in rods and UV cones (Ratscho, Scholten, & Koch, 2009). *gc3* mutants have been isolated by the Baier lab and named *zatoichi* (zat^{s125} and zat^{s376}) (Muto *et al.*, 2005). *zat* mutants show abnormal OKR and OMR, although their retinal morphology is normal during larval stages. However, retinal phenotypes in the *zat* mutant have not been studied in detail. We knocked down *gc3* with morpholino antisense oligos and found that, like *zat*, retinal morphology, including photoreceptors is normal at 4–5 dpf (Iribarne *et al.*, 2017). In addition, it was reported that PDE6c activity is reduced or absent in retGC1 knockdown mice (Baehr *et al.*, 2007). We found that Pde6c activity was drastically reduced in *gc3* morphants (Iribarne *et al.*, 2017). Furthermore, interestingly, Gc3 levels are also drastically reduced in zebrafish *els* and *gosh* mutants at the embryonic stage before cones undergo degeneration. Thus, stability of Aipl1, Pde6c, and Gc3 is reciprocally interdependent in zebrafish cone photoreceptors. Since it was reported that PDE6c fails to be transported to the cone OS in *retGc1* mutant mice (Baehr *et al.*, 2007; Karan, Zhang, Li, Frederick, & Baehr, 2008), the coupling mechanism between cGMP metabolic enzymes may be conserved between fish and mice.

At present, the molecular basis of this coupling mechanism is unknown. One possibility is that Gc3 and Pde6c are co-transported from the ER to the OS in the same vesicles. However, there is conflicting evidence. *Retinal degeneration 3* (*RD3*) mutations cause photoreceptor degeneration known as LCA12 in human. In mice, RD3 knockdown affects transport of retGC1/2 to the OS but not that of PDE6c (Azadi, Molday, & Molday, 2010), indicating that transport of PDE6c and retGC1 does not always coincide. Another possibility is that some negative feedback mechanism may keep an appropriate balance between Gc3 and Pde6c activity to prevent fluctuation of cGMP concentration. High cGMP levels lead to constitutive activation of CNG channels, resulting in high Ca^{2+} influx, which subsequently inhibits GCAPs and also cGMP production. If this negative feedback is not enough to prevent cGMP accumulation, Gc3 may be degraded by some unknown mechanism. These observations suggest that regulation of cGMP metabolic enzymes is more complex than we expected.

Mechanism of cone degeneration in the absence of cone PDE6

Previous mouse studies showed that cGMP accumulation correlates with rod photoreceptor degeneration. However, decreased cGMP concentrations were also reported in *Aipl1* mutant mice, in which rod and cone PDE6s are not maintained (Kolandaivelu *et al.*, 2014; Liu *et al.*, 2004). In addition, the *GC1* mutant chick shows low cGMP levels in photoreceptors, which were around 10–20% of those of wild-type sibling, prior to photoreceptor degeneration (Semple-Rowland *et al.*, 1998). Our analyses on zebrafish cone-specific cGMP metabolic enzymes suggest reciprocal interdependence of the stability of Pde6c, Aipl1b, and Gc3. Therefore, it is important to investigate cGMP levels in degenerating cones. Anti-cGMP antibody has been used for monitoring cGMP levels in photoreceptors (Michalakis, Xu, Biel, & Ding, 2013). Immunohistochemical analyses using the anti-cGMP antibody revealed that cGMP level is increased in photoreceptors of the zebrafish *pde6c* null mutant, $pde6c^{w59}$, at 4 dpf (Viringipurampeer *et al.*, 2014). However, we found that cGMP level is not elevated in photoreceptors in either *els* or *gosh* mutants at 4 and 7 dpf, although there are a few cells with higher cGMP levels (Iribarne *et al.*, 2017). It is possible that the anti-cGMP antibody does not have enough sensitivity to detect subtle increases in cGMP levels, which possibly occur in *gosh* and *els* mutants. It is also interesting that higher cGMP level is only observed in the severe *pde6c* allele, which show rapid cone degeneration, but not in the weak *pde6c* allele, *els*, which shows slow cone degeneration. These observations suggest that intracellular cGMP levels correlate with the severity or speed of cone degeneration.

No accumulation of cGMP in *els* and *gosh* mutant photoreceptors raises the possibility that some negative feedback mechanism prevents cGMP accumulation. Since Gc3 level is reduced in *els* and *gosh* mutants at 7 dpf, the loss of Gc3 activity may prevent chronic elevation of cGMP in these mutants (Iribarne *et al.*, 2017). In *Aipl1;Nrl* double knockout mice, PDE6c was not maintained, and cGMP level was reduced under dark-adapted conditions (Kolandaivelu *et al.*,

2014). Interestingly, the retGC1 level is also reduced in these double mutant mice. Thus, the absence of PDE6c activity links to instability of GC proteins throughout vertebrate animals. If this is the case, such a homeostatic mechanism may function as a safeguard to protect photoreceptors against fluctuating cGMP levels. An acute reduction of Pde6 activity may occur at early embryonic stages in the zebrafish *pde6c* null mutant, *pde6c*^{w59}, elevating cGMP levels despite such homeostatic mechanisms. On the other hand, in the weak allele of *pde6c* mutants, *els*, suppression of Gc3 activity may more effectively prevent cGMP accumulation, resulting in slower cone degeneration. Further study will be necessary to unravel the molecular basis for the coupling of PDE6 and GC activity.

The next important question is what molecular mechanism induces cone degeneration in the absence of PDE6c activity. Although a failure of Pde6c-mediated GMP hydrolysis must start cone degeneration process, we did not observe accumulation of cGMP in *els* or *gosh* mutants at early embryonic stages (Iribarne *et al.*, 2017). At the moment, we do not know whether cGMP levels of *els* or *gosh* mutant cones are kept in the normal physiological range or chronically in lower levels. Abnormal low cGMP levels are thought to chronically maintain lower Ca^{2+} levels by closing the CNG channels, which is toxic to neuronal cells (Fain, 2006). Thus, lower cGMP levels mimic the situation where constant light illumination triggers photoreceptor degenerations, which was proposed as 'the equivalent light hypothesis'. (Fain & Lisman, 1993). However, the photoreceptor degeneration mechanism coupled with low cGMP levels is still unclarified. In mouse *Pde6b* mutants, rod cell death is induced by apoptosis in a cell-autonomous manner, whereas cone cell death is induced cell non-autonomously by a new programed type of necrosis, called necroptosis, which depends on receptor-interacting protein kinases 1 and 3 (RIP1 and RIP3) (Murakami, Matsumoto, *et al.*, 2012). Recently, it was reported that photoreceptor cell death in the zebrafish *pde6c* null mutant, *pde6c*^{w59}, depends on Rip3 (Viringipurampeer *et al.*, 2014). The authors showed that Rip3 knockdown rescues cell death and visual responses in zebrafish *pde6c* mutants. They speculated that rod Pde6 subunits may be artificially expressed in *pde6c* mutant cones, where they mediate cone phototransduction even in the absence of Pde6c. However, we did not observe OKR in zebrafish *els* or *gosh* mutant embryos injected with morpholino *rip3* antisense oligos (data not shown). Furthermore, it was reported that cone cell death in zebrafish *pde6c*^{w59} mutants is induced cell autonomously (Lewis *et al.*, 2010), in contrast to the cell-non-autonomous necroptotic cone cell death in *Pde6b* mutant mice (Murakami *et al.*, 2015). Thus, it is still unknown whether necroptosis is a major mechanism of cone degeneration in zebrafish *pde6c* mutants.

In humans and mice, mutations of PDE6c cause achromatopsia, in which cones degenerate, but rods are maintained (Chang *et al.*, 2009; Thiadens *et al.*, 2009). In addition to PDE6c, genetic mutations of cone-specific subunits of CNG channels, CNGA3 and CNGB3, cause achromatopsia (Kohl *et al.*, 1998; Kohl *et al.*, 2000). In *Cnga3* mutant mice, accumulation of cGMP causes cone photoreceptor degeneration (Xu *et al.*, 2013), confirming that high levels of cGMP cause cone cell death. Since this cone cell death is rescued by knockdown of retGC1, the authors suggested that the absence of CNGA3 results in lower [Ca^{2+}], which induces persistent activation of GCAPs so that retGC1 increases cGMP levels. These data suggest a cone cell death mechanism caused by accumulation of cGMP without excessive Ca^{2+} influx (Figure 3(C)). Expression of ER stress response markers, Grp78/Bip, phosphor-elF2α, and CHOP as well as Ca^{2+} release from ER, is enhanced in cone photoreceptors of *CNGA3* and *CNGB3* mutant mice (Thapa *et al.*, 2012), suggesting that abnormal Ca^{2+} homeostasis activates ER stress responses in cones. Furthermore, calpain, caspase 12, and AIF are also activated in these CNG mutants, implying that the ER stress response activates calpain-mediated caspase 12 and AIF pathways. Recently, it was reported that blockade of the cGMP/cGMP-dependent protein kinase (PKG) signaling pathway reduces ER stress response and cone cell death in *Cnga3* mutant mice (Ma *et al.*, 2015), suggesting that PKG mediates cone cell death, probably through the ER stress response. Interestingly, it was reported that genetic mutations of the ER stress response regulator, ATF6A, cause achromatopsia associated with no photopic vision and severe cone loss, especially in the fovea in humans (Ansar *et al.*, 2015; Kohl *et al.*, 2015). This indicates that the ER stress response is important for cone survival in humans. Since opsins are frequently mislocalized outside the OS in both *els* and *gosh* mutant photoreceptors (Iribarne *et al.*, 2017; Nishiwaki *et al.*, 2008), it will be interesting to examine whether the ER stress response contributes to cone degeneration in zebrafish *pde6c* and *aipl1b* mutants, and whether zebrafish Cnga3 or Cngb3 knockdown induces cone cell death, as in zebrafish *pde6c* and *aipl1b* mutants. Further studies will be required to elucidate the mechanism underlying cone cell death in response to cGMP metabolic dysfunctions.

Conclusions

Studies of *rd* mutant mice have contributed to understanding the pathological process of human retinitis pigmentosa caused by genetic defects in phototransduction. In the current model, accumulation of cGMP triggers rod cell death, probably through excessive Ca^{2+} influx, and activation of calpain and AIF. *rd* mutant mice also provided a good model to study the mechanism underlying bystander cone cell death in the absence of rod PDE6 activity. Zebrafish mutant screening identified many visual mutants. They include photoreceptor degeneration mutants, in which cone-specific cGMP metabolic enzymes, including Pde6c, Aipl1b, and Gc3, are mutated. In this review, we showed that these mutants provide a good model for studying mechanisms underlying cone cell death in the absence of Pde6c activity. At present, we are still investigating how cGMP levels are modulated in cones of zebrafish *pde6c/aipl1b* mutants, and also whether dysfunctions of cGMP metabolism activate the similar or different cell death pathways between rods of *Pde6b* mutant mice and cones of zebrafish *pde6c/aipl1b* mutants. Zebrafish are suitable for recently developed

genome editing technology, such as CRISPR/Cas9 and live imaging techniques, so we believe that zebrafish genetic approaches will further contribute to understanding molecular and cellular mechanisms of cGMP toxicity to retinal photoreceptors, and also therapy development for human patients suffering from retinitis pigmentosa and achromatopsia.

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