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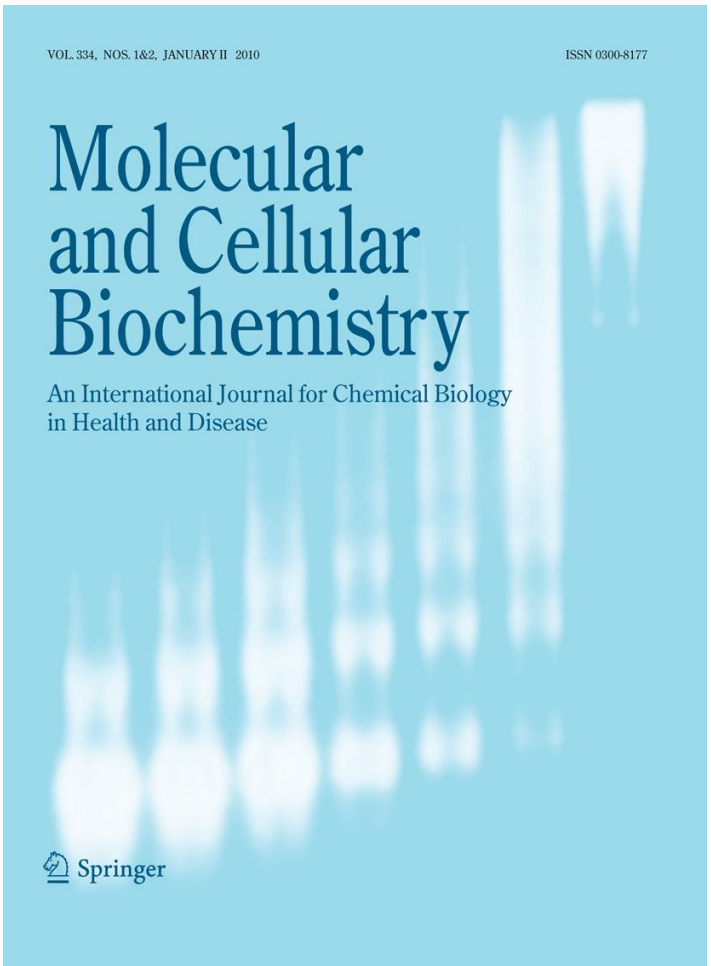

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Novel functions of photoreceptor guanylate cyclases revealed by targeted deletion

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Abstract Targeted deletion of membrane guanylate cyclases (GCs) has yielded new information concerning their function. Here, we summarize briefly recent results of laboratory generated non-photoreceptor GC knockouts characterized by complex phenotypes affecting the vasculature, heart, brain, kidney, and other tissues. The main emphasis of the review, however, addresses the two GCs expressed in retinal photoreceptors, termed GC-E and GC-F. Naturally occurring GC-E (*GUCY2D*) null alleles in human and chicken are associated with an early onset blinding disorder, termed "Leber congenital amaurosis type 1" (LCA-1), characterized by extinguished scotopic and photopic ERGs, and retina degeneration. In mouse, a GC-E null genotype produces a recessive cone dystrophy, while rods remain functional. Rod function is supported by the presence of GC-F (*Gucy2f*), a close relative of GC-E. Deletion of *Gucy2f* has very little effect on rod and cone physiology and survival. However, a GC-E/GC-F double knockout (GCdko) phenotypically resembles human LCA-1 with extinguished ERGs and rod/cone degeneration. In GCdko rods, PDE6 and GCAPs are absent in outer segments. In contrast, GC-$E^{-/-}$ cones lack proteins of the entire phototransduction cascade. These results suggest that GC-E may participate in transport of peripheral membrane proteins from the endoplasmic reticulum (ER) to the outer segments.

Keywords Membrane guanylate cyclase · Targeted deletions · Rod and cone photoreceptors · Photoreceptor membrane protein transport

Abbreviations

ER	Endoplasmic reticulum
GC	Guanylate cyclase
GCdko	GC-E/GC-F double knockout
GCAP	Guanylate cyclase-activating protein
LCA	Leber congenital amaurosis
PDE6	cGMP-specific photoreceptor phosphodiesterase
TGN	*trans*-Golgi network
WT	Wild-type

Soluble and membrane guanylate cyclases

Guanylate cyclases (GCs) synthesize cyclic GMP (cGMP), a secondary messenger in many pathways, in response to diverse signals, such as nitric oxide (NO), peptide ligands (hormones), and fluxes in intracellular Ca^{2+} mediated by Ca^{2+}-binding proteins ($[Ca^{2+}]_i$) [1, 2]. These signals use specific guanylate cyclase receptors and cofactors to initiate the conversion of cytosolic GTP to cGMP. Intracellular cGMP regulates cellular physiology by activating protein kinases, directly gating specific ion channels, or altering intracellular cyclic nucleotide concentrations through regulation of phosphodiesterases (PDEs). Guanylate cyclases are classified as either soluble or membrane (particulate),

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based on both their cellular distribution and structural domains [2, 3]. Soluble guanylate cyclases are heterodimeric proteins consisting of α- and β-subunits, and are activated by NO, another secondary messenger. Soluble guanylate cyclases are present in various cells in vertebrate retina, and maybe involved in signal transmission/modulation between cells [4]. A role in photoreceptor physiology was envisioned earlier for soluble GCs [5, 6], but no biochemical or genetic evidences are available for a role in modulation of cGMP in phototransduction. Based on phenotypes of photoreceptor GC double knockouts, a specific role for soluble GCs in phototransduction can safely be excluded.

Membrane GC isozymes (GC-A to GC-G, Table 1) exhibit highly conserved domain structures, an extracellular domain (ECD) which comprises a large part of the N-terminal part of the molecule, a single transmembrane (TM) region, an intracellular protein kinase-like homology domain (KHD), a dimerization (hinge) domain (DD), and a C-terminal catalytic domain (CAT). Based on their ligand specificities, membrane GCs have been subdivided into natriuretic peptide receptors (GC-A, GC-B), intestinal peptide-binding receptors (GC-C), olfactory uroguanylin- and guanylin-sensitive receptors (GC-D) and the so-called "orphan" receptors (GC-E/GC-F present in photoreceptors, and GC-G in testis). GC-E and GC-F have no known extracellular ligand (hence, the term "orphan"), but are stimulated by intracellular ligands, the GC-activating proteins (GCAPs). Thus, currently the only real "orphan" receptor is GC-G, a receptor of largely unknown distribution and function.

Non-photoreceptor GCs and cGMP-signaling have been reviewed extensively [1–3, 7]. However, within the last 10 years, the generation of membrane GC knockouts have led to important insights concerning their precise function (recent review: [8]). The following paragraphs attempt to briefly summarize the phenotypes of non-photoreceptor membrane GC knockouts and of naturally occurring null alleles in human.

Consequences of *non-photoreceptor GC* deletions

GC-A (natriuretic peptide receptor A, gene symbol *Npr1*, see Table 1) is expressed in the vasculature, heart, brain, testis, and other tissues, and is stimulated by atrial natriuretic peptides secreted by heart muscles. Mice lacking the Npr1 gene, produced by placing a neo-cassette in exon 4 [9] (Fig. 1), mimic many of the features of hypertensive heart disease in human patients. $Npr1^{-/-}$ mice showed multiple phenotypes, including elevated blood pressure, salt-resistant hypertension, progressive cardiac hypertrophy, and sudden death, thereby demonstrating that GC-A is essential for the maintenance of normal blood pressure [9, 10].

GC-B (natriuretic peptide receptor B, gene *Npr2*) is expressed in many different tissues, and its function had been unclear until a knockout was generated [11]. In order to generate the knockouts, exons 3–7 encoding a portion of the ECD and the TM domain, were replaced by a neo-cassette [11] (Fig. 1). $Npr2^{-/-}$ mice showed a dramatic impairment of endochondral ossification and an attenuation of longitudinal vertebra or limb-bone growth [11]. Female $Npr2^{-/-}$ mice were infertile, but male mice were not, due to the failure of the female reproductive tract to develop. Null mutations in the *NPR2* gene in humans are associated with autosomal recessive skeletal dysplasia known as "acromesomelic dysplasia, type Maroteaux" (AMDM), characterized by reduced body height. In addition, heterozygous NPR2 mutations were found to be associated with

Table 1 Nomenclature of GC enzymes and genes

Mouse (*Gene*)	Human (*Gene*)	Aliases	Ligand	Tissue/cells	Comments
GC-A (*Npr1*)	GCA, NPR-A *NPR1*)	Natriuretic Peptide Receptor A	ANP BNP	Vasculature	Regulates hypertension
GC-B (*Npr2*)	GCB, NPR-B (*NPR2*)	Natriuretic Peptide Receptor B	CNP	Brain, bone	Null alleles associated with dwarfism
GC-C (*Gucy2c*)	GCC (*GUCY2C*)		Guanylin uroguanylin enterotoxin	Intestines	Null allele insensitive to enterotoxins
GC-D (*Gucy2d*)	GCD (*GUCY2E*)	Olfactory neuroepithelia (ONE)-GC	Guanylin uroguanylin	Subset of Olfactory neurons	Pseudogene in primates
GC-E (*Gucy2e*)	RetGC-1 (*GUCY2D*)	ROS-GC1, GC1	GCAPs	Rods and cones, pineal	Null alleles associated with LCA-1
GC-F (*Gucy2f*)	RetGC-2 (*GUCY2F*)	ROS-GC2, GC2	GCAPs	Rods	Mutant alleles in cancer cells
GC-G (*Gucy2g*)	GCG (*GUCY2G*)		Unknown	Mouse testis, kidney	Pseudogene in human

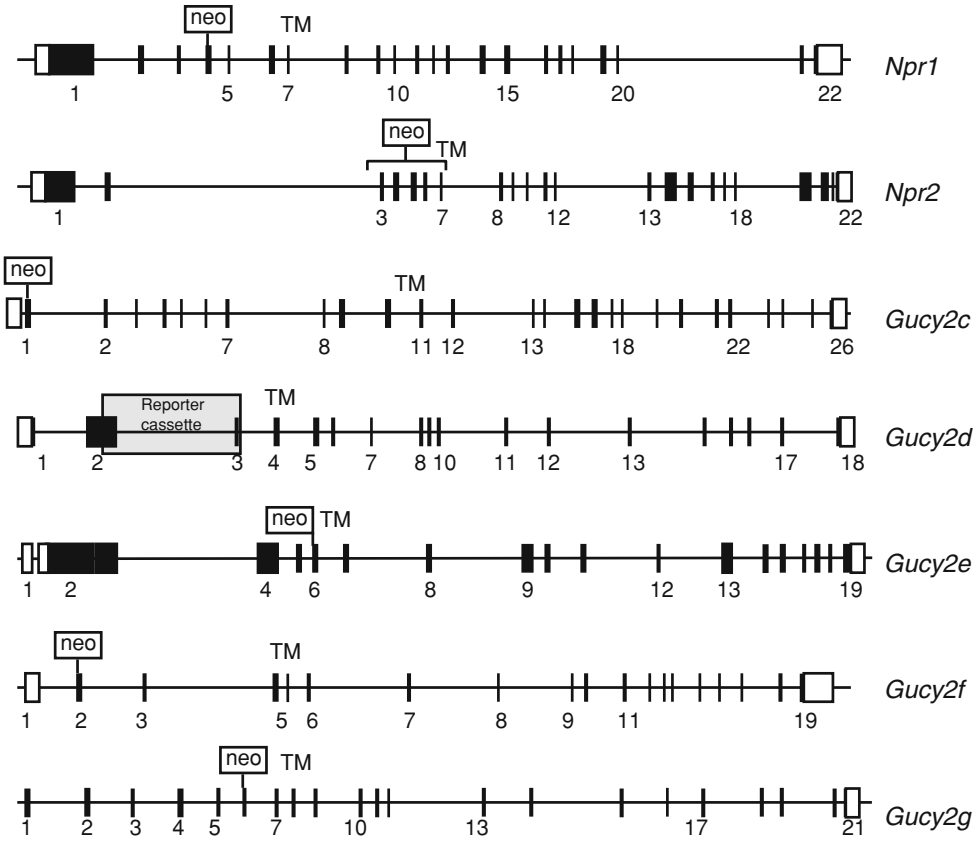

Fig. 1 Membrane guanylate cyclase knockout representations. *White boxes*—noncoding exons; *black boxes* or *vertical lines*—coding exons. *TM* transmembrane domain. Neo cassettes indicate strategies for knockout constructs. References for knockout mice are: *Npr1* [9]; *Npr2* [11]; *Gucy2c* [15]; *Gucy2d* [18]; *Gucy2e* [78]; *Gucy2f* [31]; *Gucy2g* [21]

short stature in humans [12]. Further, mutations in the Npr2 gene are responsible for dwarfism, short limbs and tail in the *cn/cn* and slw (short-limbed dwarfism) mouse [13, 14].

Heat-stable enterotoxins activate GC-C (gene symbol *Gucy2c*) in intestines and increase levels of cGMP that activate downstream targets causing acute diarrhea. The *Gucy2c* deletion was generated by inserting a neo-cassette into exon 1; the knockout mice developed normally and were resistant to enterotoxin-induced diarrhea [15]. GC-C is the major guanylate cyclase in the mammalian intestines, which has been the focus of a link to colon cancer. However, *Gucy2c* null mice show reduced polyp formation and increased apoptosis, the opposite of proliferation [16].

A small subpopulation of olfactory neurons in the main olfactory epithelium express GC-D, together with CNGA3 (cGMP-gated channel α-subunit first identified in cones) and cGMP-specific PDE2 [17, 18]. A *Gucy2d* knockout was generated by replacing exons 2 and 3 with a reporter cassette fused to a floxed neo gene [18] (Fig. 1). The knockout permitted the identification of uroguanylin and guanylin as extracellular ligands suggesting that GC-D expressing olfactory neurons may detect cues related to hunger and thirst [18, 19]. The "orphan receptor" GC-G is expressed in mouse testes [20] and kidneys [21], but its distribution in other tissues and possible function are largely unknown. A *Gucy2g* knockout suggested that in kidneys, GC-G may promote apoptotic and inflammatory responses in ischemia-reperfusion-induced renal injury [21]. Interestingly, the primate *GUCY2E* gene (encoding GC-D) and the human *GUCY2G* gene (encoding GC-G) contain multiple inactivating sequence changes and are non-functional pseudogenes [22]. These data suggest that GC-D has been lost in primates, prior to the divergence of Old World monkeys from New World monkeys (more than 40 million years ago) [22].

These gene targeting experiments clearly demonstrate the power of gene knockouts in pinpointing the exact functions of membrane GCs in vivo. Here, we will discuss the photoreceptor GCs (GC-E and GC-F) expressed in rods and cones, with particular focus on gene knockouts and their consequences for photoreceptor physiology, membrane protein transport, and photoreceptor survival.

Photoreceptor guanylate cyclases (GC-E and GC-F)

Membrane GCs are key components responsible for the production of cGMP, an essential secondary messenger of phototransduction. During phototransduction, cGMP is rapidly hydrolyzed by a membrane-associated PDE6 located on rod outer segment disk membranes, i.e., coin-like stacks of membranes unconnected to the plasma

membrane. Disappearance of the "messenger" closes cGMP-gated cation channels residing in the cell membrane, effectively hyperpolarizing the cell. In the dark-adapted photoreceptor, the basal GC activity (inhibited by Ca^{2+}-bound GC-activating proteins, termed GCAPs) is balanced by the low basal activity of PDE6, adjusting cytoplasmic cGMP to about 1–10 μM. When the intracellular calcium ions decrease from about 500 nM (dark) to <50 nM in light, the GCs are activated by the Ca^{2+}-free GCAPs, increasing the catalytic GC activity by about 10-fold. If all the components of phototransduction (R*, G*, PDE*) are "silenced" by returning to the inactive dark state, then GC stimulation leads to restoration of dark cGMP levels [23–26].

GC-E or GC-F each possesses the structural features characteristic of membrane GCs—a signal sequence preceding a large amino-terminal ECD, a single membrane-spanning region, a kinase-like homology domain, and a carboxy-terminal catalytic domain. While the nomenclature of the GCs in various species is somewhat confusing (Table 1), generally accepted abbreviations are retGC-1 and retGC-2 in human [27, 28], ROS-GC1 and ROS-GC2 in bovine [29, 30], and GC1 and GC2 in mouse [31]. Quantitative determination of GC-E and GC-F in outer segment preparations by mass spectrometry showed a ratio of GC-E/GC-F of 3.5/1 [32], but biochemical quantization showed a much higher ratio of GC-E/GC-F of 25/1 [33].

Human GC-E (retGC-1) [34] and human GC-F (retGC-2) [35] show regional localization in the photoreceptor cell bodies and inner segments by in situ hybridization with antisense probes [27, 36]. Human and monkey GC-E were associated with outer segments of rods and cones, but also to a lesser extent with IS and other cells [34, 37]. The mouse GC-E and GC-F polypeptides were shown to be present in rod outer segments, likely as homomers [38]. Mouse GC-E is prominently immunolocalized in outer segments of rods and cones [39], whereas GC-F is detectable only in outer segments of rods (Fig. 2, [31]). Neither GC-E nor GC-F is detected elsewhere in the mouse retina, at least under the conditions of Fig. 2 [31]. However, GC-E was also detected in the synaptic terminals of bovine photoreceptors [40, 41]. Outside the retina, GC-E was detected in the pineal gland [38, 42], an organ developmentally related to the retina, in the olfactory bulb [43], spermatogenic cells of bovine testes [44], as well as the cochlear nerve and the organ of Corti [45]. Interestingly, GC-F was identified in several human cancer cell lines [46] where its function is unknown.

None of the membrane GCs has an affinity for Ca^{2+} ions, or conventional Ca^{2+}-binding motifs. The activities of photoreceptor cyclases, however, are Ca^{2+}-sensitive, a sensitivity that is mediated by guanylate cyclase-activating proteins (GCAPs) at low free Ca^{2+}, as well as by S100B and neurocalcin at high free Ca^{2+}. GCAPs are Ca^{2+}-binding proteins with three high-affinity Ca^{2+}-binding sites (EF hands) stimulating GC-E and GC-F by binding to cytoplasmic sites [47–50]. S100B and neurocalcin stimulate GC-E at 17–20 μM Ca^{2+} [51, 52], a concentration which is too high for a physiological role in outer segments. The peptide ligands of non-photoreceptor GC receptors failed to activate either GC-E or GC-F [53]. GC enzymology, biochemistry, and history of cloning, are reviewed in detail in [29].

GUCY2D null alleles in human: Leber congenital amaurosis type 1

Leber congenital amaurosis type 1 (LCA-1), an autosomal recessive, early onset dystrophy defined as a "congenital

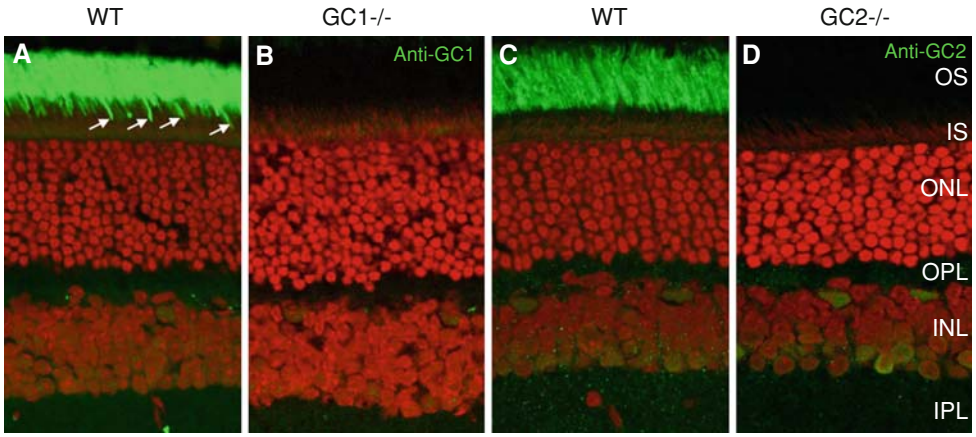

Fig. 2 Distribution of GC1 and GC2 in WT (**a**, **c**) and GC knockout (**b**, **d**) mouse retina. Cryosections **a**, **b** were probed with anti-GC1, and **c**, **d** with anti-GC2 antibodies. GC1 is present in rod (*intense green staining* in the OS area) and cone (*arrows*) outer segments (**a**). GC2 is only detectable in WT rod outer segments (**c**). GC1 and GC2 are undetectable in the ONL or OPL (synaptic terminals). Faint immunofluorescence in the INL is nonspecific (Color figure online)

Table 2 GC null alleles in human LCA-1 patients

Exon	Amino acid change	Domain	Enzyme activity	References
2	M1I	ECD	Reduced	[93]
2	W21R	ECD	Yes	[93]
2	L41F	ECD	Yes	[57]
2	C105Y	ECD		[61]
2	H109P	ECD		[93]
2	N129K	ECD	Yes	[57]
3	R313C	ECD	Yes	[57]
3	L325P	ECD		[61]
4	Y351C	ECD		[94]
4	*S448ter*	ECD	No	[93]
7	R540C	KHD		[93]
8	F565S	KHD	Reduced	[95]
10	R660Q	KHD		[60]
12	R768W	DIM		[94]
13	*Q855ter*	DIM	No	[96]
13	P858S	DIM		[61]
15	A934P	CAT		[72]
15	L954P	CAT		[61]
15	R976L	CAT		[93]
16	R995W	CAT	No	[93]
16	M1009L	CAT	No	[72]
17	H1019P	CAT	No	[93]
17	*Q1036ter*	CAT	No	[54]
18	*R1040ter*	CAT	No	[72]

Italicized mutations produce truncations

ECD Extracellular domain, *KHD* kinase homology domain, *DIM* dimerization domain, *CAT* catalytic domain

stationary cone–rod dystrophy with high hypermetropia, panretinal degeneration and highly reduced visual acuity," is a rare disease (prevalence approximately 1:100,000) [54]. Null mutations in the human GC-E gene (*GUCY2D*, on chromosome 17) are relatively frequent and distributed throughout the ECD, KHD, DIM, and CAT domains (references in Table 2). Mutations in the GC-F gene (*GUCY2F*, on Xq22) [55] have not been linked to a retina disease phenotype. Since *GUCY2F* gene is located on X, only males would be affected by disease-causing mutations. Interestingly, several missense mutations in the peptide leader sequence, the KHD and CAT domains of GC-F have been identified in panels of colon, lung and breast cancer [56]. LCA is one of the most severe forms of inherited retinal dystrophies, accounting for approximately 5% of all inherited retinal dystrophies [57, 58]. Children with LCA have greatly impaired vision and extinguished electroretinograms (ERG), despite seemingly normal fundi at birth or during the first months of life [59]. Immunofluorescent images of human GUCY2D-LCA retinas showed that all cones and rods lacked outer segments, as evidenced by the absence of labeling with anti-opsins and anti-rds/peripherin [60].

A number of nonsense and frameshift mutations in the *GUCY2D* gene have been identified in LCA-1 patients (Table 2). Missense mutations in the catalytic domain (e.g., R976L, R995W, M1009L, H1019P and Q1036Z) result in the total abolition of cyclase activity. Conversely, some missense mutations in the extracellular domain (e.g., W21R, L41F, N129, R313C) do not affect cyclase activity in vitro. Truncation mutations (e.g., S448ter) remove the CAT domain, and prevent synthesis of cGMP. Some missense mutations in the extracellular domain (C105Y, L325P) have resulted in 50% decreased cyclase activity. L325 is highly conserved and C105 has been suggested to participate in intramolecular disulfide bond formation and oligomerization of the protein. Thus, GC mutations may alter the enzyme structure and stability [61], and may affect post-biosynthetic folding or retrograde transport of peripheral membrane-associated proteins along microtubules to the cilium (Fig. 8).

Cone–rod dystrophy (CORD6)

Dominant cone–rod dystrophy (CORD6) also maps to the GC1 locus [62, 63]. Mutations causing dominant cone–rod dystrophy are restricted to the dimerization domain. Cone–rod dystrophy is a progressive disorder, characterized by the initial degeneration of cone photoreceptor cells, causing early loss of visual acuity and color vision, followed by the degeneration of rods leading to progressive night blindness and peripheral visual field loss [62]. Some of the important missense mutations of the dimerization domain are E837D, R838A, R838H, R838C, T839M [64–66]. The three disease mutations at residue 838 are non-equivalent. They exhibit GC activity equal or superior to WT GC at low free $[Ca]_{free}$ in the order R838C < R838H < R838A and showed a higher affinity for GCAP1 than WT GC [66]. Interestingly, when expressed in HEK 293 cells, (R838C)GC1 showed a decreased response to GCAP2 suggesting distinct binding sites for the GCAPs [67]. Structural studies have shown that the residue at position at 838 is a key residue that determines the extent of coiled-coil structure responsible for maintaining the retGC1 dimer. Molecular dynamics-based modeling of RetGC1 coiled-coil suggests that Arg838 forms salt bridges which maintain the configuration of the helices producing repulsion between the polypeptide chains. This causes the coils to spread apart as they extend towards the catalytic domain which then determines the dimerization [68].

A naturally occurring GC-E knockout: the *retinal degeneration* (*rd*) chicken

The *rd* (GUCY1*B) chicken is a naturally occurring blind mutant discovered in a Rhode Island Red flock about 30 years ago (reviewed in [69]). Photoreceptors in predegenerate *rd/rd* retina appeared normal at the ultrastructural level. Degeneration of rods and cones begins approximately 7 days post-hatch, and progresses relatively slowly over many months. The *rd* chicken carries a null mutation in the gene encoding photoreceptor GC-E exhibiting a disease phenotype resembling LCA [70, 71]. The gene defect is described best as a deletion/insertion event in which a gene fragment containing exons 4–7 was replaced by an inverted portion of exon 9 without interruption of the open reading frame (Fig. 3). Deletion of exon 5, encoding the transmembrane region, is predicted to result in a soluble polypeptide that, if stably expressed, would fail to traffic to the outer segments. Consistent with the deletion of the GC-E gene, levels of cGMP in the mutant retina were very low, unable to sustain phototransduction, presumably leading to permanent closure of the cGMP-gated cation channels and elimination of the dark current (constitutive hyperpolarization). In chicken, as in human LCA type 1 (*Gucy2e* null alleles) [72], only GC-E appears to contribute to the pool of cGMP essential to support phototransduction. It is unclear whether a GC-F ortholog is expressed in chicken retina. A lentivirus-based gene transfer vector carrying a bovine GUCY2D cDNA, injected into early-stage GUCY1*B embryos, rescued retinal degeneration to a large part demonstrating convincingly that the *rd* chicken phenotype is caused by deletion of GC-E [73].

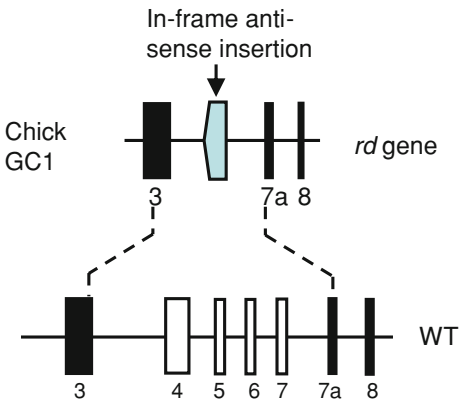

Fig. 3 The *rd* chicken gene defect. *Top*, diagram of the mutant gene in which exons 4–7 are replaced by an inverted fragment of exon 9 (*shaded*). *Bottom*, predicted WT gene representing exons 3–8. Exon 7a is an additional exon not present in mammalian GC-E genes. Adapted from [70]

Transcription of several genes involved in the phototransduction cascade, including iodopsin and cone PDEα', was shown to be normal [74, 75]. However, protein levels of GCAP1 were reduced by more than 90% in predegenerate *rd/rd* retinas compared to age-matched control retinas, while GCAP2 levels were less affected [76], similarly as seen in GC-E/GC-F double knockouts (see below, and [31]). A regulatory mechanism at the gene level was excluded because transcript levels of both GCAP genes were near normal in the predegenerate *rd* retina [77]. The observed downregulation of GCAP1 was likely caused by mistrafficking of membrane-associated proteins which relies in part on the presence of GC-E (see below).

Photoreceptor GC knockouts

The genes encoding GC-E (*Gucy2e*, on mouse chromosome 11) and the GC-F (*Gucy2f*, on the X chromosome) genes [55] are closely related in structure, each consisting of 19 exons and featuring an untranslated exon 1. The GC-E knockout mouse was generated by the group of David Garbers at UT Southwestern [78]. His group deleted the *Gucy2e* gene by targeted recombination replacing exon 5, encoding the transmembrane domain, with a neo cassette. The neo cassette effectively disrupted translation in all reading frames, preserving the extracellular domain, but deleting cytoplasmic regions including the catalytic domain so no functional cyclase can be formed. It is unknown whether an extracellular polypeptide (encoded by exons 1–4) is formed in the *Gucy2e*$^{-/-}$ photoreceptor. At 1 month of age, mouse cone responses were undetectable while rods continued to function albeit with reduced a- and b-wave amplitudes suggesting a substantial loss of rod function (Fig. 4, [78]). The phenotype of the *Gucy2e*$^{-/-}$ mice therefore resembled a recessive cone dystrophy [78, 79] while in human patients, *GUCY2E* null alleles are associated with rod/cone dystrophy (LCA-1).

In *Gucy2e*$^{-/-}$ mouse rods, a second cyclase, GC-F, is obviously able to substitute to some extent for the loss of GC-E. To identify the precise function of GC-F in rods, we generated *Gucy2f*$^{-/-}$ by replacing exon 2 of the *Gucy2f* gene containing the translation start codon and the leader peptide with a neo cassette. Therefore, a functional GC-F cannot be expressed. The phenotype of *Gucy2f*$^{-/-}$ mice was very similar to WT mice (Table 3), but showed slower recovery from intense illumination (Fig. 3 in [31]). In scotopic ERG analyses, the *Gucy2f*$^{-/-}$ a-wave amplitude reflecting rod photoreceptor function was identical to WT, while the b-wave amplitude was reduced (Fig. 4). The photopic ERG responses in the *Gucy2f*$^{-/-}$ mice were very similar to those of WT mice. We proceeded to generate GC double knockout mice (GCdko) by breeding *Gucy2f*$^{-/-}$

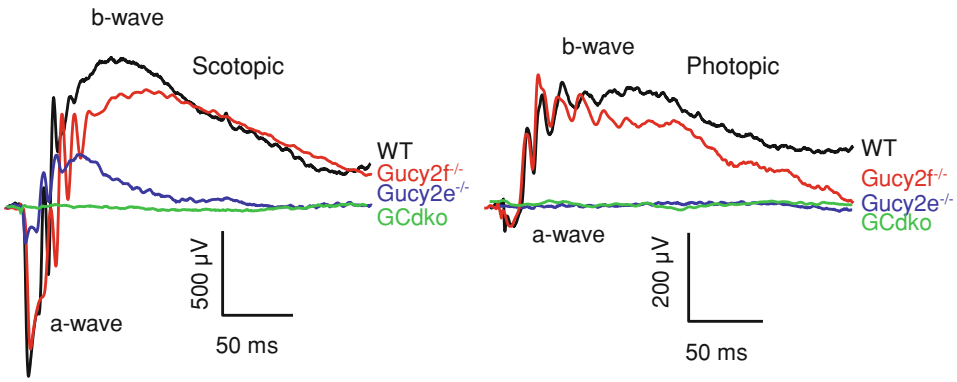

Fig. 4 Scotopic and photopic ERGs of WT, *Gucy2e*$^{-/-}$, *Gucy2f*$^{-/-}$ and GCdko mice at 2.8 log cds m^{-2}. Note lack of response in GCdko scotopic ERGs and in Gucy2e$^{-/-}$ and GCdko photopic ERGs

Table 3 Effect of GC-E deletion, GC-F deletion, and deletion of both GCs on photoreceptor physiology and outer segment constituents

Rod physiology	Cone physiology	ROS proteins	COS proteins
Gucy2e$^{-/-}$			
Functional; ERG responses attenuated	No response; cones degenerate slowly (autosomal recessive cone dystrophy)	GCAP1, GCAP2 downregulated; GC-F levels not affected	Cone Tα, cone Tγ, GRK1, cone PDEα′ undetectable; COS unstable; very little cone pigment present
No degeneration			
Gucy2f$^{-/-}$			
Slower recovery from intense illumination	Normal	Normal	Normal GC-E levels not affected
GCdko			
No response	No response Cones degenerate (recessive LCA)	PDEα, PDEβ, PDEγ, GCAP1, GCAP2 undetectable; Tα, Tγ, arrestin, rhodopsin not affected	Cone Tα, cone Tγ, GRK1, cone PDEα′ undetectable; very little cone pigment present
Rods degenerate (recessive LCA)			

mice with *Gucy2e*$^{-/-}$ mice. Both full-field ERG (Fig. 4) and rod single-cell recordings (Fig. 4 in [31]) showed that GCdko photoreceptors were insensitive to light.

Consequence of GC-E /GC-F double knockout (GCdko) in rods

At 1 month, the GCdko photoreceptors are relatively stable but outer segments are slightly reduced in length. Immunolabeling patterns of rhodopsin (Fig. 5b), arrestin (Fig. 5d), Tα (Fig. 5f) and Tγ (Fig. 5h) were apparently normal and similar to WT, but the labeling pattern of rod outer segments was "patchy" , consistent with alternating bands of tubules and dense membranes observed by electron microscopy [31]. However, expression levels of several phototransduction polypeptides were reduced severely, including those of PDE6 subunits (Fig. 5j), GCAP1 (Fig. 5l) and GCAP2 (Fig. 5n). GCAPs activate GCs in low free Ca^{2+}, are closely associated with GCs, and may be unstable in the absence of GCs. A similar downregulation of GCAP1 (but not GCAP2) was observed in the *rd* chicken carrying a *GUCY2D* null allele [77]. The diminished expression levels of GCAP1 and GCAP2 were confined mostly to the inner segments where biosynthesis occurs (Fig. 5l, n).

We were most surprised by absence of PDE6αβγ subunits in GCdko ROS (Fig. 5j). PDE6 is a peripheral membrane protein stably attached to the disk membrane surface by farnesyl and geranylgeranyl anchors. PDEα is known to be farnesylated and PDEβ geranylgeranylated at the C-terminal cysteines [80, 81]. PDEαβ subunits are prenylated by cytosolic prenyltransferases and then dock to the ER. Once docked, they are insoluble and most likely require vesicular transport to reach their outer segment destination. In contrast to PDE6, transmembrane GCs are synthesized by ER-associated ribosomes, integrate in the ER membrane, and follow the secretory pathway from the ER to the plasma membrane, mediated by IFT through the connecting cilium to the outer segments.

The posttranslational downregulation of PDE and GCAPs, observed by immunoblots [31], and the absence of photoreceptor immunoreactivity for GC-E and GC-F in GCdko retinas suggested a role in protein trafficking, comparable to that of rhodopsin-bearing vesicles [82, 83]. But in this case involving association of PDE6 and GCAPs with GC-bearing vesicles. Based on GC knockout results, enforced by PrBP/δ knockouts [84, 85], we developed a

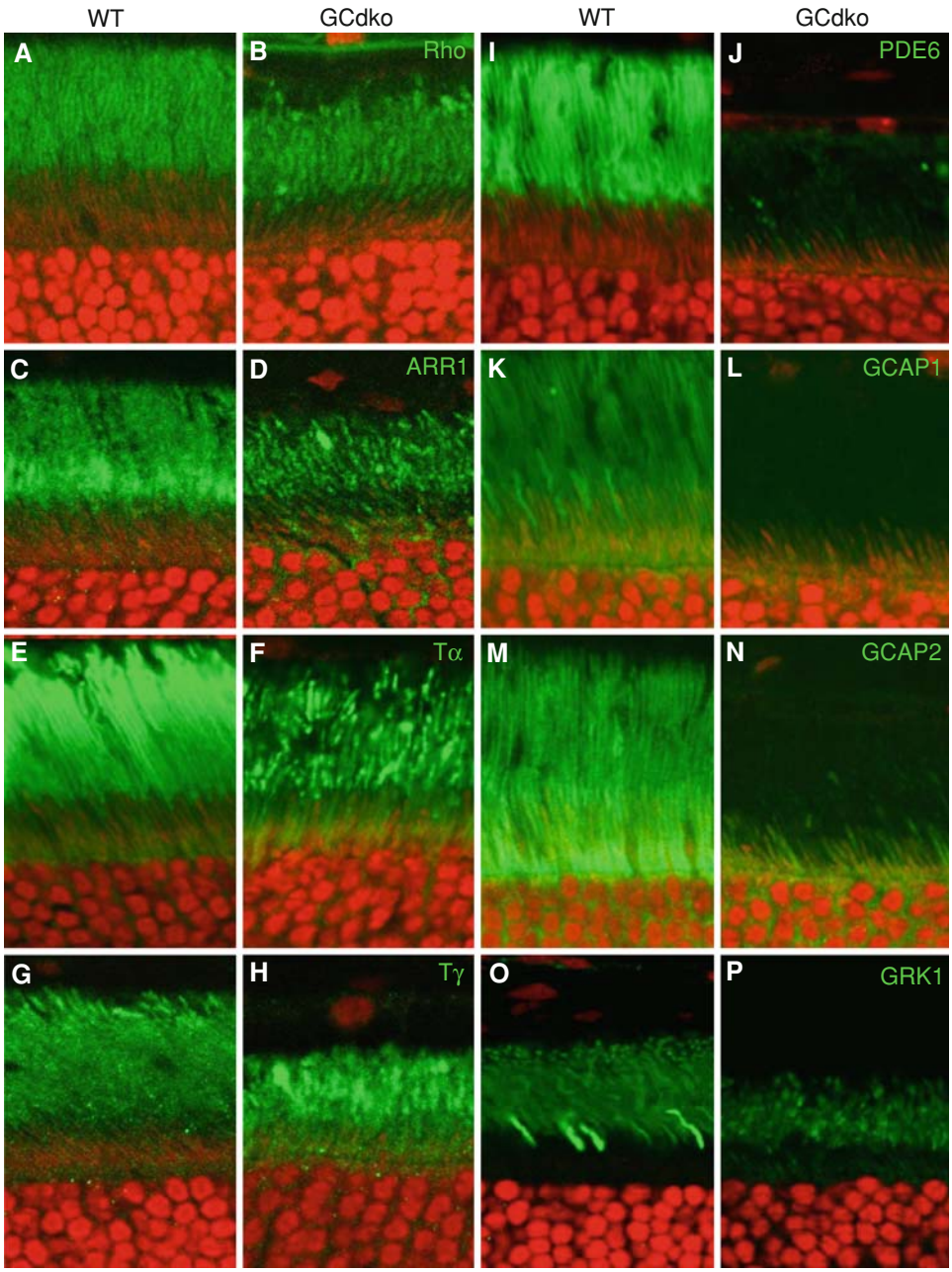

Fig. 5 Distribution of phototransduction polypeptides in WT (**a**,**c**,**e**,**g**,**i**,**k**,**m**,**o**) and GCdko (**b**,**d**,**f**,**h**,**j**,**l**,**n**,**p**) mutant outer and inner segments. Antigens targeted by antibodies (*green*) are indicated *top right* in the GCdko panels. Nuclei of the ONL are counterstained with propidium iodide (*red*). The "patchy" appearance of ROS indicates disorganization of the membrane structure [31] (Color figure online)

model describing transport of GCs in vesicles to which PDE subunits are attached peripherally (Fig. 6a).

Like other integral membrane proteins, GC-bearing vesicles are thought to traffic from the ER to the Golgi and emerge from the trans-Golgi network (TGN; Fig. 6a). We previously observed that when the prenyl-binding protein PrBP/δ was deleted in rods, traffic of GRK1 and PDE6 to the outer segments was impeded while GC-E trafficked normally. We therefore propose that the GC-bearing vesicles are loaded with PDE6 subunits, aided by PrBP/δ (and possibly additional prenyl binding proteins) that transfer PDE6 from the ER to vesicles. For simplicity, in Fig. 7 GC vesicles are charged with PDE post-TGN, but a pre-Golgi loading of the vesicles cannot be excluded. Results with either GC-E or GC-F knockouts indicate that one GC is sufficient for PDE6 (and GCAP) transport. GC-bearing vesicles loaded with peripheral membrane proteins continue retrograde transport along microtubules toward the minus end, powered by molecular motors (e.g., cytoplasmic dynein). Vesicles likely fuse with the plasma membrane at the base of the cilium where cargo is assembled for intraflagellar transport through the cilium. Heterotrimeric kinesin-2 was suggested to be the molecular motor for IFT in rods based on the observation that rhodopsin accumulated in the distal inner segment when KIF3A, the motor subunit of kinesin-2, was deleted. Intriguingly, GC-E was

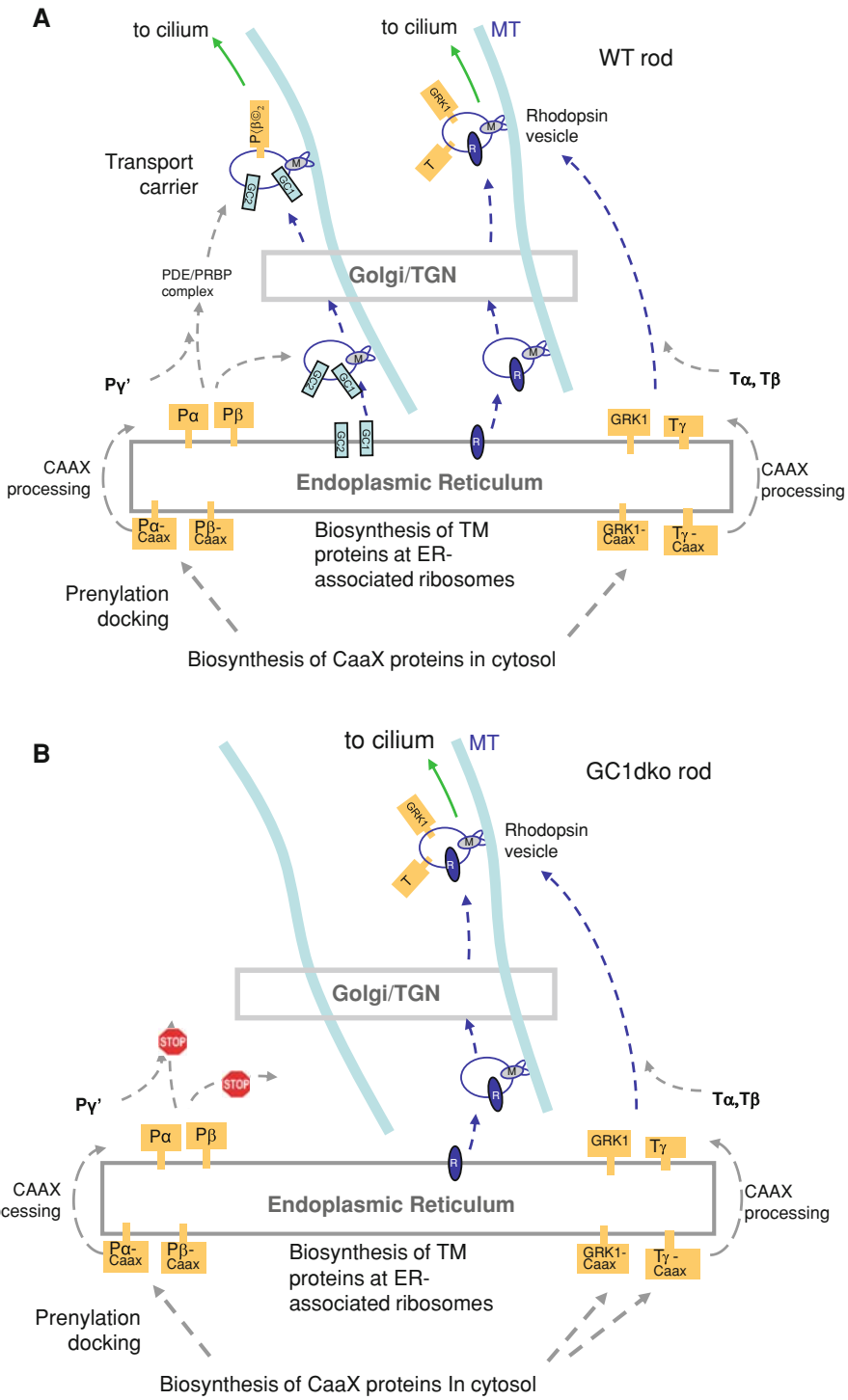

Fig. 6 Putative model of GC and rhodopsin transport following synthesis at the ER. **a** WT rod photoreceptor. **b** GCdko photoreceptor. **a** Integral membrane proteins traffic from the ER to the Golgi/TGN by retrograde transport. Vesicles emerge from the TGN and are charged with the peripheral membrane proteins PDE and GCAPs. Retrograde transport continues to the base of the cilium. **b** Since GC-E and GC-F are not produced, GCAPs and PDE cannot traffic, and are degraded. Rhodopsin, transducin, and GRK1 transport is not affected. For details, see text

suggested to be mislocalized in mice expressing a hypomorphic mutation in IFT88, a protein associated with the axoneme and IFT complexes, but the fate of PDE6 was not investigated [86, 87].

Knockout of both GCs (Fig. 6b) prevents formation of GC-bearing vesicles at the ER and coordinately prevents transport of PDE6 and GCAPs. Since GC-deletion does not affect rhodopsin and transducin transport to the rod outer segment. GC transport and rhodopsin transport may occur independently. PDE docked and retained in the ER due to the absence of GC carriers, likely presents prolonged cellular stress leading to PDE6 degradation by eliciting the unfolded protein response (UPR) with removal by the proteasome/ubiquitination pathway.

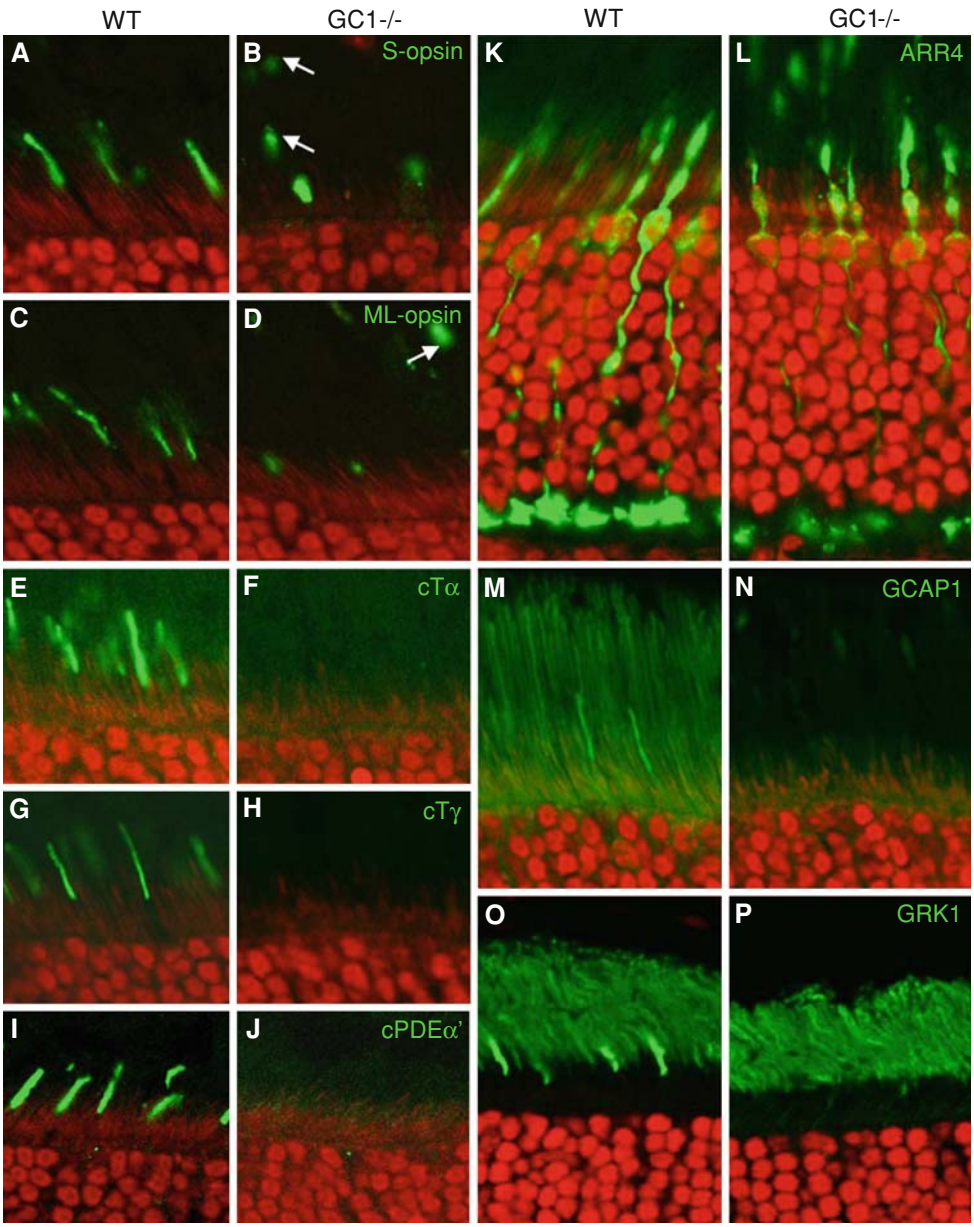

Fig. 7 Distribution of cone phototransduction polypeptides in WT (**a**,**c**,**e**,**g**,**i**,**k**,**m**,**o**) and GC1-/- (**b**,**d**,**f**,**h**,**j**,**l**,**n**,**p**) mutant outer and inner segments. Antigens targeted by antibodies (*green*) are indicated top right in the GCdko panels. *Arrows* (**b**, **d**) indicate disconnected membrane packets containing pigments. Visual pigments, cone transducin, cone PDE, GCAP1 and GRK1 are undetectable in mutant COS (Color figure online)

Effect of GC-E deletion in cones

It was shown that *Gucy2e*$^{-/-}$ cone photoreceptors degenerate gradually [78, 88]. Figure 7l shows that at 1 month of age, *Gucy2e*$^{-/-}$ cone outer segments are still present and of near-normal length, but S-opsin and M/L-opsin were present at reduced levels and mislocalized throughout the mutant cone cell. Further, acylated cone Tα (Fig. 7f), farnesylated cone Tγ (Fig. 7h), geranylgeranylated cone PDEα′ (Fig. 7j), and farnesylated GRK1 (Fig. 7p) were undetectable in Gucy2e$^{-/-}$ COSs. These results show that essential cone phototransduction proteins are either absent or below the threshold of detection in *Gucy2e*$^{-/-}$ COSs. At 6 months of age, cone cell remnants, particularly synaptic pedicles and somata, can be identified, but outer segments are absent. This finding is consistent with a postmortem study of an 11-year old LCA patient with a null mutation in the *GUCY2D* gene. The macula and peripheral retina of this patient revealed a substantial number of cones and rods, but both photoreceptor types lacked outer segments [60].

In contrast to GCdko rods where only GCAPs and PDE mistraffic, proteins of the entire phototransduction cascade are absent in *Gucy2e*$^{-/-}$ outer segments. Therefore the GC-E polypeptide, or its enzyme activity, must play a key role in retrograde trafficking of peripheral membrane proteins to the base of the cilium. An identical phenotype was observed in *Lrat*$^{-/-}$ and *Rpe65*$^{-/-}$ cones in which the retinoid cycle is blocked [89, 90]. In these mutants, the

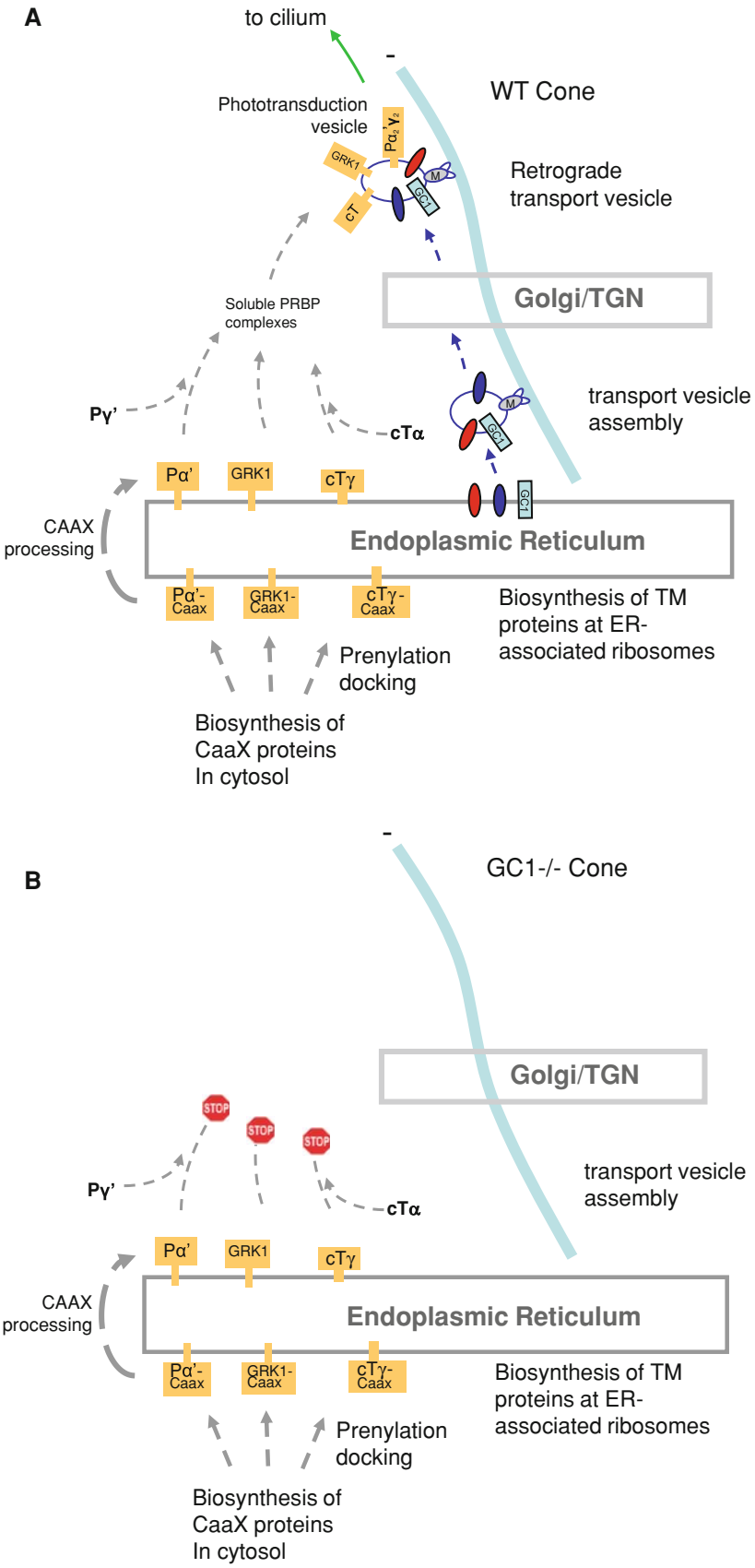

Fig. 8 Putative model for transport of peripheral membrane proteins in cones. **a** WT cone photoreceptor. **b** GC-E knockout cone photoreceptor. **a** Visual pigments and GC-E traffic from the ER to the Golgi/TGN by retrograde transport along microtubules. Vesicles emerge from the TGN and are charged with the peripheral membrane proteins PDEα', cone T, GRK1, and GCAPs. Retrograde transport continues to the base of the cilium, followed by IFT to the outer segment. **b** GC-E is not produced. Peripheral proteins remain in the ER owing the absence of GC-E and are degraded

11-*cis*-retinal chromophore cannot be recycled and opsins are synthesized as apoproteins. Central $Lrat^{-/-}$ cones degenerate rapidly between 2 and 4 weeks postnatally, and immunocytochemistry showed that proteins of the entire phototransduction cascade, including GC-E, GCAPs, and R9AP, are absent in mutant cones. Thus, cone pigments require 11-*cis*-retinal as a cofactor to be competent for post-synthesis trafficking to the outer segments. The interdependence of GC-E, visual pigments, and peripheral membrane proteins supports the idea that a large cargo is assembled at the post-ER or post-TGN levels for co-transport and trafficking to cone outer segments. Further, we recently observed that cone-specific deletion of KIF3A, the obligatory motor subunit of heterotrimeric kinesin-2, prevents intraflagellar transport of visual pigments, GC1, R9AP, and peripheral membrane proteins through the cone cilium [91]. Thus, a common phenotype, i.e. absence of peripheral membrane protein transport, was replicated in four independent deletion models ($Gucy2e^{-/-}$, cone $Kif3a^{-/-}$, $Lrat^{-/-}$ and $Rpe65^{-/-}$ mice); this result argues for the coordinated insertion/assembly of several phototransduction-associated proteins into vesicular membrane for transport from the cone inner segment.

Our working model for cone transport is predicated on the interpretation that cone pigments and GC-E apparently traffic to the Golgi together (Fig. 8). That cone opsins and GC-E co-transport is supported by mislocalization of pigments in the GC-E knockout, and by mislocalization of GC-E in the LRAT/RPE65 knockouts. Emerging from the TGN, vesicles may be formed and loaded with peripheral membrane phototransduction polypeptides, which have been extracted from the ER by prenyl binding proteins. This mechanism is supported by a PrBP/δ knockout in which cone PDE and GRK1 are unable to traffic (they are permanently docked to the ER), while cone transducin traffics unimpeded. In WT cones, GC-E and pigment-bearing vesicles are then transported along microtubules to the cilium, where IFT-cargo is assembled. Intraflagellar transport through the cone cilium is powered by the heterotrimeric kinesin-2, an anterograde motor moving towards the distal end of the outer segment (the plus end of microtubules) [91]. Knockout of the motor subunit KIF3A specifically in cones leads to a phenotype mimicking the GC-E and LRAT/RPE65 knockout, thereby preventing integral membrane proteins and membrane-associated proteins of the phototransduction cascade from trafficking to the cone outer segment.

Conclusion

Deletion of one or both retinal GCs has very different effects on the physiology of rod and cone photoreceptors. Particularly, the cone phenotype argues that GC-E is essential for trafficking several transmembrane and peripheral membrane proteins to the outer segment. We postulate that absence of GC-E in GUCY2D-LCA accounts for weakening of the outer segment membrane structure and secondarily, that accumulation of peripherally-associated proteins at the ER (mistrafficked) constitutes stress that initiates apoptotic pathways mediated by UPR [92].

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