Molecular property, manipulation, and potential use of Opn5 and its homologs

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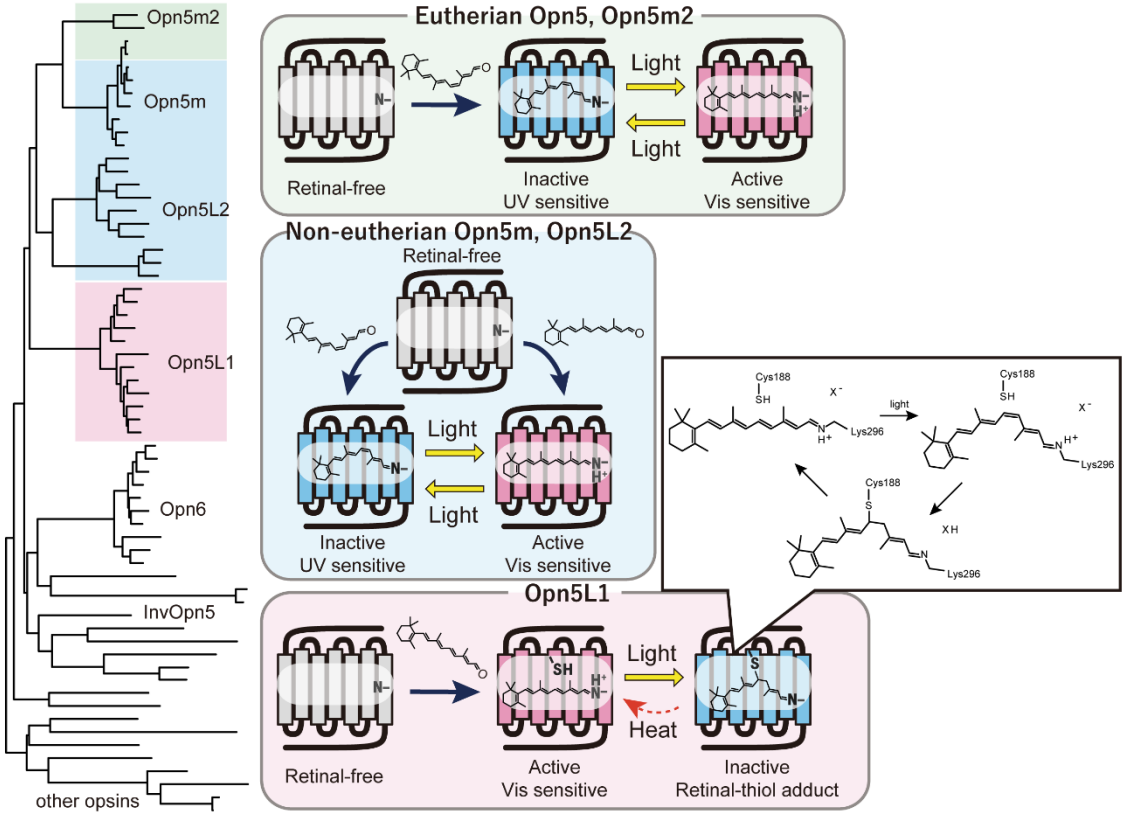
Abstract



Animal opsin is a G-protein coupled receptor (GPCR) and binds retinal as a chromophore to form a photopigment. The Opsin 5 (Opn5) group within the animal opsin family comprises a diverse array of related proteins, such as Opn5m, a protein conserved across all vertebrate lineages including mammals, and other members like Opn5L1 and Opn5L2 found in non-mammalian vertebrate genomes, and Opn6 found in non-therian vertebrate genomes, along with Opn5 homologs present in invertebrates. Although these proteins collectively constitute a single clade within the molecular phylogenetic tree of animal opsins, they exhibit markedly distinct molecular characteristics in areas such as retinal binding properties, photoreaction, and G-protein coupling specificity. Based on their molecular features, they are believed to play a significant role in physiological functions. However, our understanding of their precise physiological functions and molecular characteristics is still developing and only partially realized. Furthermore, their unique molecular characteristics of Opn5 related proteins suggest a high potential for their use as optogenetic tools through more specialized manipulations. This review intends to encapsulate our current understanding of Opn5, discuss potential manipulations of its molecular attributes,

and delve into its prospective utility in the burgeoning field of animal opsin optogenetics.

From bacteria and archaea to animals and plants, numerous organisms living on Earth are engaged in vital activities while utilizing external light as a source of energy and information.[1] Photoreceptor proteins absorb light ranging from ultraviolet (UV) to near-infrared wavelengths, converting it into energy and information usable by organisms. Understanding which wavelengths of light these proteins absorb and the mechanism by which they manifest their functions is essential for comprehending how organisms utilize light and for applying photoreceptor proteins in various applications. Photoreceptor proteins typically contain a molecule called a chromophore. Chromophores possess a π-conjugated system within the molecule and serve as the centerpiece of light reception. Examples include flavin, bilin, heme, retinal, p-coumaric acid, and the tryptophan side chain. Photoreceptor proteins that function with retinal, an aldehyde form of Vitamin A, as the chromophore are generally termed retinal proteins, or rhodopsins. Retinal proteins are ubiquitous across various biological species and can be broadly classified into microbial rhodopsin (type-I rhodopsin) and animal opsin (type-II rhodopsin).[2] In this review, we aim to provide an overview of Opsin 5 (Opn5), a type of animal opsin that forms an independent group in the molecular phylogeny. We will discuss its molecular characteristics, manipulations, and potential uses in optogenetics.

Phylogeny of animal opsins

Animal opsins are membrane proteins that belong to the superfamily of G-protein coupled receptors (GPCRs). Their activity is regulated by the binding of retinal and by light reception, and some have lost their G-protein activation ability and function as retinal photoisomerases.[3] The most extensively studied animal opsin is the visual pigment of vertebrates, particularly the rod visual pigment rhodopsin.[4] Since the research on rhodopsin by Boll and Kühne, rhodopsin has been actively studied as one of the models in the research of photo-receptive proteins.[5,6] Advances in genome analyses have revealed that many animals possess various types of opsins, including rod and cone visual pigments. Among them, some opsins are expressed in cells other than the retinal photoreceptor cells. They are believed to be involved in non-visual photoreception functions, and are referred to as non-visual opsins or non-image-forming opsins. Vertebrates possess non-image-forming opsins known as pinopsin, parapinopsin, parietopsin, VA opsin, tmt-opsin, Opn3, Opn4, Opn5, RGR, and peropsin, each of which forms a cluster on the molecular phylogenetic tree (Figure 1a).[7–16] In

animal opsins functioning as GPCRs, the 11-*cis* isomer of retinal acts as an inverse agonist, and the all-*trans* isomer functions as an agonist. Visual pigments initially bind with 11-*cis*-retinal and remain in an inactive state. Photoisomerization of 11-*cis*-retinal generates an agonist all-*trans* retinal. As a result, the opsin becomes active and catalyzes the exchange of a guanosine diphosphate (GDP) for a guanosine triphosphate (GTP) on the corresponding trimeric G protein (Figure 1b). In opsins such as rod and cone visual pigments, all-*trans*-retinal dissociates from opsin, and the photosensitivity is restored when newly supplied 11-*cis*-retinal is bound. Opsins with such properties are referred to as monostable opsins or bleaching opsins. On the other hand, there are opsins that can undergo mutual photo-conversion between 11-*cis*-retinal bound and all-*trans*-retinal bound forms. Opsins with such properties are referred to as bistable opsins (Figure 1c).

Opn5, identified in humans and mice in 2003, is a type of non-image-forming opsin.[14] Later in 2008, it was reported that other vertebrates, such as chickens and zebrafish, also possess Opn5 and that non-mammalian vertebrates have multiple genes forming a sister group on the molecular phylogenetic tree in addition to the ortholog of the Opn5 that mammals have.[17] These Opn5-like genes have been named Opsin 5-like 1 (Opn5L1), Opsin 5-like 2 (Opn5L2), and the ortholog of mammalian Opn5 has been particularly named Opn5m, derived from the initial letter of ‘mammalian type’ (Figure 1d).[17] Additionally, in ray-finned fishes, an Opn5m2 has been identified that forms a close sister group with Opn5m.[18] Meanwhile, Davies et al. have named the genes of opsins closely related to Opn5 seen in zebrafish as Opn6, Opn7, Opn8, and Opn9.[19] Figure 1d shows the molecular phylogenetic tree of Opn5 and the homologous protein groups from various organisms including vertebrates, non-vertebrate deuterostomes, and protostomes. Opn7 is equivalent to Opn5L1, Opn8 to Opn5L2, and Opn9 to Opn5m2. Opn6, although its function is unknown, is considered to belong to the Opn5 group because it forms one clade with these Opn5 and related genes (Figure 1d). Also, based on several more detailed molecular phylogenetic analyses, this Opn5 group, along with RGR, retinochrome, peropsin, and Go-opsin, forms a monophyletic clade. These groups are considered to have evolved from a common ancestral protein, and collectively, they are referred to as ‘Group 4 opsin’ or ‘tetraopsin’ (Figure 1a).[20,21] In the following, we will provide an overview of the research so far on Opn5m and related proteins (Opn5L1, Opn5L2, Opn6, invertebrate Opn5).

Opn5m (Opn5)

As far as we know, marsupials and eutherians among mammals have only one Opn5 homologous gene on their genome.[22] The chicken ortholog of mammalian Opn5, Opn5m, was the first protein in the Opn5-related protein group to have its molecular characteristics revealed.[23] Chicken Opn5m binds to 11-*cis*-retinal and all-*trans*-retinal to gain photosensitivity in the UV wavelength region and in the visible wavelength region, respectively. When light is irradiated in each state, the two mutually convert, showing chicken Opn5m is a bistable opsin. Additionally, purified chicken Opn5m in the all-*trans*-retinal bound state activates Gi-type heterotrimeric G protein.[23] That is, Opn5m has been shown to be a UV-light-sensitive opsin because UV light converts Opn5m from a resting state to a G protein activating state. Later studies have shown that a variety of vertebrate Opn5m, including those of humans and mice, are UV-sensitive in the 11-*cis*-retinal-bound state.[22,24] Therefore, Opn5m is the only UV-light-sensitive opsin commonly conserved in vertebrates from fish to humans, indicating that humans would also perceive and utilize UV light through opsin, separate from processes such as DNA damage, active oxygen, or vitamin D production.[24,25] Initially, from the study of chicken Opn5m and human OPN5, Opn5m was thought to be mainly coupled to Gi-type G protein [23,24]. However, subsequent studies suggest that Opn5m also activates Gq-type G protein.[26–28] Especially, Opn5m preferentially activates the G14 subtype of the Gq group.[29] Though the canonical pathway downstream of G14 and other Gq-type G proteins is generally the PLCβ-IP_3-Ca^{++} pathway, several studies have suggested the presence of subtype specific downstream signaling.[30,31] Opn5m would give UV-sensitivity to those signaling pathways in some specific cell populations. For the bistable nature of Opn5m, it has been shown that threonine at position 188 (bovine/human rhodopsin numbering system) is essential (Figure 2b).[32] A mutational study has shown that replacement of this residue into asparagine, glutamine, or methionine drastically hindered tautomeric conversion between inactive and active states and even binding selectivity for retinal isomers.[32] Because the introduction of serine has less effect than other residues, moderate size and hydrophilicity, or hydroxyl group itself of Ser/Thr, are necessary for proper isomerization and incorporation of retinal in Opn5m. This may be the reason why Ser/Thr at the 188 position is highly conserved in Opn5m, Opn5m2, and Opn5L2 lineages, whose members are bistable opsins (Figure 2c).

Within Opn5m proteins, human and mouse Opn5 can bind to 11-*cis*-retinal but

cannot bind to all-*trans*-retinal. On the other hand, chicken, *Xenopus tropicalis*, and zebrafish Opn5m can bind both 11-*cis*- and all-*trans*- isomers of retinal.[22] The selectivity for retinal isomers can be reciprocally altered by a single amino acid mutation; the mouse Opn5 T168A mutant binds to all-*trans*-retinal, while *Xenopus tropicalis* Opn5m A168T mutant fails to bind to all-*trans*-retinal, which shows that the amino acid at position 168 determines the selectivity to 11-*cis* or all-*trans* retinal isomer in Opn5m.[22] Thr168 is conserved in eutherian Opn5m, but all Opn5m of marsupial opossums, monotreme platypuses, and non-mammalian vertebrates have Ala at the 168 position (Figure 2c). Therefore, the selective binding of Opn5m to 11-*cis*-retinal by Thr168 would have been acquired in the common ancestor of eutherians after the divergence of eutherians and marsupials.

Regarding the physiological function of Opn5m, more research has been done for this protein than for its relatives, revealing its involvement in various functions in mammals, birds, and amphibians. In mice, Opn5 is reported to play roles in photoentrainment of the local circadian rhythms of the retina, cornea, and skin by UVA reception and in managing the central clock.[33–36] Additionally, mouse Opn5 is implicated in controlling brown fat tissue thermogenesis via short-wavelength light reception in the hypothalamus, inhibiting myopic ocular axis extension in the retina, and regulating vitreous vascular development in the eye.[37–39] Studies in chicken tissues and cells suggest that inhibition of myopic ocular axis extension is correlated to the upregulation of *Egr1* through photoreception by Opn5m.[40,41] Furthermore, research using human epidermal melanocytes has demonstrated that OPN5 is involved in melanin production triggered by the reception of UV light.[42] In *Xenopus laevis*, Opn5m is suggested to control activity levels by sensing light in the deep brain regions of larvae.[46] In the chicken retina, Opn5m, localized in Müller glial cells, is proposed to sense light to trigger a calcium response.[47,48] In quails, Opn5m has been shown to directly perceive light in the hypothalamic paraventricular organ, inducing thyroid-stimulating hormone (TSH) β expression in the pituitary pars tuberalis in long-day condition and, consequently, regulating photoperiodic gonadal development.[43,44] In canaries, conversely, antisense knockdown of Opn5m in the mediobasal hypothalamus was found to increase the mRNA level of TSHβ after a single long-day exposure.[45] Although those studies showed that Opn5m is instrumental in regulating TSHβ in avian species, further studies are warranted to resolve discrepancies likely due to species-specific mechanisms, differences

in experimental conditions, or other unknown factors.

In all but a few lineages of ray-finned fish, an opsin known as Opn5m2 (or Opn9) can be found in their genomes. This opsin forms a sister group with Opn5m in a molecular phylogenetic tree (Figure 1d).[18,19,49] This molecule binds 11-*cis*-retinal to receive UV light and transforms into an active form sensitive to visible light, showing bistability.[18] Like Opn5m, Opn5m2 is an opsin that has the ability to activate a heterotrimeric G protein of the Gq type.[29] It has lost the ability to directly bind all-*trans*-retinal, like eutherian Opn5, but the amino acid at the 168 position is conserved as Ala, suggesting that Opn5m2 maintains this binding specificity to 11-*cis*-retinal through a different mechanism (Figure 2c). One characteristic of the Opn5m2 amino acid sequence is its extremely long extracellular loop 3, compared to other opsins in general, although its function is not well understood yet (Figure 2d).

Opn5L1 (Opn7)

Opn5L1 is an opsin conserved among vertebrates, except for mammals, and forms a separate cluster from Opn5m in the phylogenetic tree (Figure 1d). Analysis using chicken Opn5L1 has revealed that Opn5L1 exhibits markedly different molecular characteristics compared to Opn5m.[50] Firstly, Opn5L1 does not bind to 11-*cis*-retinal, but selectively binds to all-*trans*-retinal, showing an absorption maximum in the visible region (λmax=510 nm in chicken Opn5L1) (Figure 3a). In this state, Opn5L1 activates a Gi-type G protein, thereby effectively acting as a chemoreceptor with all-*trans*-retinal as the agonist though Opn5L1 conserves non-canonical Val134 and Cys135 at the “DRY” triad position well known in rhodopsin-like GPCR (Figure 2c). When all-*trans*-retinal bound to Opn5L1 absorbs light, it isomerizes to 11-*cis*-retinal, and then forms a covalent bond with the thiol side chain of Cys188 in the proximity of retinal (Figure 3b). In this state where retinal-thiol adduct is formed, the ability to activate G protein is largely reduced, and this is the inactive state of Opn5L1 (Figure 3a). Because the conjugate system of the retinal is disconnected by addition of thiol, the absorption in the visible and near-UV regions disappears, and the absorption near 270 nm, which is close to that of aromatic amino acids, slightly increases (Figure 3a). Normally in opsins, retinal can be extracted by cleaving the Schiff base bond with hydroxylamine, but retinal in this 270 nm-absorbing state of Opn5L1 cannot be extracted by the standard method because a covalent bond is present between cysteine and retinal. Finally, this state spontaneously reverts to the dark state,

which is bound to all-*trans*-retinal and has the ability to activate G protein, without subsequent light irradiation. Based on these properties, Opn5L1 has been termed as a “reverse and self-regenerating” photoreceptor.[50] In the retinal-thiol adduct, C_{11}-C_{12} is single-bonded, so it can freely rotate. After rotating from the 11-cis conformer to the all-*trans* conformer in the state absorbing 270nm, it is thought to recover to the dark state bound to all-*trans*-retinal with the dissociation of thioether bond (Figure 3b). The necessity of Cys188 was demonstrated by the C188T mutant, which stops the reaction at the 11-*cis*-retinal-bound state and does not form the 270nm-absorbing state. Furthermore, the presence of a covalent bond was proven by inserting two lysines near Cys188, treating the protein with hydroxylamine and trypsin digestion to isolate the peptide bound retinal oxime at Cys188, and direct observation with liquid chromatography mass spectrometry (Figure 3c). This formation of a covalently bonded chromophore-cysteine induced by light is also found within other photoreceptor proteins such as phytochromes and cyanobacteriochromes that use a bilin-based chromophore, and LOV proteins that use flavin as a chromophore.[51,52] Additionally, there is also evidence in organic chemistry that thiol can add reversibly to the conjugate system in an electron-deficient state due to the protonated Schiff base.[53] In other words, the formation of covalent bonds could be a common reaction in general for photoreceptor proteins and chromophores. In Opn5L1, it is thought that light isomerization to the 11-*cis* form promotes the adduct formation between retinal and thiol in addition to the electron-deficient property of the protonated retinal Schiff base, and the thermal structural change to the all-*trans* form drives the dissociation. Furthermore, recent research with resonance Raman spectroscopic measurements of Opn5L1 bound to isotope labeled retinal and DFT calculations have shown that the Schiff base is deprotonated in the retinal-thiol adduct of Opn5L1 (Figure 3b).[54] This is important information for understanding the reaction mechanism of Opn5L1 because the electronic state of the retinal Schiff base would determine the formation/dissociation of retinal-thiol adduct. The existence of Opn5L1 indicates that opsins can be functionally controlled not only by isomerization of retinal, but also by the addition reaction, a common chemical nature of unsaturated systems.

Although the physiological role of Opn5L1 has not yet been elucidated, tissue distributions were analyzed in zebrafish, *Paralichthys olivaceus*, crimson snapper, *Xenopus laevis* and chicken at the mRNA level.[19,50,55–57] Opn5L1 is found in the eye, brain, pineal gland, heart, muscle, skin, and testis of zebrafish [19], in

the eye, brain, heart, testis, and fin of *Paralichthys olivaceus* [57], in the skin, intestine, and heart of crimson snapper [55], in the eye and tail fin of *Xenopus laevis* [56], and in the mesopallium and paraventricular nucleus of the brain, and the retinal inner nuclear layer of chicken.[50] The widespread distribution in extra-retinal tissues leads us to speculate that Opn5L1 is involved in a variety of non-image-forming photoreception systems.

Opn5L2 (Opn8)

Opn5L2 is an opsin conserved among vertebrates except for mammals, and forms a separate cluster from Opn5m and Opn5L1 in the phylogenetic tree (Figure 1d). This opsin has been found to be a UV-sensitive bistable opsin, like Opn5m.[58] Opn5L2 also conserves Thr/Ser at the 188 position, which would be closely related to its bistable nature (Figure 2c). Unlike Opn5m, it activates Gi but does not have the ability to activate Gq.[29]

The distribution of Opn5L2 has been analyzed in chicken, *Xenopus laevis*, zebrafish, and *Paralichthys olivaceus*. It is found in the hypothalamus, retinal ganglion cells, and adrenal catecholamine-producing cells of chicken [58], in the retinal amacrine cells, ganglion cells, and tail fin of larval *Xenopus laevis* [56,59], in the eyes, pineal gland, heart, muscle, skin, and testis of zebrafish [19], and in the eyes, brain, testis, and fin of *Paralichthys olivaceus*. [57] The in vivo function of Opn5L2 is not yet well understood.

Opn6 and invertebrate Opn5-homologs

Opn6 was first discovered in zebrafish and is a group of opsins found in vertebrates, excluding eutherians and marsupials.[19,49]. In the molecular phylogenetic tree, it forms a cluster as a group closely related to Opn5. The members of Opn6 are known to be expressed in the eyes, pineal gland, and testes in zebrafish.[19] In *Xenopus laevis* larvae, expression of Opn6 orthologs are found diffusely in the retinal photoreceptor cells of early larvae and localized to the ciliary marginal zone in mature larvae [56,59]. Interestingly, Glu/Asp at position 181, putative counterion of protonated retinal Schiff base is not conserved, suggesting molecular characteristics different from other Opn5-related proteins, but these are not yet clarified, and its physiological function is also unknown (Figure 2c). [49]

In invertebrates as well, Opn5-like genes in terms of deduced amino acid sequence are widely seen in protostomes and deuterostomes, suggesting that

Opn5 is an opsin acquired in the common ancestor of bilateral animals.[21] These opsins are included in the Opn5 group of the molecular phylogenetic tree, but are not clustered with vertebrate Opn5m, Opn5L1, Opn5L2, and Opn6 (Figure 1d). Currently, there are no reports on molecular property analyses using recombinant proteins, but there is a report on recombinant slug Opn5 protein expressed in 293 cells detected by western blot.[60] Also, invertebrate Opn5 has been shown to be expressed in the rhabdomeric photoreceptor of the eyes and several neurons in the brain of slug *Limax valentianus*, and in some brain cells of marine annelid *Capitella teleta* larvae.[60–62] Future studies using genome editing and gene knockdown techniques are expected to elucidate functions in these *Opn5*-expressing cells.[62–64]

Possible modification of molecular properties and potential optogenetic use of Opn5 related proteins

Similar to the most frequently used channelrhodopsin-2 and microbial rhodopsins that function as light-driven ion transporters, animal opsins can be used as optogenetic tools to endow cells with light sensitivity and control their functions.[65,66] The use of animal opsins in optogenetics began with the use of well-understood vertebrate rhodopsins and their intracellular loop chimeras. [66–68] Recently, a wide variety of animal opsins including non-visual opsins and their mutants and chimeras have been used based on a detailed understanding of the molecular characteristics of animal opsins.[69–71] Here, we outline the general methods of manipulating molecular characteristics in GPCR-type animal opsins and methods related to the molecular nature of Opn5 homologs, and discuss their potential for use in optogenetics (Figure 4, 5).

Manipulation of the molecular property of animal opsins

In animal opsins, which are class A GPCRs, coupling specificity for heterotrimeric G proteins can be manipulated by substituting the intracellular loops, particularly the third intracellular loop.[72,73] There are also reports that the proximal region of the C-terminal is important for the coupling specificity.[74,75] In addition to activation of various G proteins by replacing the intracellular loops of vertebrate rhodopsins [66,76,77], there have been reports on loop replacements in cone opsins, melanopsin, Opn3, and peropsin [78–81], indicating that loop replacement is a typical way to manipulate molecular functions applicable to animal opsins (Figure 4a). In fact, by replacing the third

intracellular loop of chicken Opn5L1 with that of mouse β2 adrenergic receptor, the resultant chimera molecule preferentially activates Gs more than Gi and shows the reversed effect on the intracellular cAMP levels compared to chicken Opn5L1 WT (Figure 4a).

In optogenetic applications, the first requirement is that a sufficient amount of photoreceptor protein is functionally expressed on the target cell membrane. Compared to the robust expression of vertebrate rhodopsin, many animal opsins are not expressed sufficiently in cultured cells and are often unanalyzable. In the case of microbial rhodopsins, the insertion of ER export and membrane trafficking signal sequences is commonly used to improve trafficking to the cell membrane.[82,83] In the GPCRs such as olfactory receptors, the expression on the cell membrane is improved by inserting a sequence derived from the N-terminus of bovine rhodopsin.[84] Additionally, the expression level of functional recombinant proteins and the efficiency of purification are in some case improved by shortening the long C-terminus of animal opsins.[85,86] In fact, the amount of recombinant protein expression of *Xenopus tropicalis* Opn5m and human OPN5 is improved by shortening the C-terminus.[22] In the case of chicken Opn5L1, the amount of expression is greatly improved by replacing the N-terminus and C-terminus with those of *Xenopus tropicalis* Opn5m (Figure 4b).[50]

In retinal proteins in general, the absorption wavelength can be manipulated by substituting amino acids near the chromophore retinal. Proteins that absorb longer wavelengths are expected to be used as tools for activation even in deep tissue, avoiding absorption and scattering by the tissue component. In addition, by using several opsins with different absorption wavelength characteristics, it is possible to stimulate different cell populations or provide different inputs for each wavelength, enabling more complex optogenetic control.[87] The control of absorption wavelengths in 11-*cis*-retinal-bound forms, mainly in vertebrate and arthropod visual pigments, has been extensively studied, and it is somewhat known which amino acids govern the absorption spectral property [88–90]. Particularly in the group of short-wavelength-sensitive cone visual pigments, F86Y in rodents and C90S in birds can change the protonation state of the Schiff base from the deprotonated state to the protonated one, shifting absorption maxima from UV wavelength to violet wavelength by about 80 nm [91,92]. If amino acids controlling the protonation state of the Schiff base can be found in Opn5m and Opn5L2, it may be possible to control largely the absorption wavelength of the inactive 11-*cis*-retinal-bound form. On the other hand, control

of the absorption wavelength of the all-*trans*-retinal-bound form has been barely studied in animal opsins. However, in chicken Opn5L1, mutations in the amino acids Leu90, Ile122, and Ala292 in the proximity of retinal, change the absorption maximum of the all-*trans*-retinal-bound form by about -24 to +20 nm, indicating that the absorption wavelength can be somewhat controlled by substituting these amino acid residues (Figure 4c). [50].

Optogenetic tools including microbial rhodopsins and animal opsins are typically expressed as a fusion protein with a fluorescent protein at the C-terminus. This allows for the easy visualization of their expression and localization to the cell membrane (Figure 4d). In addition, certain methods have been reported that allow for the simultaneous visualization of cellular responses by fusing sensor proteins such as GCaMP (Figure 4d).[93] There are also examples of creating proteins that can be activated not only by light but also by the administration of a luminescent substrate by fusing luciferase to a microbial rhodopsin.[94] This could also potentially be applied to animal opsins (Figure 4d). Advanced methodologies often hinge on the understanding of opsins as photosensitive GPCRs. There are reports of achieving targeted light control for the Gβγ-GIRK system by fusing either RGS8 or Gαs to the C-terminus.[95,96]. Because inhibition of adenylyl cyclase by Gαi is slower than activation of GIRK by Gβγ, acceleration of GTP-GDP hydrolysis in Gαi by fusion of RGS8 to C-terminus of *Platynereis* c-opsin1 reduces Gα-dependent cAMP response while maintaining Gβγ-dependent GIRK response (Figure 4d).[95] In the case of jellyfish opsin, the fusion of Gαs prevents the Gα-dependent activation of adenylyl cyclase while allowing Gβγ to diffuse freely from the trimeric Gs.[96] As our understanding of the molecular characteristics of Opn5-related proteins progresses, it is expected that manipulation of molecular characteristics by fusion with such functional proteins will become more precise.

Utilization of molecular characteristics of Opn5 homologs

Introducing the reaction mechanism that forms a covalent bond between Cys188 and retinal as observed in Opn5L1 into other animal opsins would substantially alter the molecular characteristics of opsins (Figure 5).[50] The amino acid at the 188 position is located near the conserved Cys187 in class A GPCRs on ECL2 and in the proximity of retinal. In vertebrate rhodopsin research, the G188R/E mutation is recognized as a cause of retinitis pigmentosa.[97,98] In *Xenopus tropicalis* Opn5m WT, both 11-*cis*-retinal and all-*trans*-retinal can bind

and convert to each other by light. This bistable property is altered by mutation of Thr188. Specifically, the T188C mutant only binds to all-*trans*-retinal. Furthermore, after the trans-cis photoisomerization to 11-*cis*- and 13-*cis*-retinal, retinal returns to the all-*trans* form by thermal isomerization without additional light irradiation (Figure 5a).[32] This suggests that the formation of the adduct between the introduced cysteine residue and the retinal causes transient breakage of conjugated double bonds and rotation of C-C bonds like Opn5L1. Also, in experiments with vertebrate rhodopsin, although the introduction of Cys188 does not change the binding specificity of retinal, the active all-*trans*-retinal-bound state is converted to the inactive 11-*cis*-retinal-bound state, which is not seen in the wild type, by photoreaction in addition to thermal reaction (Figure 5b).[99] While these mutations did not introduce the molecular properties identical as Opn5L1, it could bring very dramatic changes in molecular characteristics of opsins. The results of the introduction of cysteine in these proteins suggest that retinal in opsins can be affected by chemical modification with nearby reactive residues in addition to the steric hindrance and electrostatic interaction.

At present, two independent groups have reported that Opn5m can be utilized as an optogenetic tool, given its function as a Gq-activating opsin.[27,28] Chicken Opn5m can drive the activity in astrocytes and neurons and has been reported to be more sensitive compared to opto-a1AR and mouse Opn4, optogenetic Gq manipulation tools that have been used so far.[27,66,100]. Furthermore, human OPN5 specifically activates Gq, not Gi, and has been shown to be capable of increasing contraction frequency with light when expressed in cardiomyocytes and inducing contraction with light when expressed in gastrointestinal smooth muscle.[28] The study also suggests that human OPN5 can be used for high throughput screening of TRPC6 inhibitors, focusing on the activation of TRPC6 by DAG produced in the Gq-PLCβ system, because use of OPN5 can reduce nonspecific effects compared to pharmacological activation of the Gq system mediated by acetylcholine receptor.[28] As a UV-sensitive Gq-coupled opsin, Opn5m is a valuable opsin due to its relatively good expression compared to insect UV visual opsins.[23,101] Additionally, preferential activation of G14 in Gq-type G proteins would be a notable characteristic of Opn5m for use as an optogenetics tool (Figure 2a).[29] Because the precise roles of Gq, G11, G14, and G15 remain not fully understood, employing Opn5m and its modifications to enhance selectivity for G14 could prove beneficial in shedding light on the cellular functional role of the G14 subtype. Opn5L1 is also considered to be an animal

opsin with further potential for optogenetics as an animal opsin that is exclusively inactivated by light like peropsin (Figure 3).[50,79] Actually, Opn5L1c (Opn7b) of zebrafish, an ortholog of chicken Opn5L1, has been used as a tool that continuously activates the Gi/o system and is inactivated upon light stimulation.[102] Under the dark condition, zebrafish Opn7b constantly activates the GIRK channel via Gβγ from the Gi/o system, thereby suppressing neural activity. Thus, light-dependent interruption of Opn5L1-Gβγ-GIRK leads to an increase in neural activity.[102] This example showed the potential of Opn5L1 as a constitutively active GPCR capable of photo-inactivation. Therefore, with the appropriate modification of intracellular loops to mimic those of GPCRs, Opn5L1 could be leveraged for exploring diseases and physiological states closely associated with constitutive activity of GPCRs.[103] Additionally, many of these Opn5 proteins can incorporate all-*trans*-retinal directly as their chromophore like microbial rhodopsins do, which would be advantageous compared to 11-*cis*-retinal selective opsins such as visual pigments and would be the basis of higher sensitivity than other opsins as described above. We will try to further explore optogenetic applications that utilize the unique molecular properties of these Opn5 homologs.

Research on Opn5 has led to groundbreaking findings that challenge established knowledge, such as the possibility that humans would actively utilize UV light and the potential use of addition reactions, not just isomerization of retinal, in controlling the function of opsins. However, much remains to be explored, including the physiological functions of Opn5 homologs other than Opn5m and the molecular characteristics of Opn6 and invertebrate Opn5, which are largely unrevealed. Additionally, the structural basis for the loss of all-*trans*-retinal binding capacity in Opn5m2 or Opn5m with Thr168, and the mechanism controlling and stabilizing the covalent binding of retinal and Cys188 in Opn5L1 remain unknown. The diverse molecular characteristics of these molecular groups hold the potential to serve as optogenetic tools that play roles different from those of microbial rhodopsins, and we believe that further progress in basic research and analysis will open up new applications.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used GPT-4 in order to translate the first draft of the manuscript, originally written in Japanese, into English. Following this, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Figures

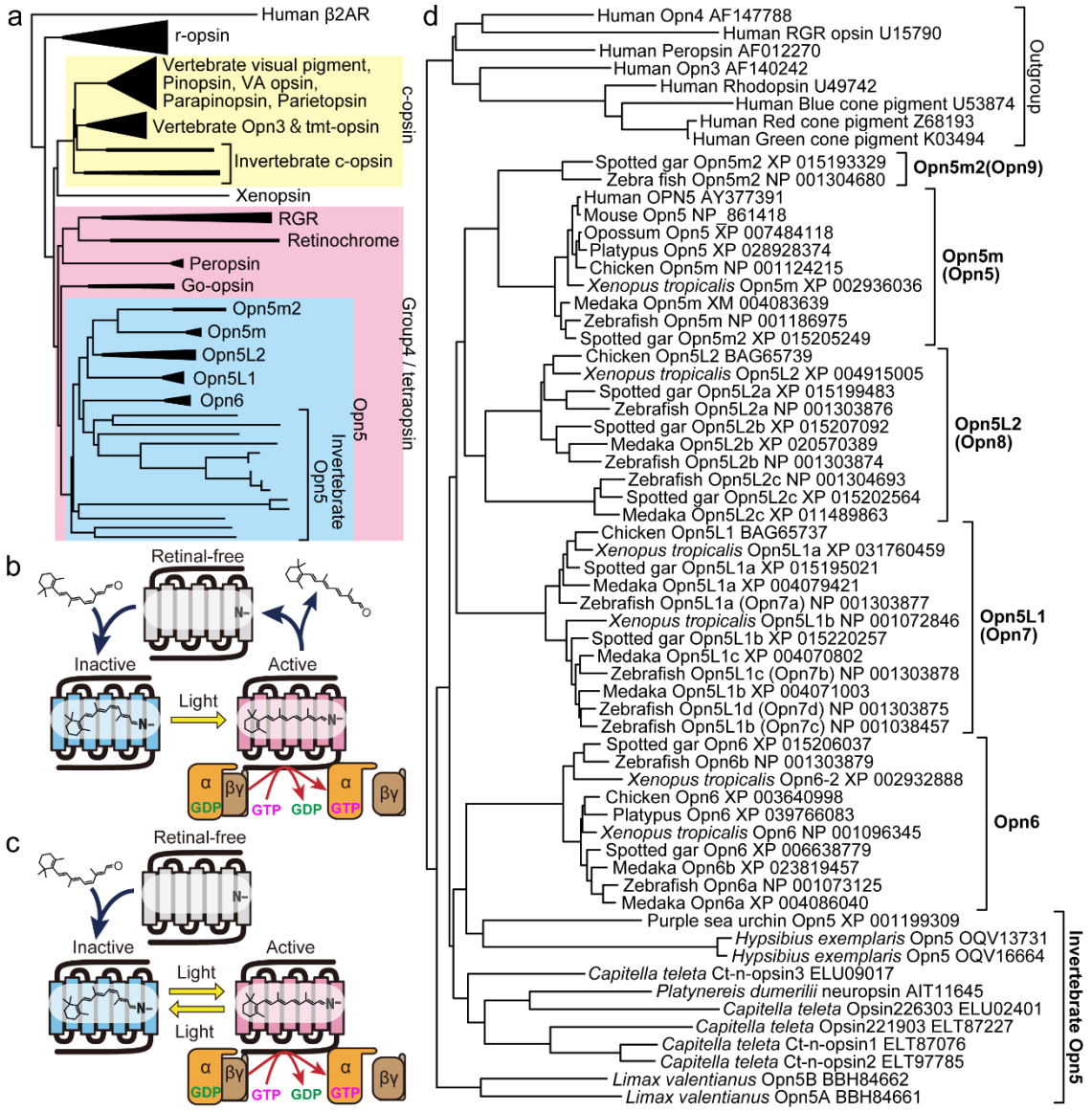

Figure 1: Phylogenetic tree of animal opsins

(a) Phylogenetic tree constructed based on the amino acid sequences of animal opsins. Amino acid sequences of opsins from human, mouse, opossum, platypus, chicken, *Xenopus tropicalis*, spotted gar, zebrafish, medaka, purple sea urchin, *Capitella teleta*, *Hypsibius exemplaris*, *Platynereis dumerilii*, and *Limax valentianus* were aligned by MAFFT.[104] Phylogenetic tree was inferred in MEGAX by the neighbor joining method.[105] Some opsin clades were compressed and shown as black triangles. The clades of c-opsin, group4/tetraopsin, and Opn5 are highlighted in yellow, magenta, and cyan, respectively. The sequence of human β2AR was included as an outgroup. (b, c)

Schematic drawings of molecular natures of bleaching opsin (b) and bistable opsin (c). Both facilitate GDP-to-GTP exchange of heterotrimeric G protein in their all-*trans*-retinal bound active state. (d) Phylogenetic tree of the opsins related to Opn5. Multiple alignment of the amino acid sequences and tree inference were performed with MAFFT and neighbor joining, respectively. Clades of vertebrate Opn5-related proteins are denoted.

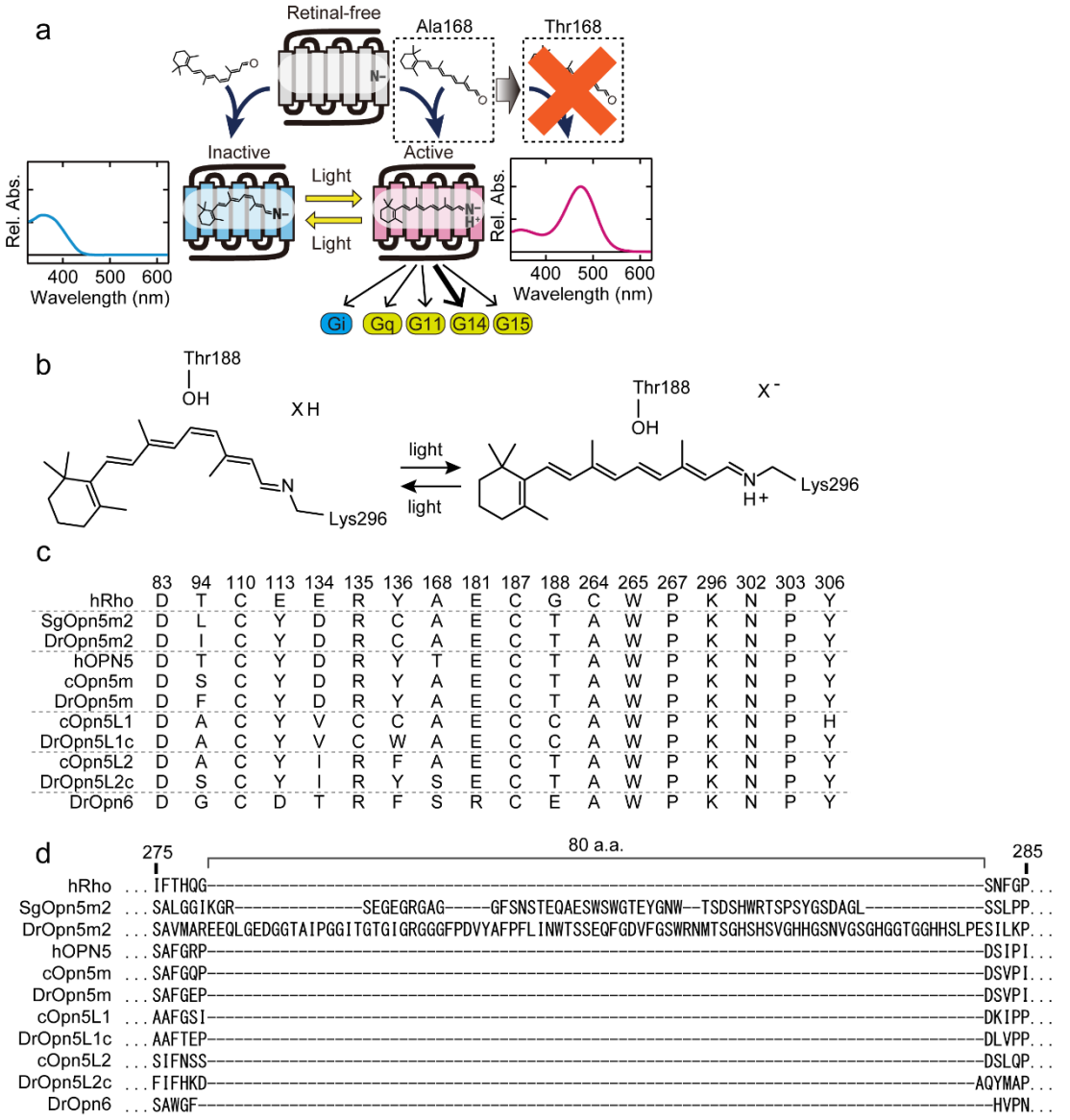

Figure 2: Molecular property of Opn5m and amino acid signature sequences in Opn5-related proteins

(a) Schematic drawings of the overall molecular nature of Opn5m. Amino acid residue numbering is based on the bovine/human rhodopsin system. Thr168 found in eutherian Opn5 hinders direct incorporation of all-*trans*-retinal. Ala168 found in non-eutherian Opn5m allows direct binding of all-*trans*-retinal. Opn5m activates Gq-type G proteins, preferentially G14, in addition to Gi. (b) Retinal isomerization in Opn5m. Retinal in Opn5m is photoconvertible between the 11-*cis* and all-*trans* forms. The Schiff base is deprotonated and protonated in 11-*cis*- and all-*trans*-retinal bound states, respectively. Thr188 has been shown to be required for proper tautomeric photoisomerization of these two configurations.

The putative proton acceptor and counterion of the Schiff base are denoted as X. (c) The functionally important amino acids in Opn5 homologs. Names of animal species are abbreviated as: h, human; Sg, spotted gar; Dr, zebrafish (*Danio rerio*), c, chicken; and added to the head of opsin names. Cysteines at 110 and 187 constitute the conserved disulfide bond in class A GPCRs. The residues at 134, 135, and 136, those at 264, 265, and 267, and those at 302, 303, and 306 are in the conserved DRY, CWxP, NPxxY motifs, respectively. The residue at 83 is related to color tuning and stability of animal opsins. The residues at 94, 113 and 181 work as counterions of the protonated Schiff base in some opsins.[106–108] The residue at 168 is a determinant for retinal binding selectivity of Opn5m.[22] The residue at 188 is functionally essential for the photoreaction of Opn5m and Opn5L1.[32,50] The lysine at 296 forms the covalent Schiff base bond with retinal. (d) Multiple alignment of Opn5 homologs in the extracellular loop 3 (ECL3) region. Positions 275 and 285 are the amino acid numbers of human rhodopsin (hRho). Only Opn5m2 proteins show extended ECL3.

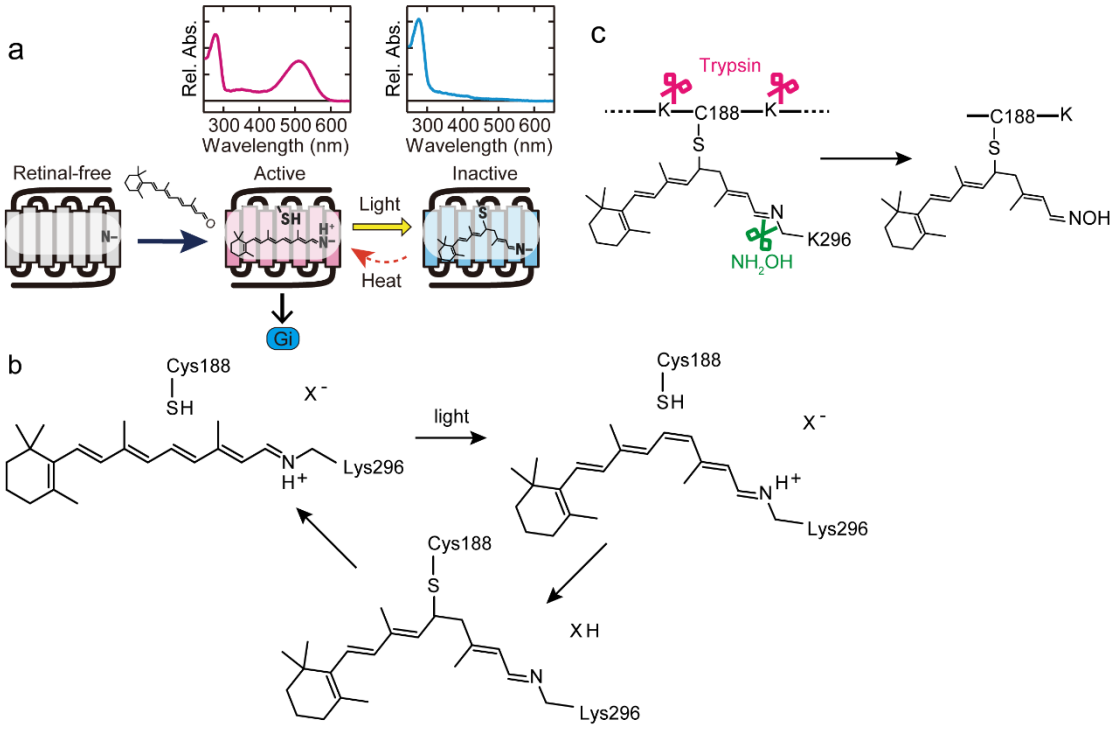

Figure 3: Molecular property of Opn5L1.

(a) Schematic drawings of overall molecular nature of Opn5L1. Opn5L1 activates Gi in the all-*trans*-retinal bound active state. Opn5L1 absorbs visible wavelength light in the active state. The absorption of the visible wavelength region nearly completely disappears with a slight increase of absorption at 270 nm in the inactive state. This state is thermally converted back to the all-*trans*-retinal bound state. (b) The light-driven reaction of retinal in Opn5L1. Opn5L1 binds all-*trans*-retinal in the dark state. Retinal is photo-isomerized into the 11-*cis* form. After that, nearby Cys188 attacks the 11th carbon in retinal to form a thioether bond. Accordingly, the Schiff base is deprotonated. Re-isomerization and thermal dissociation of the retinal-thiol adduct proceed to revert to all-*trans*-retinal. The putative proton acceptor and counterion of the Schiff base are denoted as X. (c) Schematic representation of the isolation of the retinal-thiol adduct. Trypsin cleaves the C-terminal side next to arginine and lysine. Hydroxylamine cleaves the Schiff base linkage to form oxime. With the introduction of two lysine residues near Cys188, the peptide covalently bound to retinaloxime through the thioether bond at Cys188 could be excised from Opn5L1 by trypsin and hydroxylamine treatment.

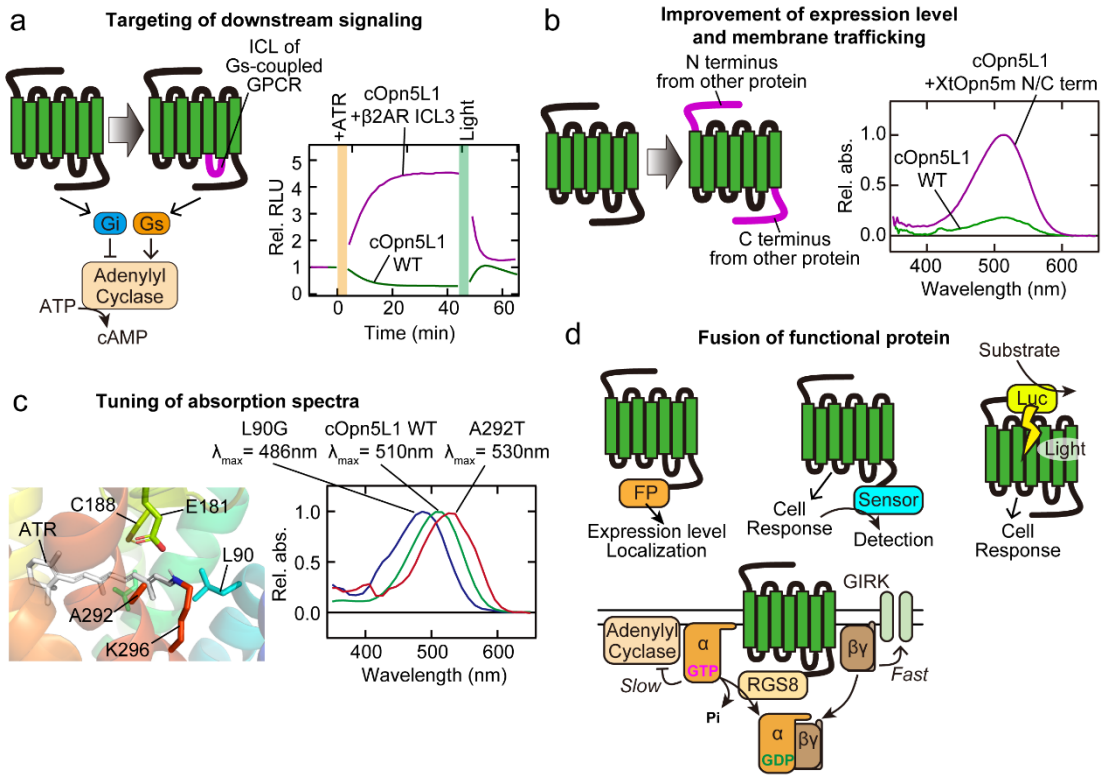

Figure 4: Possible molecular modification of animal opsins for optogenetic use.

(a) Manipulation of target downstream signaling by swapping intracellular loops. The example shows the conversion of Gi- to Gs-coupled activation by introduction of the intracellular loop (ICL) from Gs-coupled GPCR. The right graph shows the result of luminescent cAMP measurement with Glosensor 22F. In 293T cells transfected with WT cOpn5L1 (green curve) or cOpn5L1 introduced with mouse β2AR ICL3 (magenta curve), activation of receptor with addition of all-*trans*-retinal and inactivation with light irradiation showed opposite response. (b) Improvement of plasma membrane expression by replacement of N- and C-termini. Replacement or truncation of the N- and C- terminal region even with leaving the transmembrane region unchanged greatly improves functionally expression of animal opsins in some cases. The right graph shows the estimation of the functional expression level of Opn5L1 by light-dependent changes in absorption spectra of cell extracts. Replacement of the N- and C- termini of Opn5L1 with those of *Xenopus tropicalis* Opn5m increased the expression level of Opn5L1 about 8-fold without changing the photoreactive property. (c) Spectral shifting by mutation of residues near retinal. The left panel shows the amino acid residues nearby retinal in a homology model of cOpn5L1 constructed based on

the X-ray crystallographic structure of metarhodopsin II (pdb: 3pxo).[109] Amino acid residue numbering is based on the bovine/human rhodopsin numbering system. The right panel shows the absorption spectra of WT, L90G, and A292T cOpn5L1. Absorption maxima of those mutants showed spectral shift of -24 to +20 nm in the all-*trans*-retinal bound state of cOpn5L1. (d) Possible introduction of functional fusion proteins into animal opsins. Fusion of fluorescent protein (FP) enables direct estimation of expression and intracellular localization. Fusion of sensor protein enables simultaneous observation of cellular response by the optogenetic actuator. Fusion of luciferase (Luc) enables activation of opsin by addition of luciferase substrate, not by direct photo irradiation. Because inhibition of adenylyl cyclase by Gαi is slower compared to activation of GIRK channel by Gβγ, acceleration of the shutdown process of heterotrimeric Gi with facilitation of hydrolysis of GTP by RGS8 hinders the adenylyl pathway much more and induces GIRK-biased signaling.

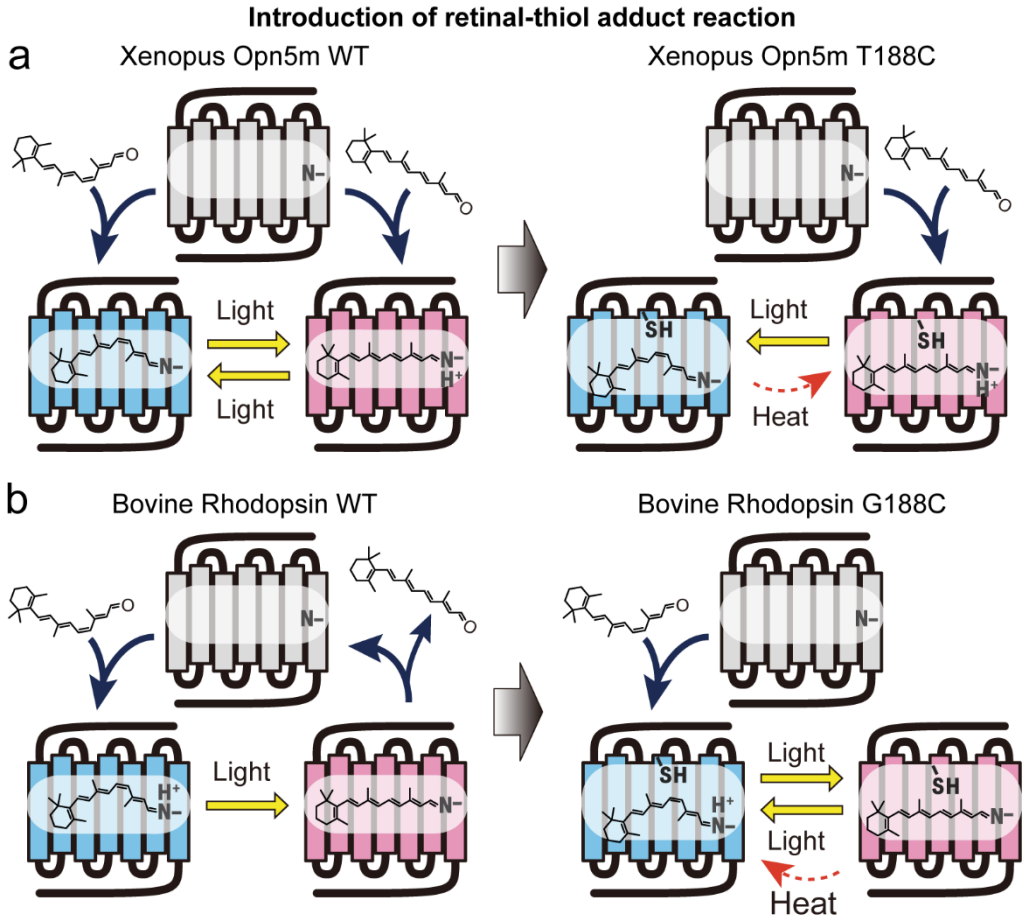

Figure 5: Introduction of Cys188 into animal opsins causes drastic changes in molecular properties.

(a) *Xenopus tropicalis* Opn5m (XtOpn5m) WT can directly incorporate both 11-*cis*-retinal and all-*trans*-retinal. Additionally, 11-*cis*-retinal bound and all-*trans*-retinal bound states are mutually photoconvertible. In contrast, XtOpn5m T188C stably binds exclusively to all-*trans*-retinal. Furthermore, 11-*cis*- and 13-*cis*-retinal generated by photoisomerization of retinal in XtOpn5m T188C is thermally converted back to all-*trans*-retinal without additional light irradiation. (b) Bovine rhodopsin (BvRh) WT selectively incorporates 11-*cis*-retinal. BvRh is activated by photoisomerization of retinal into all-*trans* configuration. Subsequently, BvRh releases all-*trans*-retinal, and newly incorporates 11-*cis*-retinal again. In the BvRh G188C mutant, binding selectivity for retinal isomers is unaffected. However, all-*trans*-retinal generated by photoisomerization of 11-*cis*-retinal could autonomously and thermally revert to 11-*cis*-retinal in BvRh G188C. Additionally, UV irradiation of the active state of BvRh G188C converts back to the dark state more efficiently than WT.