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Article

Novel Modular Rhodopsins from Green Algae Hold Great Potential for Cellular Optogenetic Modulation across the Biological Model Systems

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Abstract: Light-gated ion channel and ion pump rhodopsins are widely used as optogenetic tools and these can control the electrically excitable cells as: (1) they are a single-component system i.e., their sensor and effector functions are encoded by the 7-transmembrane domains and (2) they show fast kinetics with small dark-thermal recovery time. In cellular signaling, a signal receptor, modulator and effector components are involved for attaining synchronous multicomponent regulation. Optical modulation of this network requires either receptor to effector encoded in a single ORF or direct modulation of the effector domain through bypassing all upstream players. Recently discovered modular rhodopsins like rhodopsin guanine cyclase (RhoGC) and rhodopsin phosphodiesterase (RhoPDE) paves the way to establish proof of concept. Light sensor coupled modular system could be expressed in a precise cell type and which holds great potential in the advancement of optogenetics 2.0. It would enable manipulating entire relevant cell signaling system. Here, we had identified 50 novels modular rhodopsins with variant rhodopsins domain and its diverse cognate signaling cascades encoded in a single ORF, which are associated with specialized functions in the cells. These novel modular algal rhodopsins have been characterized functionality based on their sequence and structural homology with previously characterized rhodopsins. Presented novel modular rhodopsins with various effector domains hold potential to expand optogenetics tool kit to regulate various cellular signaling pathways across the diverse biological model systems.

Keywords: Enzymerhodopsin; Channelrhodopsins; Optogenetics; Two-component system; Cyclase; Phosphodiesterase

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Abbreviations: Cop-Chlamyopsin (rhodopsin from *Chlamydomonas reinhardtii*), Vop-Volvoxopsin (rhodopsin from *Volvox carteri*), GpRh 1-5 (rhodopsin from *Gonium pectorale*), AsRh1-4 (*Asterochloris sp.*), KnRh1-3 (*Klabsormidium nitens*), OtRh1-2 (*Ostreococcus tauri*), MpuRh1&2 (*Micromonas pusilla*), MspRh1&2 (*Micromonas species*), OlRh1-4 (*Ostreococcus lucimarinus*), CsRh1 (*Chlorella sorokiniana*), ApRh1 (*Auxenochlorella protothecoides*) , BgRh1&2 (*Bigelowiella natans*), GtRh1-10 (*Guillardia theta*), DsRh1 (*Dunaliella salina*), TsRh1 (*Tetraselmis subcordiformis*)

1. Introduction

Many photobehavioural responses are mediated by rhodopsin-based photoreceptor(s) that are distributed across almost all clades of life. Rhodopsins are seven transmembrane helical proteins which use retinal as a chromophore. Based on the isoforms of the retinal bound in the ground state,

rhodopsins are classified into two broad categories i.e., Type I or microbial type (MTR) and Type II or animal-type rhodopsins (ATR). MTRs are widely distributed across all kingdoms of life and perform diverse physiological functions, such as the light-activated ion pumps- Bacteriorhodopsin (BR) [1] and Halorhodopsin (HR) [2], light-gated channels- Channelrhodopsins (ChR1 & ChR2) [3,4], and sensory photoreceptors (SRI & II) [5]. Light-gated ion pumps and channels cause alterations in the membrane potential in a light dependent manner whereas sensory rhodopsins mediate downstream signaling. SR I and II in halobacteria communicate with the flagellar motor via transducer proteins HtrI and HtrII respectively [5].

ATR or type II rhodopsins are broadly classified as vertebrate and invertebrate rhodopsins on the basis of variation in their amino acid sequences [6]. The ATRs (both vertebrate and invertebrate) mediate the downstream signaling cascade through the G-protein coupled receptor (GPCR) proteins that involves multiple steps and protein complexes. Both the ATRs and SRs of MTRs are multi-component systems which require a series of protein complexes to mediate the light-activated signalling. This poses the limitation to use them as an optogenetic tool for regulating intracellular signaling process. The success of MTRs as an optogenetic tool is mainly attributed to its property that both the light sensing and the ion channel activity of the Channelrhodopsins (ChR) are encoded in a single protein. Recent advancements in the genome database has led to the discovery of many new MTRs which are directly coupled to effector domains e.g. two-component system and cyclase in enzyme-rhodopsins [7,8]. This structural diversity imparts great precision, fast kinetics and low off-target effects that provides an edge to the MTR to target and regulate specific cellular processes simply by illumination. cAMP and cGMP, the key modulators of cell signaling, are the secondary messengers that regulate many cellular, metabolic and developmental processes. However, it is difficult to target/modulate cGMP and cAMP levels precisely in specific cell types with spatial-temporal resolution using the animal-type rhodopsin signaling cascade because of the involvement of many player(s) in the cascade. In addition, pharmacological targeting has the limitation of specificity and temporal issues at the cellular level.

Enzyme-rhodopsins (Rhodopsin phosphodiesterase; RhoPDE and Rhodopsin cyclase; RhoGC) have emerged as promising optogenetic tools for the precise and non-invasive spatiotemporal control of cyclic nucleotide signaling pathways. The heterologous expression of RhoPDE [9,10] from *Salpingoeca rosetta* in *Xenopus* oocyte and HEK293 cell lines demonstrated the light-activated cGMP and cAMP-phosphodiesterase activity [11]. Similarly, RhoGC [12,13] isolated from fungi *Blastocladiella emersonii* and *Catenaria anguillulae* when expressed in various mammalian cell lines, could generate substantial cGMP, and were used as an optogenetic tool [14,15]. Since then significant interest has developed towards the identification, characterization and testing of novel modular rhodopsins [7,16,17] as optogenetic tool candidates for tweaking the cell signaling process. The identified modular rhodopsins coupled with other domains in a single ORF have shown the potential to overcome the limitation of SRs to be used as an optogenetic tool. Characterizing the physiological role of the existing and newly identified multidomain rhodopsins is tempting but limited because of their large transcript size, poor heterologous expression of transmembrane domain and lack of the established functional assays for these modular rhodopsins. Recently, we have identified 24 new modular rhodopsins from different algae [7]. In the present study, we have identified many new modular rhodopsins and ChRs fused with new domains that were previously unknown and analysed their evolutionary pattern and sequence homology as well as the structural and functional potential of these domains coupled to rhodopsin (based on available experimental evidences). We have also investigated the diversity of multidomain rhodopsins and the recruitment of signaling component in a single ORF in relation to its prokaryotic counterpart. This extensive analysis of MTRs defines a future roadmap towards the involvement of modular rhodopsin-based photoreceptors in the photophysiological response of the relevant organism. Evolutionary pattern analysis of the MTRs suggests the evolution of multi-domain rhodopsins in the microalgal system after evolution of the ChRs with extended C-terminus of unknown function by lateral gene transfer. Moreover, these novel modular rhodopsins with different effector domains hold potential to

expand optogenetics tool kit 2.0 to regulate various cellular signaling pathways across the manifold biological model systems.

2. Materials and Methods

2.1. Identification of rhodopsin domain, homology and structural analysis.

Extensive genome database search for MTRs and modular rhodopsins were performed on JGI genome database, metagenome database and NCBI portal using BR and Chlamydomonas rhodopsin as template. The rhodopsin identity, sequence accession number, homology, conserved domains are summarized in Table S1. Multiple sequence alignment was performed using Clustal_X program [18] and BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). All colour editing was done by using the BioEdit program. The rhodopsin domains of new MTRs were identified by sequence alignment with canonical rhodopsins and analysis with conserved domain architecture retrieval tool (CDART) [19] and conserved domain database [20] program. The rhodopsin with conserved seven transmembrane helices and retinal binding motif in the seventh helix was considered for further analysis. The number indicating the position of amino acid is referred with respect to BR unless mentioned in the text.

2.2. Evolutionary analysis of rhodopsin domains of modular proteins

Molecular evolutionary analysis of typical MTR and rhodopsin domains of modular proteins were performed computationally with protein sequences. Multiple sequence alignment of rhodopsin domain was done on Clustal X 2.0 [18]. Phylogenetic analysis was performed by Neighbour – joining (NJ) method using MEGA X [21] with a thousand bootstrap replicates. The same was also verified by maximum likelihood ML method on MEGA X and topology was viewed by MEGA X as well as tree view and NJ plot [22].

2.3. Protein-protein interaction analysis of novel domains from modular algal rhodopsins

The interactomes for domains associated with ChRs, i.e. FimV, MED15 and UL36, were constructed. The interacting partners for each of the effector domains were predicted using the String version 11 [23] and the output was further used to generate the network by employing Cytoscape 3.7.2 [24].

3. Results and Discussion

3.1. Microbial rhodopsins with modular domain organization

Mining the genome database of the organisms from diverse taxa and strata has revealed the presence of MTRs from archaea to algae inhabiting in diverse habitats from freshwater to terrestrial environments. The phototactic green alga C. reinhardtii has been extensively studied for learning various aspects of cell biology from photobehavioural responses (especially ChR-mediated) to photosynthesis, cilia biology, intraflagellar transport to vesicle, and membrane-bound trafficking and dynamics [25,26]. The early modular rhodopsins were identified in this green alga and since then very few have been reported in other organisms. Owing to its cellular optogenetic potential, a thorough and extensive genome database search was performed to identify novel rhodopsin(s) with modular nature, better kinetics and fast recovery time.

Here, we have identified new microbial modular ChRs (Figure 1A and table 1A&B) and SRs (Figure 1B-D and table 2A&B) across different taxa and analysed their critical features that segregate MTRs from other seven transmembrane protein families. Based on the modular domain coupled to the rhodopsin, we evaluated the possible function of these proteins in the respective organism and their potential optogenetic application in cell and developmental biology of the different model systems.

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3.2. Modular Channelrhodopsins and their optogenetic potential

Our targeted search for the modular ChR yielded three modular ChRs as shown in Figure 1A. These are (i) KnRh3 from Klebsormidium nitens (terrestrial alga) which is coupled with the peptidoglycan binding protein, FimV, (ii) the blue-shifted ChR, TsRh1 from Tetraselmis subcordiformis, for which the rhodopsin domain has been characterized [TsRh1 is coupled with the mediator subunit, MED15 (Mediator of RNA polymerase II subunit 15)] [27], however its modular nature has not been discussed and (iii) GpRh1 from Gonium pectorale, which is coupled with UL36 (large tegument protein). The optogenetic potential of these modular domains (FimV, MED15, and UL36) is summarized in table 1A. The Rhodopsin domains of KnRh3, TsRh1 and GpRh1 were aligned with well characterized ChRs taken as the reference for sequence analysis (Figure 2). The conserved residues essential for photocycle are marked in Figure 2, and the same have been analysed for four main functionalities namely: (1) retinal-binding lysine, (2) counter ion/proton acceptor of RSB, (3) proton-release complex and (4) DC-gate present in helix 3 and 4. Based on these amino acid residues, we evaluated the rhodopsin domain and summarized the details in table 1B and 2B for modular ChRs and SRs respectively.

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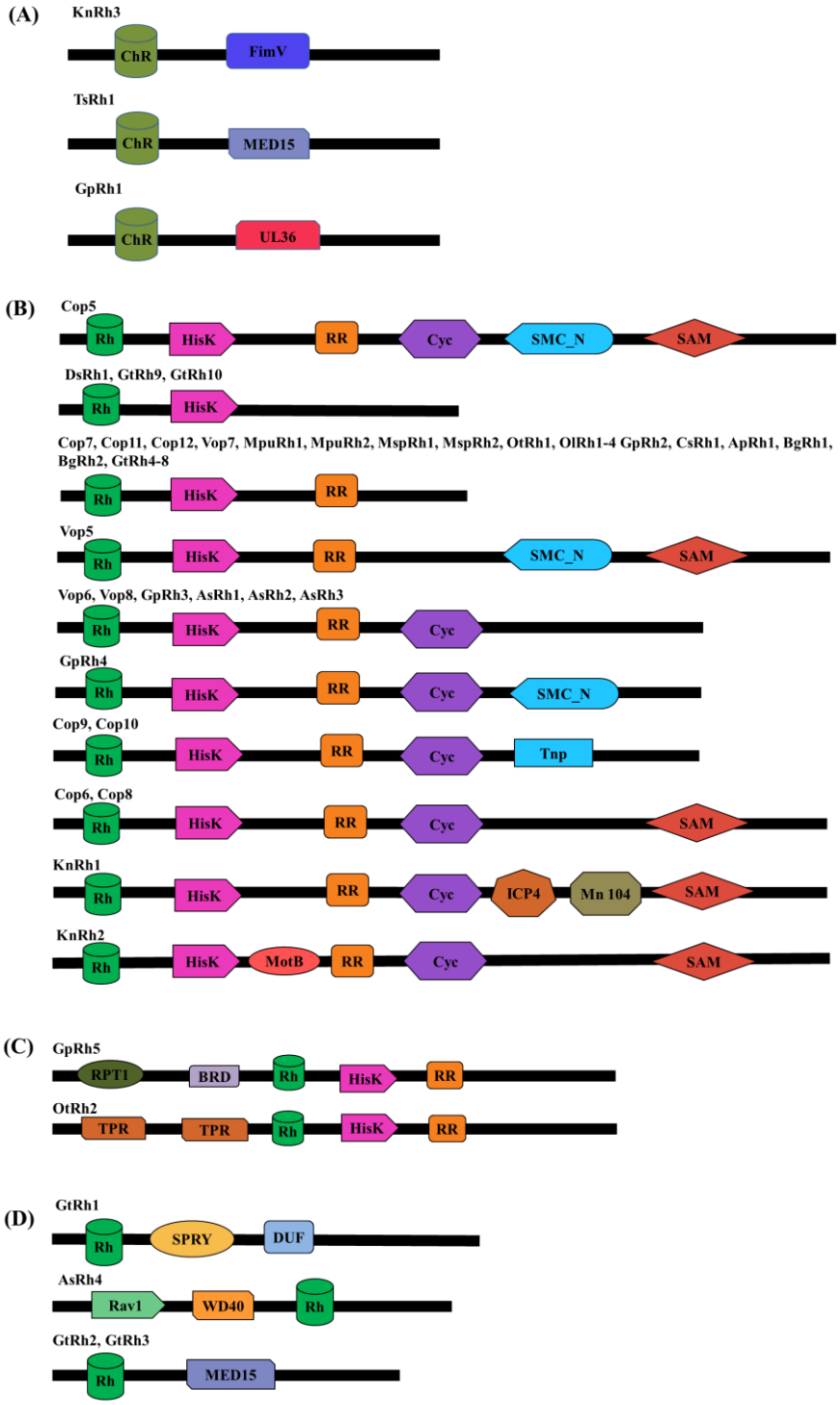

Figure 1. Schematic representation of domains present in modular microbial type rhodopsins: The schematic representation shows rhodopsin with modular domain(s), the black line represents full-length protein and domains are depicted by geometrical structures (Figure not to scale). (A) Domain organization of modular ChRs. ChR coupled with FimV (peptidoglycan binding protein), MED15 (mediator of RNA polymerase transcription factor subunit 15) and UL36 (large tegument protein) were found in three different algae. (B) Rhodopsin coupled HisK and RR forms the largest group of modular domain and other having additional unique effector domain like cyclase (Cyc), sterile alpha subunit (SAM), structural maintenance of chromosome_N terminus (SMC_N), transposase (Tnp2), major viral transcription factor ICP4 homolog (ICP4), 104kDa microneme/rhoptry (Mn 104)

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and bacterial flagellar motor protein (MotB). (C) Modular rhodopsin with rhodopsin preceded by unique domain at N-terminus; ATP-dependent 26S proteasome (RPT1) and bromodomain (BRD) in GpRh5 and tricopeptide (TPR) in OtRh2. (D) Modular rhodopsin lacking HisK and RR; GtRh1 possess SPRY (regulate innate and adaptive immune response) and DUF (domain of unknown function), GtRh2 and 3 possess MED15. AsRh1 possess RAV1 (regulator of V-ATPase of vacuolar membrane protein 1) and WD40 at N-terminus.

All the three ChRs have the conserved seven transmembrane domains and the lysine motif at seventh helix that forms a covalent linkage with retinal (Figure 2 and table 1B). Asp253 (in ChR2) accepts proton from retinal Schiff base (RSB) during deprotonation and Asp156 (in ChR2) donates proton to the RSB during re-protonation. Both these sites are conserved in modular ChRs (Figure 2 and table 1B). Arg82 (in BR) stabilizes the negatively charged proton acceptor Asp85 (in BR) and is hydrogen bonded to Tyr83 via water 405 in M state and together play primary role in deprotonation. This site is highly conserved among MTRs including modular ChRs (Figure 2 and table 1B). Asp156 (in ChR2) is hydrogen bonded to Cys128 to form a DC gate that acts as a switch for the movement of ions [28]. Mutation of Cys128 to Thr (C128A) delays the closure of the ion channel gate and therefore remains in the conducting state for a longer period [29]. This mutation has enhanced the property of ChR2 to be used as an optogenetic tool. Cys128 is also conserved in newly identified modular ChRs (Figure 2 and table 1B).

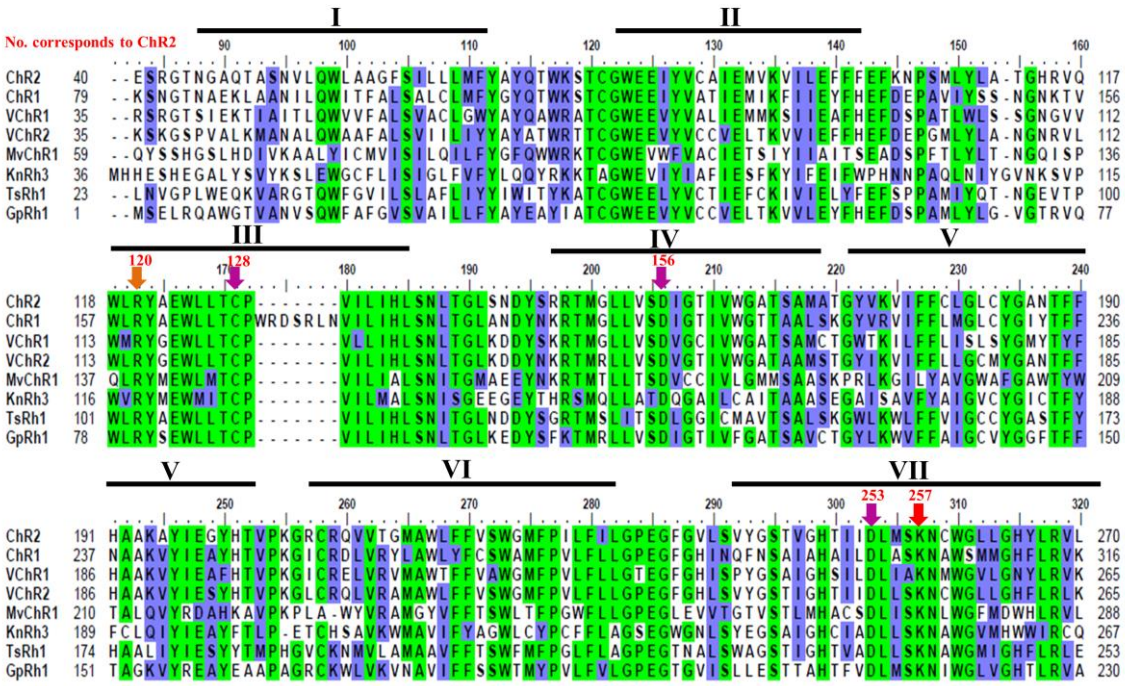

Figure 2. Comparison of novel channelrhodopsin and mapping of the important amino acid residues: Modular ChRs (KnRh3, TsRh1 and GpRh1) were aligned with other ChRs (ChR1 & ChR2 from C. reinhardtii, VChR1 & VChR2 from V. carteri, MvChR1 from M. Viride. Helix 2-7 are depicted by black bar and marked in roman numbers. Retinal binding lysine is marked by red arrow; proton acceptor/donor and cysteine hydrogen bonded to proton donor (DC pair) are marked by pink arrow; and arginine important for primary translocation of proton is marked by orange arrow.

The conservation of important amino acids reflects their functionality and could be engineered to enhance their properties. Thus, newly identified modular ChRs hold potential to be used as optogenetic tools for controlling new biological pathways.

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Table 1. A. Modular domains coupled with Channelrhodopsins.

Modular Domain	Channelrhodopsin	Functional role and optogenetic potential
FimV (Peptidoglycan binding protein)	KnRh3	In bacteria: Controls bacterial pathogenesis by indirectly activating adenylyl cyclase and hence cAMP level.
MED15 (Subunit of mediator complex)	TsRh1	In mammals: Regulates cholesterol and lipid homeostasis. Promotes cancerous growth and used as a biomarker for malignancies.
UL36 (Large tegument protein)	GpRh1	Regulates viral entry to the cells.

Apart from the three modular ChRs, the genome database search also led to the identification of many SRs from diverse alga. A diverse set of domains fused with SRs were identified in a single ORF, which suggests multiple light mediated cellular signaling pathways in these algae. Most of the identified rhodopsins are coupled with two component histidine kinase (HisK) and response regulator (RR) system. The first modular rhodopsin identified and characterized was Chlamyopsin-5 (Cop-5/HKR1) of *C. reinhardtii* [30].

Table 1. B. Conserved amino acid residues of modular channelrhodopsins.

Function of the residue	Proton acceptor	Proton donor	DC gate	Proton-release complex	Retinal attachment
No. in ChR2	**253**	**156**	**128**	**120**	**257**
ChR2	D_{253}	D_{156}	C_{128}	R_{120}	K_{257}
KnRh3	D_{250}	D_{154}	C_{126}	R_{118}	K_{254}
TsRh1	D_{236}	D_{139}	C_{111}	R_{103}	K_{240}
GpRh1	D_{213}	D_{116}	C_{88}	R_{80}	K_{217}

3.3. Modular sensory rhodopsins and their optogenetic potential

In the Cop-5 modular organization, rhodopsin was coupled with HisK and RR domain along with Cyc, SMC_N and SAM (Fig 1B). Experimental evidence suggests that Cop-5 localizes in the eyespot of C. reinhardtii, with dichromic absorbance maxima in UV range [30], however, their native functional role is still not clear. Followed by Cop-5, many other rhodopsins with similar domain architecture were identified in C. reinhardtii and other algae as well. Cop 6-8 expression were further confirmed in C.reinhardtii and Cop-8 was localized in cilia and eyespot in a light dependent manner [7]. Similar homologs of the modular rhodopsin were identified in another closely related colonial green algae Volvox carteri and other algae (Figure 1B). Along with HisK and RR, other domains like Cyc, SMC_N, Tnp, SAM were also coupled in some modular rhodopsins as shown in Figure 1B. Interestingly, GpRh5 and OtRh2 possess domains (RPT1 and BRD in GpRh5; TPR in OtRh2) at the N- terminus of rhodopsin and the two-component system at the C-terminus of rhodopsin (Figure 1C, table 2A). Another group of modular rhodopsin lacks two-component system but are coupled to a unique domain like SPRY, DUF, and MED15 (Figure 1D), respectively. AsRh4 is unique among this group in possessing Rav1 and WD40 at the N-terminus of rhodopsin (Figure 1D). We have summarized the modular SRs according to their domain architecture, cellular function and possible optogenetic applications in table 2A.

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Table 2. A: Modular domains coupled with sensory rhodopsins.

Modular Domain	Modular Rhodopsins	Cellular role and optogenetic potential
HisK	DsRh1, GtRh4-10, Cop5-12, Vop5-8, AsRh1-3, GpRh2-5, KnRh1 & 2, OtRh1&2, OlRh1-4, MpuRh1&2, Msp1&2, CsRh1, ApRh1, BgRh1&2	Part of two-component signaling; regulates gene expression
HisK-RR (Histidine kinase-response regulator) Two-component signaling system	GtRh4-8, Cop5-12, Vop5-8, AsRh1-3, GpRh2-5, KnRh1 & 2, OtRh1&2, OlRh1-4, MpuRh1&2, Msp1&2, CsRh1, ApRh1, BgRh1&2	Regulates gene expression and various other cell processes via output domain like helix-turn-helix (HTH), RNA, enzyme or ligand-binding domain.
Cyc (Cyclase)	Cop5, 6, 8, 9 &10, Vop6&8, AsRh1-3, GpRh3&4, KnRh1 & 2	Regulates the level of secondary messengers: cAMP and cGMP.
SMC_N (Structural Maintenance of Chromosome _N terminal)	Cop5, Vop5, GpRh4	Stabilizes the chromosome, helps in its proper segregation during cell division and DNA repair.
Tnp (Transposase)	Cop9 & 10	Recognizes the transposable elements in DNA and catalyses their movement to another DNA.
SAM (Sterile alpha motif)	Cop5-8, Vop5, KnRh1 & 2	Mediate protein-protein interactions, RNA and lipid binding; regulates transcription factor
ICP4 (Infected-cell polypeptide 4)	KnRh1	Major transcription factor of herpes simplex virus type1 (HSV-1)
Mn104 (Microneme/rhoptry)	KnRh1	Helps in invading host cell by apicomplexan parasites; N-terminal region proposed to serve as signal peptide for ER
MotB (Flagellar motor protein)	KnRh2	MotB acts as a stator in proton pump.
RPT1 (Regulatory Particle Triple ATPase)	GpRh5	Forms a part of 26S proteasomal complex
BRD (Bromodomain)	GpRh5	Modulate gene expression by associating with acetylated lysine on histone
TPR (Tetracopeptide repeat)	OtRh2	Regulates virulence in bacteria; translocation of receptors to their respective organelles in different systems
SPRY [Spore lysis A (Spl A) in Dictyostelium discoideum and mammalian Ryanodine receptor (RYR)]	GtRh1	Substrate binding for ubiquitination in ubiquitin ligase family proteins; involved in various immune response

DUF	GtRh1	Mediate protein-protein interaction
Rav1 (Regulator of V-ATPase of vacuole membrane protein 1)	AsRh4	Regulates the assembly of V-ATPase (ATP powered H+ pump in vacuole forming organelles)
WD40	AsRh4	Mediate protein-protein interaction

3.4. Light-gated ion pump and photo-sensory function prediction based on conserved residues of rhodopsins

Amino acids in the proximity of retinal are the key determinants in the activation and function of rhodopsin. The crystal structure of BR suggests that Asp85 is the proton acceptor from RSB during deprotonation. Thr89 is hydrogen bonded to Asp85 (Figure 3 and Table 2B). Asp212 also remains protonated and thus plays a role during the primary proton transfer event. Asp96 donates proton to the RSB during reprotonation. Glu194 and 204 are the terminal amino acids responsible for the outward release of into extracellular side. These positions were analysed in the modular rhodopsins to assign their functionality. Out of 47 modular rhodopsins at position 85, 14 had conserved Asp/Glu while 17 had Gln (Figure 3 and Table 2B). Position 89 is well conserved with 43 out of 47 modular rhodopsins possessing Ser/Thr at this position (Figure 3 and Table 2B). Asp96 is only conserved in AsRh4 (Table 2B). Asp212 is well conserved among modular rhodopsin except 6 of them which possess Asn at this position (Figure 3 and Table 2B). Only 4 modular rhodopsins possess Asp at 194th position while 25 modular rhodopsins have Glu at 204th position (Figure 3 and Table 2B). Since the retinal attachment lysine is conserved among all modular rhodopsin, these rhodopsins seem to be functional (Figure 3 and Table 2B). AsRh4 is the only modular rhodopsin with an amino acid conserved for proton pump. Other modular rhodopsins seem to form a new group with different mechanism for activation and relay of signals. Despite lacking the proton acceptor Asp85, Cop5 was found to be active in UV A and blue light (Figure 3 and Table 2B). Cop6/Vop6 was suggested to be a light inhibited guanylate cyclase upon supplementation of ATP when expressed in Xenopus oocyte [31] Although it lacks Asp85, Asp96 and Asp212 (Figure 3 and Table 2B). Signal relay in Cop6/Vop6 proceeds through HisK and RR. OtRh1/Ot-HKR is a green absorbing modular rhodopsin controlling the circadian clock of O. tauri. The photophysical properties of OtRh1/Ot-HKR are affected by salt concentration indicating this rhodopsin might provide input for adaptation in salt environment [32]. These examples suggest that the important amino acids are substituted but these rhodopsins are functional. Unique domains coupled with rhodopsin might regulate specific function in cell/organism and hold potential to be used as optogenetic tool and therefore should be explored in detail.

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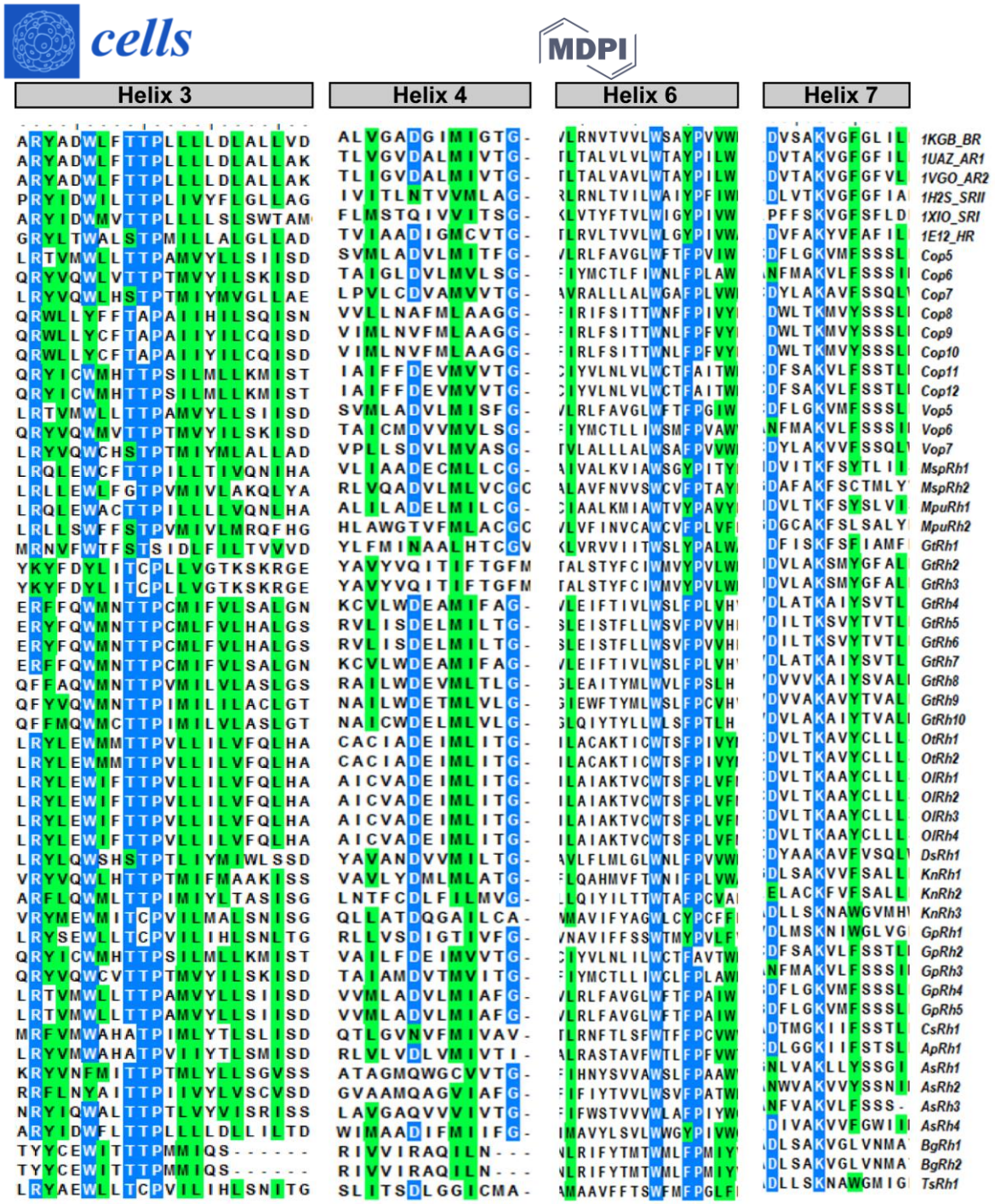

Figure 3. Comparison of light sensor domain of the modular rhodopsin among different algae: Most conserved third to seventh helices of rhodopsin are depicted here. Numbering was adapted according to the protein of BR. 1kGB: Bacteriorhodopsin, 1UAZ: Archaerhodopsin-1, 1VGO: Archaerhodopsin-2, 1El2: Halorhodopsin, 1H2S: Sensory Rhodopsin II, 1XIO: Anabaena sensory rhodopsin.

Table 2. B. Conserved amino acid residues of sensory rhodopsins.

Function of the residue	Ion pumping				Proton-release to outside		Retinal attachment
No. in BR	**85**	**89**	**96**	**212**	**194**	**204**	**216**
BR	D	T	D	D	E	E	K
HR	T_{90}	S_{94}	A_{101}	D_{217}	E_{198}	T_{209}	K_{221}
KR2 (Na+)	N_{112}	D_{116}	Q_{123}	D_{251}	L_{227}	R_{243}	K_{255}

 doi:10.20944/preprints202009.0015.v1

SR1	D_{75}	T_{79}	S_{86}	P_{206}	S_{188}	D_{198}	K_{210}
SR2	D_{75}	T_{79}	F_{86}	D_{201}	L_{188}	D_{193}	K_{205}
RhGC	E_{254}	T_{258}	L_{265}	D_{380}	S_{364}	A_{372}	K_{384}
RhPDE	E_{164}	T_{168}	W_{175}	D_{292}	Q_{276}	G_{284}	K_{296}
AsRh4	D_{2593}	T_{2597}	D_{2604}	D_{2718}	G_{2701}	E_{2710}	K_{2722}
GtRh1	F_{152}	S_{156}	I_{163}	D_{297}	G_{280}	K_{289}	K_{301}
GtRh2/3	D_{95}	T_{99}	T_{106}	D_{248}	T_{232}	E_{240}	K_{252}
Cop5	M_{113}	T_{117}	L_{124}	D_{239}	M_{223}	E_{231}	K_{243}
Cop6	Q_{170}	T_{174}	I_{181}	N_{294}	V_{279}	-	K_{298}
Cop7	Q_{161}	S_{165}	M_{172}	D_{287}	W_{271}	E_{279}	K_{291}
Cop8	L_{67}	T_{71}	I_{78}	D_{194}	D_{178}	S_{186}	K_{198}
Cop9-10	L_{141}	T_{145}	I_{152}	D_{268}	D_{252}	S_{260}	K_{272}
Cop11	C_{95}	T_{99}	L_{106}	D_{279}	L_{263}	E_{271}	K_{283}
Cop12	C_{95}	T_{99}	L_{106}	D_{221}	L_{205}	E213	K_{225}
Vop5	M_{157}	T_{161}	L_{168}	D_{283}	L_{267}	E_{275}	K_{287}
Vop6	Q_{153}	T_{157}	I_{164}	N_{278}	L_{263}	-	K_{282}
Vop7	Q_{147}	S_{151}	M_{158}	D_{272}	W_{256}	E_{264}	K_{276}
Vop8	-	-	-	D_{72}	D_{56}	S_{64}	K_{76}
MspRh1	E_{140}	T_{144}	I_{151}	D_{284}	F_{268}	Q_{276}	K_{288}
MspRh2	E_{142}	G_{146}	L_{153}	D_{299}	S_{283}	L_{291}	K_{303}
MpuRh1	E_{140}	T_{144}	I_{151}	D_{300}	F_{284}	Q_{292}	K_{304}
MpuRh2	S_{151}	S_{155}	L_{162}	D_{328}	A_{312}	A_{320}	K_{332}
GtRh4	Q_{92}	T_{96}	V_{103}	D_{225}	S_{209}	Y_{217}	K_{229}
GtRh5	Q_{222}	T_{226}	V_{233}	D_{355}	G_{339}	Y_{347}	K_{359}
GtRh6	Q_{234}	T_{238}	V_{245}	D_{367}	G_{351}	Y_{359}	K_{371}
GtRh7	Q_{116}	T_{120}	V_{127}	D_{249}	S_{233}	Y_{241}	K_{253}
GtRh8	Q_{226}	T_{230}	V_{237}	D_{359}	L_{343}	Y_{351}	K_{363}
GtRh9	Q_{229}	T_{233}	I_{240}	D_{362}	L_{346}	Y_{354}	K_{366}
GtRh10	Q_{192}	T_{196}	V_{203}	D_{325}	L_{309}	F_{317}	K_{329}
BgRh1/2	E_{173}	T_{177}	S_{184}	D_{302}	L_{286}	E_{294}	K_{306}
OtRh1	E_{181}	T_{185}	L_{192}	D_{314}	M_{298}	E_{306}	K_{318}
OtRh2	E_{476}	T_{480}	L_{487}	D_{609}	M_{593}	E_{601}	K_{613}
OlRh1	E_{204}	T_{208}	L_{215}	D_{337}	L_{321}	E_{329}	K_{341}
OlRh2	E_{260}	T_{264}	L_{271}	D_{393}	L_{377}	E_{385}	K_{397}
OlRh3	E_{188}	T_{192}	L_{199}	D_{321}	L_{305}	E_{313}	K_{325}
OlRh4	E_{115}	T_{119}	L_{126}	D_{248}	L_{232}	E_{240}	K_{252}
DsRh1	Q_{140}	S_{144}	M_{151}	D_{268}	L_{252}	E_{260}	K_{272}
GpRh2	C_{91}	T_{95}	L_{102}	D_{217}	L_{201}	E_{209}	K_{221}
GpRh3	Q_{85}	T_{89}	I_{96}	N_{209}	A_{194}	-	K_{213}
GpRh4	M_{67}	T_{71}	L_{78}	D_{193}	L_{177}	E_{185}	K_{197}
GpRh5	Q_{1412}	S_{1416}	M_{1423}	D_{1537}	L_{1521}	E_{1529}	K_{1541}
CsRh1	M_{144}	A_{148}	T_{155}	D_{269}	L_{253}	E_{261}	K_{273}
ApRh1	M_{67}	A_{71}	T_{78}	D_{192}	A_{176}	E_{184}	K_{196}
AsRh1	N_{122}	T_{126}	L_{133}	N_{248}	L_{232}	T_{240}	K_{252}
AsRh2	N_{123}	T_{127}	L_{134}	N_{249}	L_{233}	S_{241}	K_{253}
AsRh3	Q_{78}	T_{82}	V_{89}	N_{203}	L_{187}	C_{195}	K_{207}

KnRh1	Q_{166}	T_{170}	M_{177}	D_{292}	L_{276}	E_{284}	K_{296}
KnRh2	Q_{95}	T_{99}	L_{106}	E_{221}	T_{205}	E_{213}	K_{225}

3.5. Spectral tuning of the new microbial rhodopsins

The amino acid residues surrounding the chromophore are primarily responsible for tuning the absorbance maxima of the holoprotein rhodopsin. The significant role of amino acids in spectral tuning was studied in case of green and blue proteorhodopsins (GPR & BPR respectively). The amino acid at 105th position of highly homologous green absorbing proteorhodopsin (GPR: AY210898) and blue absorbing proteorhodopsin (BPR: AY210919) have nonpolar leucine and polar glutamine residue, respectively. Substitution of either converts it into other form and vice versa [33]. The four rhodopsins of halobacteria BR, HR, SRI and SRII have the same bound chromophore but SRII shows a blue-shifted absorbance at 498 nm as compared to BR, HR and SRI by 60 to 80 nm. Point mutations of all residues in retinal pocket in phoborhodopsin corresponding to BR did not shift the maxima of phoborhodopsin to BR [34,35]. This suggests spectral tuning is also regulated by other structural feature(s) of rhodopsin, probably by residues present at the flanking sides of the retinal binding pocket. The absorption spectrum of animal rhodopsin covers the entire visible range from UVA to NIR. Absorbance maxima of MTRs are largely confined to the blue and green region of the spectra. But the recently characterized Cop5 modular rhodopsin coupled with HisK, RR and Cyc, suggests its tuning to UV A & blue light (bi-stable switch). The chromophore isomerisation and counterion distance was involved in spectral shift [30,36,37]. Based on the sequence analysis and comparison of residue corresponding to 105th position (proteorhodopsin), the spectral shift (blue or green) of the modular rhodopsin has been analysed and summarized in table 3. This analysis suggests that newly identified modular rhodopsins are green tuned due to presence of a non-polar amino acid at a position corresponding to 105th position (proteorhodopsin) except GtRh1which possesses an acidic amino acid.

Table 3. Comparative analysis of residues determining spectral tuning of the rhodopsin.

Rhodopsin	105th position/ Corresponding amino acid	Polar/Non-Polar aa	Green/Blue shifted
Green PR	Leucine	Non-Polar	Green
Blue PR	Glutamine	Polar	Blue
KnRh3, TsRh1 and GpRh3	Isoleucine	Non-polar	Green
Cop8-12, GpRh2, ApRh1, AsRh2	Isoleucine	Non-polar	Green
MspRh1, MpuRh1, AsRh3-4, OtRh1-2, OlRh1-4, DsRh1, GtRh2,3	Leucine	Non-polar	Green
Cop5-7, Vop5-7, GpRh3-5, GtRh4-10, AsRh1, MspRh2, MpuRh2, CsRh1, BgRh1-2, KnRh1-2	Methionine	Non-polar	Green
GtRh1	Aspartate	Acidic	unknown

3.6. Evolutionary pattern of the modular microbial rhodopsins

MTRs provide a smart alternative pathway of ATP production other than photosynthesis in archaea and help in the survival of the organism in harsh conditions. Many reports have been published for the evolutionary pattern of MTRs [38,39] but the descent of modular rhodopsins is not yet known. Since, this is the first report of modular rhodopsin from diverse organisms, it is noteworthy to analyse the evolutionary pattern of these rhodopsins from different taxa of life.

FimV, UL36 and MED15 coupled Channelrhodopsins (KnRh3, GpRh1 and TsRh1) were grouped with ChR and VChR (Figure. 4) while rhodopsins from proteobacterium, proton pumping BR, chloride pumping HR and SR clustered in separate clades (Figure 4). Interestingly, AsRh4

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preceded by Rav1 and WD40 domain at N-terminus was the only modular rhodopsin grouped with algal proton pump CsR from Chlorella subllipsoedea. Sequence alignment also confirmed the presence of important residues required for pump activity in AsRh4 (Figure 2 and 3, see text). Surprisingly, modular rhodopsins clustered together independent of SRs. Close analysis of branching pattern shows ChRs to group together with modular rhodopsins more closely than proton pumping algal rhodopsins. Close analysis of ChRs and modular rhodopsins reveals their unique functional properties. Among the ChRs, the best-characterized one is the light-driven ion channel. The spectroscopically characterized modular rhodopsin domain, Cop5, is a UV and blue light absorbing rhodopsin [30,36,37]. Cop6 expressed in Xenopus laevis behaves as a light-inhibited guanylate cyclase in the presence of ATP [31]. Photophysical properties of histidine kinase rhodopsin Ot-HKR (referred here as OtRh1) from O. tauri are affected by salt concentration indicating that this rhodopsin might provide input for adaptation in salt environment [32]. OtHKR/OtRh1 speculated to regulate circadian clock genes TOC1 and CCA shows higher expression during dusk [32]. Characterization of further multidomain rhodopsin is tempting because it may unearth an entirely new class of rhodopsins not known yet. At the same time, it is limiting because of long transcript and high molecular weight protein, poor heterologous expression of full length and transmembrane domain and lack of established functional assay.

Figure 4. Sequence relatedness of the microbial type modular rhodopsin: Rhodopsin domain phyletic topology shows clustering of typical MTR and extended C-terminus rhodopsins in a separate clade. Modular rhodopsins formed a different clade. KnRh3, GpRh1 and TsRh1 grouped with ChRs. AsRh4 with Rav1 domain is the only modular rhodopsin grouped with proton pumping algal rhodopsin CsR (Rhodopsin from *Coccomyxa subllipsodea*). GtRh1 was unique and separated from all lying between BR and HR. Gtrh2/3 grouped with modular rhodopsin.

3.7. Cyclase domain a canonical secondary messenger of modular sensory rhodopsin

Cyclases are a lyase class of enzymes that catalyse the formation of cyclic nucleotides. Cyclic nucleotide monophosphate (cNMP) serves as a signaling molecule in many prokaryotes and eukaryotes. Based on the substrate specificity, there are two class of cyclases- adenylyl cyclase (AC) and guanylyl cyclase (GC). Multidomain cyclases are generally composed of a receptor domain at the N-terminus, a kinase homology domain in between and a cyclase domain at the C-terminus. A similar architecture is found in modular rhodopsin coupled cyclases. Sequence analysis suggests that most cyclase domains have a conserved amino acid residue to perform the enzymatic activity. Cop5 and Vop5 lack the conserved aspartate involved in metal binding (Figure 5). Substrate binding and transition state stabilizing residues are also absent in Cop5 and Vop5 (Figure 5). This implicates inactive cyclase which was also confirmed by SMART domain analysis program. Cyclases generally function in the dimer state with the active sites being located at the dimer interface. The activity requires a divalent cation, either Mg2+ or Mn2+. The conserved motifs especially transition state stabilizing residues of the cyclase are also missing that suggests other transition state stabilizing molecules might be involved in signaling (Figure 6). Both monomers work in tandem to carry out cyclase activity where substrate specificity is determined by one and metal-binding sites are provided by another monomer. The inactive cyclase might probably be the form of regulation and activity of cyclase may be complemented by another functionally active monomer partner.

In *C. reinhardtii*, cAMP induces rapid mobilization of membrane adhesion receptor protein from cell membrane to ciliary membrane in gametes [26] which leads to the adhesion and fusion of gametes to form zygote and hence promotes the sexual life cycle of *C. reinhardtii* [40]. In the phototaxis mutant strain of C. reinhardtii, cyclase level biases the photobehavioural response and carotenoid biosynthesis [41]. Modular rhodopsin in conjunction with two-component and cyclase might be performing diverse light-regulated physiological functions in the green alga. Sequence analysis suggests degenerate cyclase in Cop5 and Vop5. Apart from the ciliary signaling, cilia beating pattern, phototaxis and communication with eyespot, some modular rhodopsin(s) must have a diverse physiological role and be localized elsewhere than the eyespot [7]. These above-mentioned hypotheses get strong support from the fact that homologous modular rhodopsins are also present in the non-flagellated, eyespot devoid, unicellular green algae *Ostreococcus lucimarinus*, symbiotic algae and in colonial algae *Volvox carteri*. Rhodopsin coupled guanylyl cyclase from fungus *Blastocladiella emersonii* is required for phototactic behaviour of the zoospore and had shown *in vitro* functional activity as well. Rho-GC from other fungi had shown promising results in modulating light dependent cGMP level in the cell. It will be interesting to investigate the functional modulation of cAMP/cGMP in cell by the modular algal rhodopsins as well.

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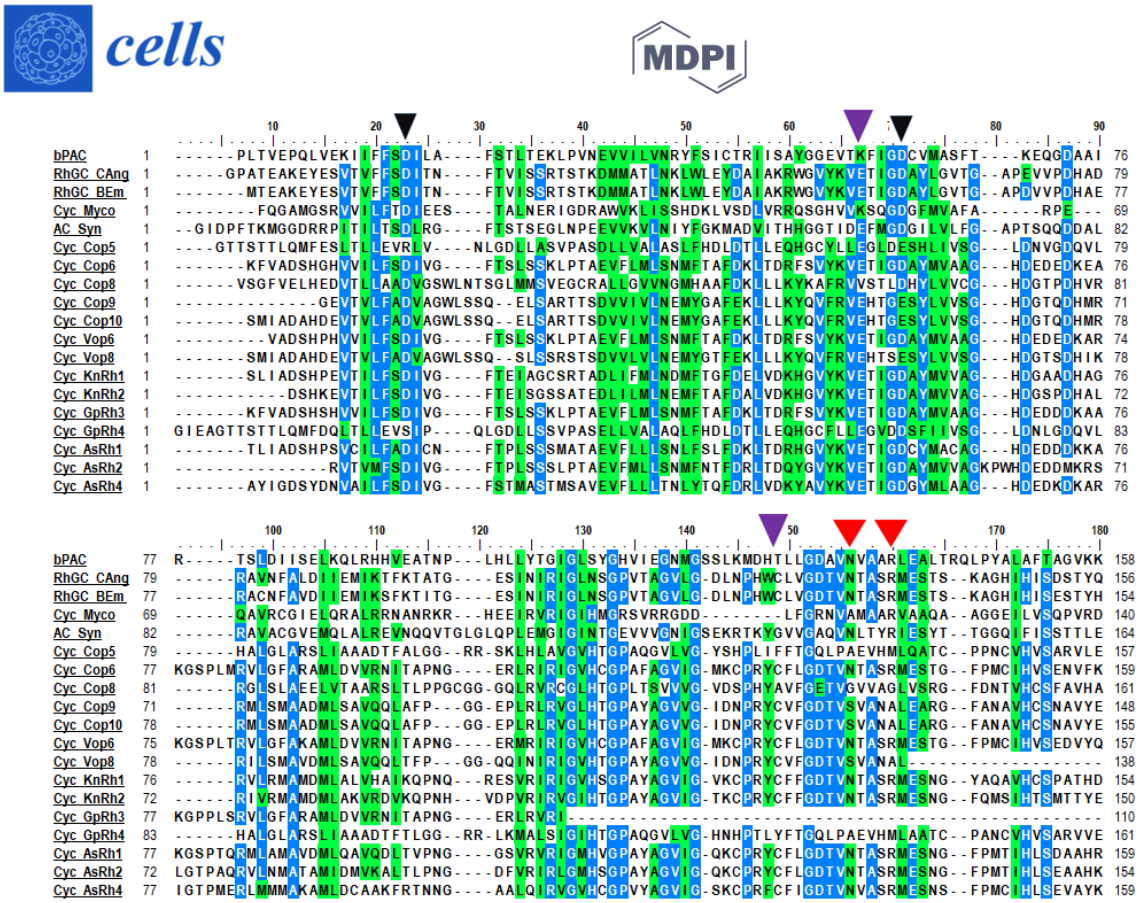

Figure 5. Multiple sequence alignment of the cyclase domain of modular rhodopsins: Cyclase domains of modular rhodopsins were aligned with canonical cyclase proteins. Black arrowhead depicts metal-binding residue, purple arrowhead shows substrate binding residue and red arrowhead shows transition state stabilizing residues of the cyclases.

3.8. Optogenetic potential of the novel modular rhodopsins

Among a variety of effector domains coupled with the ChRs, we selected FimV, MED15 and UL36 domains of functional importance, which have not yet been characterized in the algal system. We subjected these domains for protein-protein interaction network analysis and identified their potential partners and associated pathways. The protein-protein interaction analysis for FimV domain revealed its association in regulating bacterial pathogenesis machinery (Figure S1A). In the opportunistic pathogen Pseudomonas aeruginosa, FimV is an inner membrane hub protein which controls type IV pilus (T4P)-mediated twitching motility by regulating intracellular cAMP levels via activating the adenylate cyclase (CyaB) [42,43]. Factors like pili, flagella, toxin etc., that determine virulence/pathogenicity are controlled by cAMP, an allosteric activator of the virulence factor regulator, Vfr [44]. However, FimV and the Chp system (PilG, PilJ, PilN and PilF) also regulate twitching motility in a cAMP-independent manner in P. aeruginosa, where PilG may regulate directional movement, while FimV appears to localize both structural and regulatory elements to cell poles for optimal function [43]. So, based on the protein network analysis, we could conclude that ChR coupled FimV domain could be used for the optogenetic control of cAMP-dependent as well as cAMP-independent pathways to regulate twitching motility that may elucidate the molecular signaling pathways of pathogenic invasion.

MED15 (a co-activator) has a crucial role in the regulation of transcription of RNA polymerase II-dependent genes [45]. The protein-protein interaction analysis of MED15 domain showed its interactions with other mediator complex subunits (Figure S1B). MED15 was identified as regulator of mammalian sterol regulatory element-binding protein 1α (SREBP1α) which controls genes involved in cellular cholesterol and lipid homeostasis [46]. MED15 has a "KIX domain fold" responsible for binding to SREBP1α and this fold is also conserved in the Caenorhabditis elegans

orthologue, MDT15 and Yeast orthologue GAL11p [46,47]. It has also been reported that dysregulation of MED15 expression promotes human malignancies and inactivation of MED15 may inhibit the progression of several types of cancers [45,48]. Several studies found MED15 as an important prognostic biomarker for patients with various types of carcinomas [45,48] . In breast cancer and few epithelial cancers, inactivation of MED15 inhibits aberrant transforming growth factor β (TGFβ)-induced epithelial-mesenchymal transition (EMT), as it acts as a crucial cofactor for TGFβ signaling [49]. Localized tumor specific expression of ChR coupled MED15 could be used to target tumor cell signaling and eventually induce the tumour for autophagy or growth arrest in conjunction with other engineered proteins, in a light dependent manner.

The UL36 domain, associated with modular ChR, GpRh1 from G. pectorale is a the largest tegument viral protein found in herpes simplex virus 1 (HSV-1) and its homologues are well distributed across the members of Herpes viridae [50]. UL36 protein is an ubiquitin-specific protease [51] which is evident from our protein-protein interaction analysis of UL36 protein (Figure S2A). Most of the interacting partners like Ubiquitin, 26S proteasome regulatory subunit S5A, proteasome regulatory particle subunit (RpnC) and DSS1/SEM1 family protein belongs to the ubiquitin-dependent proteolysis machinery [52–54]. Proteasome subunit S5a (the human homologue of Rpn10) functions in conjunction with hHR23a/b (the two human homologues of Rad23) to recruit ubiquitylated substrates to the proteasome for their degradation [55]. In humans, DSS1/SEM1 is related to a tumour suppressor protein (BRCA2), which has a crucial role in the recombinational DNA repair in association with RAD51 [56,57]. UL36 deubiquitinating activity has a role in inhibiting the interferon-mediated immune defense upon viral invasion in the host [51]. Interestingly, the UL36 domain coupled to GpRh1 showed similarity to the C-terminal segment of HSV-1 UL36 protein (Figure S2B). Böttcher et al. (2005), in a mutation analysis with UL36 homologues from Pseudorabies Virus, constructed several truncations and showed that the extreme C terminus of UL36 having proline/alanine rich region is crucial for viral replication [58]. Overall, as observed from the protein-protein interaction analysis, it may be assumed that, ChRs coupled effector domain can be utilized as the next generation optogenetic tools, which might help in controlling processes ranging from lipid metabolism, ubiquitin-mediating proteolysis, and pathogenesis to carcinogenesis. Apart from the natural variant, the modular rhodopsins could also be genetically engineered for enhanced kinetics, better spectral tuning and modulation to precisely controlled diverse cellular physiological responses.

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