SUMOylome Modulates Intraflagellar Transport Machinery and Eukaryotic Cilia Motility

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Article

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Abstract

Translocation of channelrhodopsins (ChRs) is mediated by intraflagellar transport (IFT) machinery. However, the functional role of the network containing photoreceptors, IFT and other proteins in controlling cilia motility of the alga is still not fully delineated. In the current study, we identified two important motifs at the C-terminus of ChR1. One of them is similar to a known ciliary targeting sequence that specifically interacts with a small GTPase, and the other is a SUMOylation site. For the first time, experimental data provide an insight into the role of SUMOylation in the modulation of IFT & ChR1. Blocking of SUMOylation affected the phototaxis of *C. reinhardtii* cells. This implies SUMOylation based regulation of protein network controlling photomotility. The conservation of SUMOylation site pattern as analyzed for the relevant photoreceptors, IFT and its associated signaling proteins in other ciliated green algae suggested SUMOylation based photobehavioural response across the microbes. This report establishes a link between evolutionary conserved SUMOylation and ciliary machinery for the maintenance and functioning of cilia across the eukaryotes. Our enriched SUMOylome of *C. reinhardtii* comprehends the proteins related to ciliary development and, photo-signaling, along with homologue(s) associated to human ciliopathies as SUMO targets.

Introduction

Rhodopsins, a major class of photoreceptors for responding to several light-associated environmental cues, exists in all the three domains of life i.e., archaea, bacteria and eukaryote (1, 2). Microbial type and animal type rhodopsins are classified based on different bound retinal in their dark state. Animal type rhodopsins are mainly involved in visual and non-visual phototransduction in vertebrates and invertebrates (3). Rhodopsin functioning is associated with its dynamic localization and modifications during the photochemical signaling in metazoans (4-8). *Chlamydomonas reinhardtii*, comprises probably ten microbial type rhodopsins (named as Chlamyopsins, Cop3-12), among which Cop3 (Channelrhodopsin 1, ChR1) and Cop4 (Channelrhodopsin 2, ChR2) are light-gated cation channels. ChR1 and ChR2 are primarily responsible for the photo-orientation of this alga (9-12). Photoactivation of ChRs generates two types of photocurrents, a “photoreceptor current” in the eyespot followed by a “flagellar current” that results in calcium (Ca^{2+}) flux across the ciliary membrane (12-16) leading to the ciliary beating pattern that help cells to move towards ambient light (14). ChR1 and Cop8 were found to be localized to the eyespot and cilia in a light dependent manner (17). It is known that the trafficking of proteins to specialized compartments like chloroplast, mitochondria, nucleus or cilia is dependent on some specific signature motif(s) encoded in the relevant cargo proteins (18). In case of cilia, these signature motifs include ciliary targeting sequences (CTS) or a SUMOylation motif. CTS includes sequences like VXPX and Ax[S/A] xQ, and the mutations in these motifs could alter their localization pattern leading to ciliary trafficking and associated disorders (19-22). Vertebrate rhodopsins, containing the CTS motifs (VXPX and FR), are targeted to the modified cilium of rod cells by polarized vesicular trafficking using several GTPases (23, 24). These CTS interacts with a small GTPase, ARF4 and its activator in the vesicles while budding off from Golgi bodies, which recruits other protein complexes

required in the transport (24, 25). CTS assisted trafficking of rhodopsins in mammalian system is well studied (26).

Recently, SUMOylation mediated ciliary localization of proteins like small GTPase, ARL13 and an olfactory signaling protein, adenylyl cyclase 3 (AC3) have also been reported (27, 28). SUMOylation is known for modulating the expression and organellar localization of the targets (29). SUMO (small ubiquitin like modifier) modification also regulates expression and function of many cytoskeletal proteins and hence it is emerging as a current interest for its involvement in ciliary protein trafficking and related disorders (30, 31).

In the presented study, a CTS motif, VMPS, has been identified in the C-terminus of ChR1, which is required for its interaction with a small GTPase. This is also the first report demonstrating a microbial type rhodopsin (i.e., ChR1), conserved IFT components, ciliary targeting machinery are modulated by cellular SUMOylation. Blocking of SUMOylation partially affects the photomotility of *C. reinhardtii*. Proteins related to ciliary maintenance, ciliary signaling and ciliopathies associated candidates along with photoreceptors were identified from SUMOylome using Nano-LC-MS/MS with the eluents of immunoprecipitant sample(s). Orthologues of channelrhodopsin and its trafficking associated machinery components in other ciliated algae found to possess a conserved SUMOylation pattern. In essence, the similar pattern of SUMOylation in evolutionarily conserved IFT machinery and its associated components indicates its functional importance across the relevant eukaryotic organisms, and a defect of the same may lead to certain ciliopathy disorder in human.

Materials And Methods

Bio-informatics analysis

The Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html#!search?show=BLAST>) BLAST tool was used to identify sequences orthologous to the human ARF4 sequence. The SUMOylation sites and the SUMO interaction motif (SIM) were predicted with SUMOplot (<http://www.abcepta.com/sumoplot>) and GPS-SUMO (<http://sumosp.biocuckoo.org/online.php>) (33, 34). Clustal W was used for multiple sequence alignment and edited with the BioEdit tool (35). The protein-protein interaction network was predicted using STRING (string-db.org/) and was edited and analyzed with the Cytoscape_3.7.2 (36).

Constructs used and site directed mutagenesis

ChR1Ct (960-2136 bp) and ChR2Ct (945-2211 bp) cloned in the pETSUMO vector (Champion pET SUMO Expression System, Thermo Fisher Scientific) were provided by Dr. Mayanka Awasthi. The CrARL11GST fused construct cloned in pGEX4T1 was provided by Dr. Peeyush Ranjan. ChR1Ct was mutated at V643XP645X site to M643XS645X and ChR2Ct at V628XP630X site to M628XS630X using QuickChange II Site-Directed Mutagenesis kit, Agilent. SDM-PCR cycling parameters were adjusted according to the instruction of manual. The sequence of the primers used for SDM are as follows:

ChR1CtV643M;P645S Fw: CATGCAGGCCATGGGTGGCATGATGTCCAGCCCCGCCCC, Rev: GGGGGCGGGGCTGGACATCATGCCACCCATGGCCTGCATG and

ChR2CtV628M;P630S Fw: ATGAGCTCCGGCGTGGTGGCCAACATGACGTCTCCGCCG

Rev: CGGCGGAGGGCGTCACGTTGGCCACCACGCCGGAGCTCAT.

Chlamydomonas reinhardtii cell culture

Chlamydomonas reinhardtii wild type strain CC-124 was procured from Chlamydomonas resource center (www.chlamycollection.org). Culture was grown using TAP (tris-acetate-phosphate media, pH 7.4 and supplemented with Hutner trace elements) at 25 °C in a shaker incubator (120 rpm) in a light synchronized manner (14 hrs light and 10 hrs dark condition). Light exposure was provided using white fluorescent light (2300 lux).

Preparation of Chlamydomonas total cell lysate (CrTCL) under different experimental conditions and immunoblotting

The total cell lysate of *C. reinhardtii* culture (CC-124 strain) was prepared using exponential phase cells that were harvested by centrifugation at 5000 rpm for 5 min. The cell pellet was resuspended in 10 ml of 1X TBS (50mM Tris-Cl pH 8.0, 150mM NaCl) supplemented with 1 mM PMSF and recommended amount of protease inhibitor cocktail (Protease inhibitor cocktail, for plant cell extracts, Sigma Aldrich) in the presence or absence of 20 mM n-ethylmaleimide (NEM, Cat. No. E3876, Sigma Aldrich), as required for the SUMOylation detection experiments. NEM has been used for the detection of SUMO conjugated proteins in *C. reinhardtii* as described earlier (37, 38). The cell suspension was homogenized using the following sonication parameters: 35% amplitude, 8 sec. ON/OFF cycle lasting for 3 min. After sonication, the concentration of protein in the CrTCL was estimated by the Bradford assay. CrTCL was also incubated with a purified ULP-1 enzyme (Concentration: 8µg/µl) preparation as per the mentioned experimental design: Different concentrations of the ULP-1 enzyme (1:100, 1:500 and 1:1000) were incubated with 30 µg of CrTCL in the absence or presence of NEM. The reaction was incubated at two temperatures, 22°C (Optimal temperature for growth of *C. reinhardtii* cells) for 2 hrs and 37°C for 1 hr. Finally, samples were solubilized in standard 2X Laemmli buffer and subsequently analyzed with SDS-PAGE. Then, western blotting using ChR1Ct or SUMO antibody, was done according the protocol established earlier (17).

Co-immunoprecipitation (Co-IP) and Nano LC-MS/MS

C. reinhardtii cells were harvested in their exponential phase (O.D.₇₀₀: 0.6-0.7) and resuspended in IP lysis buffer [50 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.5% Sodium deoxycholate, 1% NP-40, 1 mM PMSF (added freshly), and PIC (Protease inhibitor cocktail)] in the presence or absence of 20 mM NEM. Sonication was performed for 3 min. (8 sec. ON/OFF cycle with 35% amplitude) to homogenize the algal cells and cell debris was removed by centrifugation at 14000 rpm for 10 min. The supernatant from the previous spin

was precleared with pre-immune serum (rabbit) along with protein A beads (Protein A Sepharose beads, Invitrogen, USA). 1 ml of the precleared lysate was then incubated overnight at 4°C with 1 µg/ml of primary antibody with end to end rotation. The treated lysate was then incubated with 25 µl protein A Sepharose beads for 2 hours at 4°C with rotation. After removing the CrTCL supernatant, the antibody-antigen complex bound beads were washed once with 1X PBS and 3 times with IP lysis buffer. For Nano-LC-MS/MS analysis, the complex was eluted from the bound protein A beads with 0.1 M glycine, pH 2.8 (pre-sterilized with 0.2-micron filter), which were then neutralized with 1 M Tris-Cl pH 8.0. The elutions were then solubilized in a buffer containing 6 M guanidinium hydrochloride and 50 mM ammonium bicarbonate. The reduction and alkylation were done with 10 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and 55 mM iodoacetamide (IAA) in 25 mM ammonium bicarbonate, respectively. These samples were trypsinized overnight and then desalted. After speed vac, these samples were resuspended in 0.1% formic acid and loaded on C18 reverse phase column (Central Instrument Facility, UDSC). Raw data was then analyzed using the Proteome Discoverer 2.2 (Thermo Scientific, USA). For detecting co-immunoprecipitation of a specific protein in the IP elution using immunoblotting, the protein-antibody complex was eluted from the beads by directly adding 2X Laemmli buffer and heating at 95°C for 10 min. Western blotting was done with the protein specific antibody (annotation and dilution of antibody have been provided in the respective Fig. legend).

Expression and purification of the recombinant proteins

Expression constructs containing the target genes (ChR1Ct, ChR2Ct, CrARL11GST, ChR1CtVP mutant, GST Only) were transformed into *E. coli* strain BL21 (DE3λ). Luria-Bertani broth (LB) was supplemented with 50 µg/ml of kanamycin (for pET-Sumo constructs) or 100 µg/ml ampicillin (for pGEX4T1 constructs), during bacterial cultivation. Cultures were incubated overnight at 37°C with shaking at 200 rpm. The culture was then scaled up (secondary culture) using the overnight grown primary culture, in TB media (Terrific Broth) with appropriate antibiotic, incubated at 37°C with shaking till an O.D₆₀₀ of 0.6-0.8 was attained. Flasks were incubated on ice for 45 minutes prior to induction. Recombinant protein expression was induced with 0.3 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) at 16°C with shaking at 200 rpm. Cells were harvested after 48 hrs of induction by centrifugation at 6000 rpm for 10 min. and further resuspended in 1XPBS containing 50 µg/ml lysozyme and 200 µM PMSF. The solubilized cell were homogenized by sonication at 35% amplitude and 30 sec. ON/OFF pulse for 5 min. Lysed cells were then centrifuged at 12000 rpm for 55 min. The total cell lysate was separated into soluble and insoluble fraction. The soluble fraction, containing the protein of interest, was filtered using a 0.45 µm filter. The protein with a histidine (His)-tag (in case of pET-SUMO based constructs) was purified using a Co²⁺ immobilized talon beads column (TALON® Metal Affinity Resins, Clontech Laboratories, Inc., USA) by immobilized metal ion affinity chromatography (IMAC). Protein with a GST tag (for pGEX-4T1 based constructs) was purified using Glutathione Sepharose beads 4 Fast Flow (GE Healthcare, USA). The protein of interest was eluted with 250 mM Imidazole (Fisher Scientific, USA) in case of Co²⁺ immobilized talon beads. The GST beads bound protein complex was eluted with 30 mM reduced glutathione solubilized in 50 mM Tris-Cl pH 8.5 [Glutathione reduced (GSH), SRL].

GST pull-down assay

Recombinant protein of CrARL11GST, GST only, ChR1Ct, ChR2Ct and ChR1CtVP mutant variants were expressed into *E. coli* strain BL21 (DE3 λ). Cells were pelleted, homogenized and fractionated by centrifugation as described in the previous section of materials and methods. GST beads bound CrARL11GST was used as a bait to pull down ChR1Ct, ChR2Ct and ChR1CtVP mutant variants (prey for the experiment) from their respective soluble fractions. GST protein was used as a negative control during this experiment. For the pull down, 1 ml soluble fractions of the prey protein(s) were first precleared with 20 μ g GST protein and 20 μ l glutathione Sepharose beads. Soluble fraction was then spun at 13000 rpm, 4°C and the supernatant was taken for GST pull-down assay. Each prey protein was incubated with glutathione Sepharose beads bound to 20 μ g GST as negative control and to 20 μ g ARL11GST, separately, for 1hr at 4°C with end-to-end mixing. Beads were washed 3 times with chilled 1XPBS. Bound proteins were eluted with GST elution buffer in subsequent fractions of 15 μ l each. Eluted fractions were mixed with 2X Laemmli buffer containing 10% β -mercaptoethanol and heated at 95°C for 10 min. Eluents were analyzed using SDS-PAGE followed by immunoblotting of the elutions with the protein specific antibodies (Anti-ChR1Ct and Anti-ChR2Ct). The same western blot was stripped with stripping buffer (60 mM Tris-Cl pH 6.8, 2% SDS and 50mM β -mercaptoethanol) and then probed again with the HRP conjugated primary GST antibody as a loading control.

Treatment of Chlamydomonas cells with the SUMOylation blocker, 2D08

The SUMOylation blocker, 2D08 (Cat. No. SML1052, Sigma Aldrich, Merck), has been used for several SUMO related studies (39-41). It is a cell permeable synthetic chemical reagent that blocks the SUMO tag transfer from E2 enzyme (UBC9) to the substrate. *C. reinhardtii* cell wall mutant strain, CC3403, was grown till O.D. reached 0.6 under 14:10 hours light-dark cycle. Cells were then harvested by centrifugation at 3000 rpm and resuspended in lower volume (1/20) of fresh TAP media and 3 ml of resuspended cells was poured in two Petri dishes. Cells in one Petri dish were incubated for 8 hours with 100 μ M of 2D08 (dissolved in DMSO), while another Petri dish containing equal number of cells was incubated with equal volume of DMSO as of 2D08, as negative control. For dish assay, the Petri dishes containing the 2D08 incubated cells and DMSO incubated as negative control were exposed to white light (Cool Fluorescent Lights) from one side of the Petri dishes. The movement of cell population was observed in both the Petri dishes and the images were captured accordingly. In order to detect the ChR1 in the 2D08 incubated cells, lysis of the cells were carried out using an 8:4:3 (v/v) solution of methanol:chloroform:water with the required volume of the cells to precipitate all the proteins (32). The precipitated proteins were then pelleted down and resuspended in 100 μ l of 50 mM Tris-Cl buffer (pH 8.0), 150 mM NaCl supplemented with 20 mM NEM and PIC. Samples were then prepared from the resuspended and precipitated protein using 2X Laemmli buffer and heating at 65°C for 30 min. Samples were resolved on 8% SDS-PAGE and then immunoblotting was done with anti-ChR1Ct antibody followed by HRP-conjugated anti-rabbit secondary antibody.

Results

ChR1 ciliary targeting sequence interacts with CrARL11

Detailed sequence analysis of ChR1 from *C. reinhardtii* revealed that it contains motifs, “VMPS” (Valine Methionine Proline Serine) and “FR” (Phenylalanine Arginine), at its C-terminus (ChR1C-terminus, ChR1Ct), similar to the known CTS, VXPX and FR (Fig. 1A). The VXPX sequence of vertebrate rhodopsin specifically interacts with a small GTPase, ARF4, for its vesicular targeting towards cilia (24). This led to the search for the orthologue of ARF4 GTPase in the *C. reinhardtii* genome that might be interacting with the VMPS of ChR1. Mining of the *C. reinhardtii* genome using Phytozome [plant genomics resource platform, (phytozome.jgi.doe.gov)] was carried out with the human ARF4 sequence as a query sequence. CrARL11 showed maximum homology to the ARF4 sequence, and sequence alignment of ARF4 with CrARL11 revealed homology in all the characteristic G-protein domains (Fig. 1B). CrARL11 showed around 77% homology to ARF4. The interaction between CrARL11 and ChR1 was first confirmed by immunoprecipitation (IP) using CrARL11 and ChR1Ct antibodies in *C. reinhardtii* total cell lysate (CrTCL). CrARL11 antibody co-immunoprecipitated the ChR1 from CrTCL and vice-versa. The detection of ChR1 in CrARL11 IP eluent and CrARL11 in ChR1 IP eluent by immunoblotting showed interaction between ChR1 and CrARL11 (Fig. 1C). Recombinant ChR1Ct protein (Fig. 1D) and ChR1 from CrTCL (Fig. 1E) were pulled-down by GST-tagged-CrARL11 protein. The results demonstrated the direct physical interaction between ChR1 and CrARL11, and that the ChR1Ct is sufficient for this interaction. ChR2 and Chlamyopsin 5 (Cop5) were also found to contain the CTS similar to VXPX motif in their C-terminus (Fig. S2A). IP was carried with ChR2Ct and Cop5 specific antibody in the CrTCL to confirm their interaction with CrARL11. IP elutions of ChR2Ct and Cop5 showed the co-immunoprecipitation of CrARL11 as detected by immunoblotting with CrARL11 antibody (Fig. S2B). The result showed that both ChR2 and Cop5 also interact with CrARL11. CrARL11-GST was able to pull-down recombinant ChR2Ct (Fig. S2C). The obtained results confirmed the direct interaction between ChR2 and CrARL11 through its C-terminus.

The VMPS motif of ChR1 is crucial for the ChR1-CrARL11 interaction

It is well documented that the mutations V345M and P347S are responsible for disrupting ARF4 interaction with rhodopsin leading to autosomal dominant retinitis pigmentosa (ADRP) in vertebrates (24, 42-44). To determine the VMPS mediated interaction between ChR1 and CrARL11, the VMPS sequence in the ChR1Ct was mutated to MMSS. CrARL11-GST was used as a bait to pull-down wild type ChR1Ct and ChR1CtVP mutant (V and P residues in VMPS were mutated and the mutated protein was annotated as ChR1CtVP mutant). Detection of ChR1 with ChR1Ct antibody by immunoblotting of pull-down eluents revealed the presence of wild type ChR1Ct and absence of ChR1CtVP mutant (Fig. 1F). This specifies that the interaction of ChR1 and CrARL11 is mediated by VMPS motif present at the C-terminus of ChR1.

The C-terminus of ChR1 contains a functional SUMOylation site

The SUMOylation machinery consists of a series of enzymes for the conjugation of SUMO protein via an isopeptide bond to the ϵ -amino group of lysine residues within the consensus SUMOylation motif, Ψ KXE, of the target proteins (45). An alternate mechanism of SUMO conjugation to the target protein is through SUMO interaction motif (SIM), a small motif of amino acids that binds directly to SUMO or SUMO tagged

to target proteins. ChRs from different green algae were contained Ψ KXE as shown in the multiple sequence alignment of their C-termini (Fig. 2A). Supplementary table 1 annotates the predicted position of SUMO tagged lysine residue and SIM motif within the ChRs of diverse algae. However, *Pleodorina starrii* (PstChR1 and PstChR2) and ChR from *Klebsormidium flaccidum* was found to contain SIM motif only (Supplementary table 1). ChR1 was predicted with a single SUMOylation site at the end of its C-terminus. Experiments were performed using SUMO tag protectors, the schematic illustration of the same is provided in Fig. 2B. NEM (n-ethylmaleimide) is an isopeptidase inhibitor that inhibits SUMO tag cleaving proteases, ubiquitin-like proteases (ULPs), and hence protects the target from deSUMOylation. NEM has been used extensively for the detection of SUMO and SUMO conjugating proteins in CrTCL (37). CrTCL was incubated with NEM and a protease inhibitor cocktail (PIC). Immunoblotting of the samples with ChR1Ct antibody showed bands with higher intensity at the expected molecular weight of ChR1 and a higher molecular weight band, in comparison to the negative control (with no NEM and PIC) as shown in Fig. 2C. Quantification of the ChR1 specific band by densitometry revealed a comparatively significant increase in the ChR1 accumulation (Fig. 2D). It can be inferred from the results that ChR1 was stabilized in the presence of NEM and PIC. This indicates an involvement of SUMOylation machinery in the regulation of the ChR1.

ULP-1 enzyme mediated deSUMOylation of ChR1 in Chlamydomonas

ULP-1 (Ubiquitin-like protease 1) is involved in deSUMOylation of the SUMOylated protein targets and form an integral part of the reversible SUMOylation machinery (25, 38). CrTCL samples (with or without NEM), were incubated with different dilutions of the purified yeast ULP-1 for 2 hrs at 22°C (optimum temperature for *C. reinhardtii* growth). Samples containing only ULP-1 showed lower intensity of ChR1 specific bands upon immunoblotting with ChR1Ct antibody, in comparison to the samples incubated with both ULP-1 and NEM (Fig. 2E). Densitometry analysis of the ChR1 specific bands revealed that the amount of ChR1 significantly decreased in the presence of ULP-1 (Fig. 2F). This decreased ChR1 amount is expected to be associated with the ULP-1 mediated deSUMOylation of ChR1, which further possibly led to degradation of the ChR1. It indicates that SUMOylation of ChR1 probably is related to its stability, protecting it from ULP dependent degradation. Similar set of experiment was carried at 37°C for optimal activity of the ULP1. It also showed results similar to those obtained at 22°C, however no higher molecular weight band accumulation was seen (Fig. S3).

SUMOylation controls phototaxis of Chlamydomonas

C. reinhardtii cells were incubated for 8 hours with the SUMOylation blocker 2D08, which inhibits SUMOylation by blocking the transfer of SUMO from E2 (UBC9) enzyme to the substrate (41). The accumulation of ChR1 was apparent in comparison to the control experiment with DMSO only upon immunoblotting in the CrTCL (Fig. 3A). Quantification of the immunoblot bands specific to ChR1 in control vs 2D08 treated samples revealed a relative increase in the abundance upon treatment with 2D08 (Fig. 3B). *In vivo* experimental data support functional significance of ChR1 SUMOylation associated with ChR1 turnover or degradation. Phototaxis of *C. reinhardtii* cells incubated with or without SUMOylation

blocker was compared by the disc assay (Fig. 3C). The phototaxis of the cells was observed to be affected in the presence of SUMOylation blocker. Mean intensity from different regions of the plate was measured by ImageJ software to compare the phototaxis of the cells in terms of the cell population density (Fig. 3D). Comparatively, a greater number of cells showed no phototaxis in the presence of SUMOylation blocker. Despite of the increased ChR1 amount in the presence of SUMOylation blocker, the phototaxis of the *C. reinhardtii* cell population was partially compromised. This suggests that the SUMOylated form of ChR1 plays an important role in phototaxis, and/or blocking the SUMOylation machinery is preventing SUMOylation dependent perturbation of protein homeostasis of flagellar proteins required for the maintenance of photo-movement of *C. reinhardtii*.

SUMOylation machinery of *C. reinhardtii* modulates ciliary proteins, centriole associated proteins and photoreceptor associated network

This report also presents the enrichment and identification of endogenous SUMOylated proteins (SUMOylome) of *C. reinhardtii*. The SUMOylome of *C. reinhardtii* was immunoprecipitated using anti-SUMO2 antibody followed by identification of the peptides using Nano-LC-MS/MS. SUMO and other SUMO conjugated proteins were detected in the CrTCL by immunoblotting with SUMO2 antibody (Fig. 4A). SUMO2 IP followed by Nano-LC-MS/MS detected 520 proteins in total upon relevant database search. Of these, 66 proteins were also present in rabbit pre-immune serum IP Nano LC-MS/MS, taken as negative control and the same were excluded from SUMOylome. Proteins specific to SUMO2 Nano-LC-MS/MS showed association with vesicular and transporters (9%), photoreceptors and miscellaneous proteins (11%), cell cycle associated proteins, DNA binding proteins (6%), housekeeping and other miscellaneous proteins (62%). Interestingly, numerous identified proteins represented the ciliary/FAPs/IFT group [(12%) (Fig. 4B)]. Proteins, corresponding to which at least two peptides were identified in the MS data, were selected for further analysis and discussion. Several proteins were selected containing single peptide in proteomics data based on the established protein-protein interactions as mentioned in the relevant publications. An interactome was predicted with the proteins identified in the Nano-LC-MS/MS using STRING program. The acquired protein-protein interaction network data was visualized in Cytoscape v3.7 using the degree centrality tool. The degree of a protein centrality is shown by varying size of the nodes in the network that enlarged with increase in protein's biological importance in the cellular system (Fig. 4C). Various flagellar associated proteins (e.g., FAP148, FAP78 etc.) were in the center of interactome connecting motor proteins, vesicular proteins (Dynamin like GTPase), ion channels (VGKC) and proteasome machinery (26S). Table 1 lists selected proteins from SUMO Nano-LC-MS/MS. Detailed analysis of the proteins identified in the Nano-LC-MS/MS data are listed in supplementary table 4 including their SUMOylation sites predicted using online SUMOylation program. Proteins from both SUMOylation and ubiquitination machinery like E3 ubiquitin ligase, SUMO activating enzyme E1B (SAE1) and UBCs were present. ChR1, Phototropin and Cop5 photoreceptors were also identified. The experimental results obtained have revealed ChR1 as a SUMOylation target (Fig. 2). Among the flagella related proteins, most were the FAPs, RSPs (radial spoke proteins) and IFT machinery components [motor proteins, kinesin & dynein (DHC); IFT complex proteins]. Proteins related to centriole biology were also identified such as CEP290, Centrin-3 and microtubules associated tubulin tyrosine

ligase/polyglutamylase (TTL) etc. Various FAP's and RSP proteins were found to have a high degree of centrality indicating their importance in controlling the flagellar protein network. Occurrence of these proteins in SUMO2 IP followed by Nano-LC-MS/MS supported the functional relatedness of SUMOylation to IFT machinery and several flagellar associated proteins. Further prediction of SUMOylation sites or SIM motif in the IFT machinery components (from *C. reinhardtii*) using SUMOylation detecting online programs, corroborates the possible involvement of SUMOylation in the regulation of IFT machinery (Supplementary Table 2). IFT complex proteins were containing SUMOylation site and SIM motif consensus amino acid sequence, except a few like IFT80 and IFT140, which were lacking SUMOylation site and SIM, respectively. IFT machinery is known to be evolutionarily conserved from lower to higher eukaryotes including humans (46). It is essential for the maintenance and development of cilia or flagella. This aspect led to compare the SUMOylation site pattern in IFT complex proteins from selected model organisms representing lower to higher eukaryotes including human, and pathogenic or non-pathogenic organisms. Identified homologues of IFT proteins from the respective model organism genome with the homology search, predicted to contain the SUMOylation site. The comparison of the SUMO site location revealed similarity in the position of the lysine residue within the SUMOylation motif (Supplementary Table 3). This conserved feature of the SUMOylation pattern within the IFT proteins postulates its crucial importance in the regulation of IFT machinery and related physiology and associated human ciliopathies. The SUMOylation pattern of ChR trafficking related protein machinery that includes kinesin, dynein, IFT complexes, ARF, BBSome, SMC, DnaJ as identified in different flagellated green algae containing ChRs was also analyzed and has been presented in Supplementary Table 5. It was observed that except IFT27 and BBS2, all other proteins showed similar SUMOylation pattern suggesting that SUMOylation is an essential post-translational regulation of possible ChR trafficking machinery in other flagellated/ciliated algal systems.

Discussion

ChRs are light-gated cation channels involved in the phototile response of *C. reinhardtii* for scrutinizing an ambient light condition for the survival of the organism (10-12). Their potential of membrane depolarization, successful engineering for variable ion channel kinetics and spectral properties led to the emergence of the field of optogenetics specifically to study neural signaling (47-50). The physiological relevance of ChRs in *C. reinhardtii* is an important aspect for exploring the evolutionary link of several rhodopsin signaling pathways through *C. reinhardtii* (51). The physiological function of both ChR1 and ChR2 has been proven by siRNA antisense approach with subsequent photocurrent and photoorientation studies (9, 12, 13, 52). However, the detailed knowledge of the trafficking, processing, signaling and fate of ChRs from their synthesis to the functional sites is still obscure. Moreover, the study of the fate of microbial rhodopsins in *C. reinhardtii* would allow researchers to compare this system with other organisms containing microbial type rhodopsins. Like in flagellated fungi, *Blastocladiella emersonii*, microbial type rhodopsins (rhodopsin cyclases) are responsible for the photoorientation of their zoospores (53-55).

ChR1 has been reported to be transported into the eyespot and flagella in a light dependent manner, which involves IFT machinery and other accessory components like guanylate cyclase, DnaJ and SMC (17). IFT is involved in the trafficking of most of the proteins into the flagella or cilia (46). Ciliary trafficking of proteins utilizes other proteins or complexes in association with the IFT components like BBSome complex, small GTPases like Rabs, Arfs, Arls, and ciliary components (22, 56-58). The transport of vertebrate rhodopsin to the outer segment of rod cells requires the involvement of small GTPases like Arf, Arl and Rab proteins in their vesicular targeting to the base of the inner segment (24, 59). Several ciliary proteins possess CTS with which these protein complexes are known to interact for migration towards cilia. In addition to vertebrate rhodopsins, other transmembrane proteins like the heterotetrameric channel, CNGB1b (olfactory cyclic nucleotide-gated channel subunit 1b); ciliary cation channel responsible for ADPKD and PC-1 (polycystin-1 channel) have also been reported to contain a C-terminus VXPX motif for their ciliary localization (18, 60). Interestingly, few ciliary proteins contain CTS motif at their N-terminus like ADPKD associated PC-2 (polycystin-2 channel) and the cation transporter in sperm flagella, Na⁺-K⁺ ATPase (61, 62). GPCRs such as SR3 and Htr6 lack VXPX but are composed of a Ax[S/A]xQ motif in the loop region of the third intracellular helix for their ciliary localization (22). We hypothesized that similar machinery might be operational for the vesicular sorting of ChR1 to the flagella or eyespot along the microtubules in *C. reinhardtii* (17, 63). Our results showed a similar rhodopsin-GTPase based interaction between the CTS of Chlamyopsins (ChR1, ChR2, Cop5) and CrARL11 (Fig. 1, Fig. S3). However, further in vivo experiments would be required to establish its functional relevance. In vertebrates, the loss of VXPX sequence in rhodopsins causes the exposure of the mis-trafficking signal (CCGKN) within its C-terminus leading to mis-localization and degradation of the rhodopsin (20, 21). ChR1 variant lacking both N-terminus and C-terminus reported to be required for proper ligand-gated ion channel activity and photochemical property (64). The indispensable requirement of the VMPS motif of ChR1 C-terminus for mediating ChR1-CrARL11 interaction supports its importance in related phenomena. Certain eyespot mutants of *Chlamydomonas* exhibit photo-perception, which was explained by light dependent ciliary localization of ChR1, hypothesized to be responsible for this phototransduction through cilia (17). ChR1 interaction with the small GTPase, CrARL11, as shown in this report and IFT20 as reported by Awasthi et al. in 2016, probably suggests the possible trafficking of ChR1 from the cell body to the cilia (or eyespot), similar to mammalian system. Flagella isolated in the light to dark transition period also showed the presence of ChR1 in the flagella only fraction (Fig.S1). The transport of proteins or photoreceptors to the eyespot might exhibit some other unique mechanism yet to be explored. Similarities in the trafficking mechanisms of animal type rhodopsins and microbial type rhodopsins revealed the significance of VXPX motif for their ciliary targeting. This also indicates an evolutionary conserved mechanism for the trafficking of the widely distributed rhodopsin photoreceptor and might be relevant to other ciliary proteins. Other than vertebrates and algae, some fungi (e.g. *Blastocladiella emersonii*, *Allomyces macrogynus* etc.) also harbor canonical and modular microbial type rhodopsins i.e. rhodopsin domain fused to some functional domain like cyclase (54, 65). The genome of *Blastocladiella emersonii* was searched for the presence of IFT machinery components. Most of the IFT proteins were retrieved from the genome revealing its probable significance in trafficking of rhodopsin or other proteins for various physiological processes in the ciliated zoospores (Unpublished data).

A SUMOylation site (LKNE) was identified within the ChR1 sequence at its C-terminus. SUMOylation seems to be important for the regulation of ChR1 amount in the cells (Fig. 2). SUMOylation has been reported to play a role in photoreceptors development by the regulation of associated transcription factors in relation to their expression and localization (61, 62, 66). SUMOylation has been well known for regulating ion channels specially potassium channels (e.g. voltage gated potassium channel, Kv1.5, and leaky potassium channel, K2P1), and thereby modulating their activity (67, 68). In these ion channels, SUMOylation tightly regulates their activation and deactivation in a reversible manner. This finding had added a novel function to the SUMO machinery (69). Recently, few reports have stated the role of SUMOylation in the localization of several membrane receptors (27, 28). However, the functional role of SUMOylation has not yet been identified for any type of rhodopsin (animal type rhodopsins or microbial type rhodopsins) in nature. This is the foremost report, where SUMOylation of a microbial type rhodopsin (ChR1) and IFT machinery is established. The presence of a SUMOylation site/SIM at the C-terminus of the ChRs and IFT from other eukaryotes suggested SUMOylation mediated regulation of rhodopsin homeostasis and modulation IFT components, respectively (Fig. 2A). *In vivo* experiments with the SUMOylation blocker showed an accumulation of ChR1 in *C. reinhardtii* indicating that SUMOylation may have a role in its degradation or stability (Fig. 3A&B). Altered phototaxis of cells in the presence of SUMOylation blocker stipulates a crucial role of the SUMOylation machinery in the protein network responsible for the photomotility of *C. reinhardtii* (Fig. 3C&D), including photoreceptors, ChR1, IFT components, cell cycle and signaling proteins.

IP of SUMO tagged proteins from the CrTCL and their detection by Nano-LC-MS/MS revealed the presence of several flagella related proteins, IFT proteins and the photoreceptors (Table 1). SUMOylation mediated regulation has been previously identified for cytoskeletal proteins including ciliary, microtubular and flagellar proteins (30). The computational prediction of the SUMOylation sites of the IFT proteins showed the presence of SUMOylation site(s) and sumo interacting motif (SIM). The existence of these sites in most of the IFT components suggested that SUMOylation machinery mediates a salient role in this large protein-protein interaction network (Supplementary Table 2). The conservation of the SUMOylation site position in respective IFT machinery proteins was seen in the selected model organisms (Supplementary Table 3). These results strengthen the pivotal involvement of SUMOylation-mediated regulation of protein-protein interaction of IFT machinery and its associated cellular physiology. Possibly, defects in the SUMOylation of IFT machinery might be linked or lead to several human diseases.

SUMOylation is a key factor in controlling potassium channels and has been linked to various diseases, collectively called channelopathies, like sudden death in epilepsy (70). An imbalance in the SUMOylation-deSUMOylation cycle of targets has been associated with several diseases (71). Voltage gated potassium channel (VGKC) and TRP ion channel were identified in the presented SUMOylome, which delineate SUMO based regulation of ion channels in *C. reinhardtii* also. SUMOylation is also known to regulate cell cycle regulatory proteins in a spatiotemporal manner in cancer, and centriole associated proteins like nucleo-cytoplasmic localization of centrin-2 (65, 72, 73). Centriole biogenesis is associated with cilia generation initiating from centriole translocation (to basal bodies) to the growth and development of cilia (30). The cilia or microtubule formation from centrioles includes the crucial role of

cell cycle associated centriole proteins like C2 domain containing proteins, e.g., CEP120, and sequentially the IFT complex proteins (51). Centriole elongation and integrity is also controlled by C2 domain containing proteins (74, 75). Any mutation in these proteins can cause impairment in cilia biogenesis, leading to ciliopathy (47). The SUMOylated proteome of *C. reinhardtii* confirm association of the centriole associated proteins e.g., CEP290, centrin-3 and C2 domain containing proteins with SUMOylation (Fig. 4C). These proteins are also found to be associated with SUMOylation/Ubiquitination machinery proteins (ubiquitin like protein and 26S proteasome machinery). An E3 ubiquitin ligase, TRIM37, has been reported earlier to control centriole reduplication (76). These evidences depict the involvement of SUMO mediated regulation in centriole biogenesis, cilia growth and cell cycle. The SUMOylome contain proteins related to ciliopathy disorders that belong to the categories like DNA binding proteins, vesicular proteins & transporters and cell cycle associated proteins. For example, MYND finger and RCC domain (chromosome associated enzyme), DNA regulating proteins, have been reported to be responsible factor for ciliary dyskinesia (77). Other proteins like Cyclin B (cell cycle regulator), transporter like calmodulin binding IQ motif, guanylate cyclase, DnaJ like chaperones and SMC superfamily proteins, were reported to be involved in various ciliopathies (56, 78). The presence of these proteins in the SUMOylated proteome further lead to a direction for the involvement of SUMOylation regulation in ciliary disorders through these integrated proteins (Fig. 4C, Supplementary table 4). The current study of the identification of trafficking and SUMOylation signal in the C-terminus of algal channelrhodopsins uncovered major findings related to physiological significance of microbial type rhodopsins in *C. reinhardtii* and other ciliated organisms (Fig. 5, Supplementary table 5). In addition, this work has also led to the emergence of impulsive groundwork for novel research arenas like SUMOylation regulated IFT machinery, ciliary trafficking of cargos and association with ciliopathies.

Declarations

Conflicts of interest/Competing interests: The authors declare that they have no conflict of interest.

Ethical Standards: Not Applicable

Author Contribution: SK, PH and GKP conceptualized and designed the research plan. KS conducted the experiments. SK and KS interpreted and analyzed the data. KS prepared the figures. KS, SK and GKP wrote the manuscript. All authors have reviewed the manuscript.

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Tables

Table 1: Categorization of proteins identified in SUMO IP followed by Nano-LC-MS/MS data and their link to cilia function or related disorder (detailed analysis given in Supplementary table 4 with the full references of cilia-related or ciliopathies related proteins)

Nano-LC-MS/MS proteins categorized/ Phytozome ID	Uniprot ID	Protein name	Cilia-related or ciliopathies related
SUMOylation/Ubiquitination machinery proteins			
Cre13.g582350	A0A2K3D0H6	ubiquitin like modifier	
Cre10.g429000	A0A2K3D9Q5	Ubiquitin-conjugating enzyme E2	
Cre16.g681354	A8JAA7	E3 Ubiquitin-Protein Ligase Mdm2	(2)
Cre12.g516500	A8JHY5	Ubiquitin ligase SCF complex subunit Cullin2	
Cre06.g296983	A0A2K3DQP2	ubiquitin-like 1 (SUMO)-activating enzyme E1 B (UBLE1B, SAE2, UBA2)	
Cre02.g143600	A0A2K3E3Y1	26S proteasome machinery regulatory subunit	
Flagella associated proteins			
Cre07.g342850	Q6JIY8	Oda	
Cre16.g658650	A0A2K3CTD1	Myosin heavy chain, class XI, MYO1	
Cre17.g704300	A0A2K3CP55	FAP47	
Cre02.g078600	Q27YU6	RSP8	
Cre13.g572700	A9XPA7	IFT144	
Cre14.g627576	A0A2K3CYE9	DYNEIN HEAVY CHAIN 6	
Cre04.g217914	A0A2K3DTF4	WD domain repeats, FAP57	
Cre09.g394769	A0A2K3DEI0	Tubulin tyrosine ligase/polyglutamylase (TTL)	(4)
Cre10.g434600	A0A2K3DA34	FAP148	
DNA related proteins			
Cre10.g442650	A0A2K3DAL4	DNA helicase	
Cre07.g342350	A0A2K3DKM3	3',5'-cyclic-nucleotide phosphodiesterase	(5)
Cre16.g671050	A0A2K3CUH6	MYND finger, Ciliary dyskinesia	(6)
Cre10.g465793	A0A2K3DC92	Endonuclease/Exonuclease/phosphatase family	
Vesicular proteins and transporters			
Cre14.g616600	A0A2K3CXP1	dynamin like protein like GTPases	
Cre17.g715300	A0A2K3CPX6	POLYCYSTIN-RELATED, PKD2	
Cre02.g098750	A0A2K3E228	VOLTAGE AND LIGAND GATED POTASSIUM CHANNEL	
Cre17.g728250	A0A2K3CQS6	Vacuolar sorting-associated protein 13	(9)
Cre01.g042000	A0A2K3E7I2	calmodulin-binding IQ motif	(7)
Cell cycle associated			
Cre04.g215800	A8J9Q2	Sfi1 spindle body protein (Sfi1)	
Cre10.g441850	A0A2K3DAK0	SMC superfamily	(10)
Cre08.g370401	A8JER8	cyclin B (CCNB), CYCB1	(11)
Photoreceptors			
Cre14.g611300	A0A2K3CXC9	ChR1	
Cre03.g199000	Q8LPD9	Phototropin	
Cre02.g074150	A0A2K3E007	Chlamyopsin 5, Cop5	
Miscellaneous			
Cre05.g238332	Q39615	Photosystem I reaction center subunit II	
Cre06.g257601	Q9FE86	2-cys peroxiredoxin, chloroplastic	(12)
Cre17.g701500	A0A2K3CNY6	DNJ1,DnaJ-like protein	
Cre12.g520100	A0A2K3D405	guanylate cyclase	(13)
Cre17.g742200	A8JEA7	RCC1 domain	(18, 19)
Cre13.g561800	A0A2K3CYZ8	C2 domain	(14, 15)

Figures

Figure 1

Schematic representation of ChR1, homology analysis of ChRs, immunoprecipitation and GST pull-down for the interaction of ChR1 with CrARL11. A. Ciliary targeting sequences, VMPS (cyan oval background) and FR (green oval background) in ChR1. The VMPS sequence has been shown at the end of the C-terminus of the ChR1 representative structure. B. Homology analysis of HsARF4 with CrARL11. Dark grey and light grey color background reflects identical and similar amino acids, respectively. Homology in the characteristic G-boxes (4 G-boxes, red outlined boxes) and switches (Switch I and II, blue coloured brackets enclosed) of ARF like proteins, was observed. C. Immunoprecipitation of ChR1 and CrARL11 from CrTCL. Immunoblotting with ChR1 antibody in CrARL11 eluents (upper panel) shows the presence of ChR1 as a band at the calculated molecular weight of ChR1 i.e., around 78kDa. Similarly, immunoblotting with CrARL11 antibody shows that ChR1Ct pull down CrARL11 as a band of around

20kDa, its expected molecular weight (lower panel). Pre-immune serum containing no relevant protein band has been shown as negative control for the experiment. D. ChR1-CrARL11 direct interaction: Upper panel; GST pull-down with CrARL11GST as bait. Recombinant ChR1Ct in the elutions (E1 and E2) of CrARL11GST is shown by immunoblotting with ChR1Ct antibody. E. ChR1-CrARL11 and semi-in vivo interaction: CrARL11-GST as bait to pull down ChR1 from CrTCL. ChR1 is present in CrARL11GST elutions. Lower panel (D and E): Immunoblotting with Anti-GST for showing loading control. F. GST pull-down assay showing interaction of ChR1Ct and ChR1CtVP mutant with CrARL11: CrARL11 GST as bait to pull-down recombinant ChR1Ct and ChR1CtVP mutant. No protein bands present in the CrARL11-ChR1CtVP mutant pull-down. Lower panels (D, E and F): immunoblotting with Anti-GST serve as loading control. GST only serves as negative control. Antibody dilution: Anti-ChR1Ct: 1:3000, Anti-CrARL11: 1:1000, Anti-GST: 1:5000. Abbreviations: E: elution, M: Molecular weight marker

Figure 2

SUMOylation detection of ChR1: A. Multiple sequence alignment of C-terminus of the selected ChRs from different algae given in supplementary table 1. Red outlined box represents the SUMOylation motif. B. Schematic illustration of the mechanism of sumo specific protease inhibitor, NEM, and the mechanism of ULP-1. C. Detection of SUMOylation of ChR1 in CrTCL: ChR1 seen with increased band intensity along with higher molecular weight bands accumulation (~130kDa) in the presence of sumo specific protease inhibitor, NEM and PIC. Lane without PIC and NEM serves as negative control. Ponceau stained membrane serves as loading control. D. Intensity quantification and relative comparison of ChR1 band

using ImageJ and plotted using OriginPro8.5. E. CrTCL samples with or without NEM is presented by + or -; above each lane, ULP-1 is present in all lanes except the last one as differentiated by a line drawn over the lanes with ULP-1. Different dilutions of ULP-1 used are given above the immunoblot. Experiment was performed at 22°C for 2 hrs. F. Quantification of relative intensity of ChR1 in the lanes in the presence (+) or absence (-) of NEM and ULP-1, done using ImageJ and plotted using OriginPro8.5. Error bars denotes standard deviation derived from 3 individual experiments. Anti-ChR1Ct: 1:3000

Figure 3

In vivo SUMOylation detection of ChR1 and photobehavioural response of Chlamydomonas: A. CrTCL prepared from the *C. reinhardtii* cells (CC3403 cw-) which were incubated with (+) or without (-) 2D08. Ponceau stained membrane is acting as loading control. Concentration of 2D08 used: 100µM; ChR1Ct antibody dilution used: 1:3000. B. Relative intensity of ChR1 in the presence or absence of SUMOylation blocker was calculated using ImageJ. C. Phototaxis assay in Petri plates containing CC3403 cw- culture incubated with or without 2D08. Direction of light exposure is shown. D. Mean intensity of the area calculated, where the negative phototaxis of the cells observed in comparison to the no phototaxis region. Intensity was measured using ImageJ software and plotted using OriginPro8.5

Phototropin, and Cop5) are shown in orange-coloured nodes. The protein-protein network is based on degree of centrality determining tool in Cytoscape that predicts the biological importance of a protein in the network comparative to others. The scale for degree of centrality measurement is the size of node which is predicted as larger value corresponding to large sized node.

Figure 5

Proposed model for working of ChR1 mediated photomotile response of *C. reinhardtii*: ChR1 consist of ciliary targeting sequence (CTS) and SUMOylation motif in its C-terminus required for its functional localization and hence collectively involved in the directional photomotility of *C. reinhardtii*. Ciliary targeting sequence, VMPS, in ChR1 is interacting with a small GTPase, CrARL11, in *C. reinhardtii*. ChR1 is SUMOylated in the cellular system. IFT machinery mediated trafficking of ChR1 into the cilia shown to be interconnected through SUMOylation. Lacking SUMOylated ChR1 or IFT or both may affect the photomotility of *C. reinhardtii*. C, chloroplast; N, nucleus; V, vesicle; E, eyespot; MT, microtubule; B, basal bodies; Ci, Cilia

Supplementary Files

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