Insights into degradation and targeting of the photoreceptor channelrhodopsin-1

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Abstract

In *Chlamydomonas,* the directly light-gated, plasma membrane-localized (PM) cation channels channelrhodopsins ChR1 and ChR2 are the primary photoreceptors for phototaxis. Their targeting and abundance is essential for optimal movement responses. However, our knowledge how *Chlamydomonas* achieves this is still at its infancy. Here we show that ChR1 internalization occurs via light-stimulated endocytosis. Prior or during endocytosis ChR1 is modified and forms high molecular mass complexes. These are the solely detectable ChR1 forms in extracellular vesicles (EVs) and their abundance therein dynamically changes upon illumination. The ChR1-containing EVs are secreted via the PM and/or the ciliary base. In line with this, ciliogenesis mutants exhibit increased ChR1 degradation rates. Further, we establish involvement of two cysteine proteases in its turnover: CEP1, a papain-type C1A member, and a calpain. ΔCEP1 knock-out strains lack light-induced ChR1 degradation, whereas ChR2 degradation was unaffected. Low light stimulates CEP1 expression, which is regulated via phototropin, a SPA1 E3 ubiquitin ligase and cAMP. Further, mutant and inhibitor analyses revealed involvement of the small GTPase ARL11 and SUMOylation in ChR1 targeting to the eyespot and cilia. Our study thus defines the degradation pathway of this central photoreceptor of *Chlamydomonas* and identifies novel elements involved in its homeostasis and targeting.

1 INTRODUCTION

One peculiar behavioral response of the motile green alga *Chlamydomonas reinhardtii* is its precise positioning with respect to a defined light source. This orientation movement is called phototaxis. Its direction is determined not only by the intensity and quality of the light environment but also by the actual physiological status of the cell. Phototaxis is triggered by excitation of the two blue-green light absorbing channelrhodopsin photoreceptors, ChR1 and ChR2, which are localized in the plasma membrane (PM) region of a composite organelle commonly termed eyespot (reviewed by Kreimer et al., 2023). Both ChRs are directly light-gated cation channels. Their Ca^{2+} conductance is sufficient to initiate phototaxis via a yet largely unknown Ca^{2+}-mediated signaling cascade that involves signal amplification towards the two cilia. Subtle Ca^{2+}-dependent changes in the beating plane and frequency of the cilia finally result in a change of the swimming direction. Thus, exact positioning of the eyespot and the two ChRs relative to the cilia is of fundamental importance for the observed precision and reproducibility of the phototactic response to a given light stimulus. In contrast, the non-directional photophobic response, which is only observed upon an intense and sudden change in the light intensity, requires both, the Ca^{2+} and H^+ conductivity of the ChRs. It is accompanied by a massive depolarization of the ciliary membrane, activation of voltage-gated Ca^{2+}-channel(s) therein, and a brief Ca^{2+}-dependent change in the ciliary beating pattern (Baidukova et al., 2022; Govorunova & Sineshchekov, 2023; Kreimer et al., 2023).

Due to its central role for the cell to find optimal conditions for photosynthesis and avoiding photodamage, the regulation of the phototactic sensitivity must be tightly controlled. However, in contrast to other

photoprotective mechanisms in *Chlamydomonas* (e.g. Erickson et al., 2015; Ruiz-Sola et al., 2023), our understanding of the*in vivo* homeostatic regulation of the phototactic sensitivity and the two ChRs is still in its infancy. Besides the well-studied fast photoreceptor currents of the ChRs and their molecular properties in ectopic expression systems (Baidukova et al., 2022; Govorunova & Sineshchekov, 2023; Vierock & Hegemann, 2023) only a few additional molecular components and regulatory switches are known so far (reviewed by Kreimer et al., 2023). Briefly, both the expression of the ChRs and the phototactic behaviour exhibit a strong diurnal phasing. The highest phototactic sensitivity is observed in the morning and the ChR transcript levels peak at the end of the night phase. They decrease massively within the first hour after light exposure (Mittag et al., 2005; Zones et al., 2015). Their protein abundance also decreases in the light, with ChR1 being more sensitive and faster degraded than ChR2. Notably, the ChR1 degradation - but not that of ChR2 - is under the control of the blue-light receptor phototropin (PHOT), a COP1-SPA1 E3 ubiquitin ligase and the transcription factor Hy5. Further, the cyclic AMP (cAMP) level and the cellular redox poise affect the ChR1 degradation (Wolfram et al., 2023). Another rapid and effective sensitivity control mechanism is the regulation of its ion conductivity by reversible posttranslational modifications (PTMs). Both ChRs are phosphoproteins and it is assumed that fast reversible phosphorylation plays here a role (Wagner et al., 2008; Böhm et al., 2019). ChR1 phosphorylation responds to several key physiological stimuli known to have an impact on the phototactic sign and is regulated via a Ca^{2+}-based feedback loop. The involved kinase(s) are not yet known, but PHOT can be excluded (Böhm et al., 2019). However, PHOT is involved in the long-term desensitization by acting as a negative regulator of the eyespot size. This is most likely achieved through its regulatory function on the ChR1 level. ΔPHOT strains exhibit a decreased ChR1 degradation rate and no light regulation of the eyespot size. On the other hand, strains overexpressing the PHOT kinase domain and ChR1 deletion mutants have significantly smaller eyespots (Trippens et al., 2012; Greiner et al., 2017; Wolfram et al., 2023). Notably, several of the signalling components involved in the regulation of ChR1 levels and the phototactic sign are also central regulators of general photoprotective mechanisms in *Chlamydomonas* (Boonyareth et al., 2009; Gabilly et al., 2019; Petroutsos et al., 2011, 2016; Ruiz-Sola et al., 2023; Trippens et al., 2017; Wolfram et al., 2023).

The correct placement of the eyespot and the ChRs in relation to the cilia is a crucial prerequisite for phototaxis. Currently only a rudimental picture of the involved secretory pathway, targeting mechanism(s) and molecular components can be drawn. Of central importance is the D4 rootlet, one of the four acetylated microtubular rootlets that originate from the basal body (BB) region and are connected to the ciliary apparatus. The eyespot is always associated with this rootlet at a roughly equatorial position and the ChRs move along it towards the eyespot (Holmes & Dutcher, 1989; Mittelmeier et al., 2011; Thompson et al., 2017). ChR1 has also been detected in the cilia (Awasthi et al., 2016). The functional significance of this localization is not known. It has been suggested to be important for the photophobic response via complex formation with a voltage-gated Ca^{2+} channel, which co-localizes with ChR1 in the eyespot and cilia (Sanyal et al., 2023). Additionally, a dynamic redistribution of ChR1 between both organelles during transitions from light to dark and vice-versa has been reported (Awasthi et al., 2016). These ChR1 allocations coincides exactly with the times when the greatest fluctuations in its abundance occur either due to its light-induced degradation in the morning or eyespot disassembly at the beginning of the night phase prior to cell division (Holmes & Dutcher, 1989; Wolfram et al., 2023). It could therefore also be related to associated transport and degradation processes of this photoreceptor.

In vertebrate photoreceptors, a transport machinery called intraflagellar transport (IFT) involving the motor-proteins kinesin-2 and cytoplasmic dynein, is responsible for the transmission of opsin-containing vesicles through the connecting cilia. The C-terminus of rhodopsin is important for this transport (Tai et al., 1999; Concepcion et al., 2002; Williams, 2002; Keady et al., 2011; Trivedi et al., 2012; Kong et al., 2013). Immunofluorescence analyses and immune-precipitation data indicate that ChR1 transport and targeting also involves a kinesin-2 dependent mechanism and that CR1 interacts with LC8, a component of axonemal and cytoplasmic dyneins, and other dynein complex components. Further, components from both IFT complexes and a structural maintenance of chromosome protein (SMC) were among the co-precipitated proteins and co-localize with ChR1 (Awasthi et al., 2016). Members of the SMC protein-family are involved

in the regulation of protein transport in primary cilia of vertebrate photoreceptors and other cell types in addition to their function in chromosome dynamics (Khanna et al., 2005). An SMC is also present in the cilia of*Chlamydomonas* (Pazour et al., 2005). Notably, the ChR1 C-terminus contains the ciliary targeting motif VXPX (Sharma et al., 2023). In vertebrates the small GTPase ARF4 binds to the VXPX motif of rhodopsin and is involved in the formation of rhodopsin transport vesicles at the*trans* -Golgi network (TGN) as well as the retrograde transport from endosomes to it (Deretic et al., 2005; Mazelova et al., 2009; Li & Hu, 2011; Nakai et al., 2013; Sung & Leroux, 2013; Deretic et al., 2021). The *Chlamydomonas* orthologue of ARF4 is ARL11. Immunoprecipitation data suggest that ARL11 interacts with the C-terminus of ChR1. It might thus aid in ChR1 transport to the cilia and eyespot (Sharma et al., 2023). These authors also reported the presence of a C-terminal SUMOylation motif and that an anti-SUMO2 antibody precipitates ChR1 and PHOT. SUMO is known to regulate the trafficking and function of many membrane proteins as well as the ciliary localization of several signaling proteins (Zhao 2007; Li et al., 2012; McIntyre et al., 2015). Although the *in vivo* function of the C-terminus of ChR1 is not yet understood in detail, its importance for targeting and regulation is underlined by the following observations: (i) Disturbing it by a fluorescent tag induce ChR1 accumulation in cytoplasmic vesicles and drastically reduce its localization in the eyespot, and (ii) deletion of the last 78 AS completely inhibits light-induced ChR1 phosphorylation and reduces its abundance (Greiner et al., 2017; Böhm et al., 2019). Only a few additional proteins involved in eyespot assembly and placement have been identified and the proteins required for the specific binding and detachment of ChRs from the D4 rootlet are completely unknown (Kreimer et al., 2023).

Also, the ChR degradation pathway has not been investigated in detail. For ChR1, proteasomal proteolysis can be excluded as its specific inhibitors MG-132 and HMB-Val-Ser-Leu-VE have no effect on ChR1 degradation (Böhm & Kreimer, 2021; Fig. S1). ChR1 is quite stable in the dark, but is rapidly degraded upon illumination in an intensity and wavelength dependent manner. Degradation is affected by cAMP, the cellular redox poise and involves PHOT, a COP1-SPA1 E3 ubiquitin ligase and the transcription factor Hy5 (Wolfram et al., 2023). Gene products important for ChR1 degradation regulated by these classical light signalling elements are not yet known.

In this study, we therefore aimed to identify and characterize additional elements involved in ChR1 degradation and targeting to increase our understanding of its homeostatic regulation. For this we combined pharmacological and biochemical approaches, generated knockout mutants of a cysteine protease identified in the eyespot proteome and of ARL11, and used a variety of other already existing mutants. With respect to ChR1 degradation we were able to show that

(i) ChR1 undergoes an enhanced endocytic internalization in the light, accompanied by increased degradation involving the cysteine protease CEP1. Transcription of CEP1 is increased by low light and is controlled by PHOT, a SPA1-E3 ubiquitin ligase and cAMP.

(ii) ChR1 is increasingly modified and forms high molecular mass complexes (HMMCs) before or during its internalization.

(iii) the modified ChR1 forms and HMMCs are secreted in extracellular vesicles (EVs) in the size range of exosomes at the ciliary base and/or PM.

Further, we show that the IFT machinery, ARL11, SUMOylation and the cilia are involved in targeting and regulating the abundance of ChR1. Our study thus delineates important novel elements involved in the homeostasis and targeting of this central photoreceptor for the movement responses of *Chlamydomonas* .

2 MATERIALS AND METHODS

2.1 Strains and culture conditions

Table S1 lists all used *Chlamydomonas* strains together with their sources and abbreviations. Cultures were grown - if not otherwise stated - in liquid minimal or Tris-acetate phosphate medium (TAP; Harris, 2008) as described (Wolfram et al., 2023), except that cultures used for isolation of cilia or EVs were grown with aeration.

Generation of mutant strains with CRISPR-Cas9 and verification of Clip mutants

CEP1 and ARL11 mutant strains were generated in the CC-3403 background and screened as described (Greiner et al.,2017; Kelterborn et al., 2022). Figure S2 summarizes their characterization by next generation sequencing. Mutants LMJ.RY0402.060340 (*spa1* , characterized by Tokutsu et al., 2019) and LMJ.RY0402.239561 (*cal3*) were obtained from the *Chlamydomonas* Resource center (Li et al., 2019). For*cal3* insertion of the CIB1 cassette was verified in three randomly chosen single-clone derived colonies by genomic PCR according to the CLiP library instructions (see Table S2 for primers).

2.2 Inhibitors & Antisera

Cycloheximide (CHX, Applichem), PD 145305 and PD 150606 (Cayman) were dissolved in ethanol. 3-Isobutyl-1-methylxanthine (IBMX), 4-hydroxy-2,2,6,6-tetramethyl-piperidine 1-oxyl (TEMPOL), Colchicine (CLC), and Pitstop-2 (all Sigma) were dissolved in DMSO or TAP, respectively. CK-666, CK-689 (Merck), 2-D08, E-64d (MedChemExpress), and PR-619 (Cayman) were dissolved in DMSO. Used inhibitor concentrations refer to a cell density of 4 x 10^6 cells/mL. Cell viability was checked for all inhibitors by differential interference microscopy at the beginning and end of experiments. The antibody against acetylated tubulin (clone 6-11B-1) was from Sigma. Alexa Fluor^{TM} 488 goat anti-rabbit (H+L) and 555 goat anti-mouse IgG (H+L) were from Invitrogen. For all other used antisera see Wolfram et al. (2023).

2.3 Protein electrophoretic based methods

Determination of ChR levels and its light-induced degradation, protein extractions, SDS- and piperazine diacrylamide (PDA)-PAGE, immunoblotting and quantification, were done as described (Wolfram et al., 2023).

2.4 Isolation and sub-fractionation of cilia

Cilia, if not otherwise stated, were isolated from cultures at the end of the night phase. All manipulations prior to illumination were done under dim red safety light. Isolation of highly purified cilia using dibucaine and their subfractionation followed the protocol of Witman (1986). Proteins were precipitated according to Schmidt et al. (2006) and solubilized in 2x SDS-PAGE sample buffer supplemented with 4 M urea. For quick cilia isolation a modification of the method of Finst et al. (1998) was used. At the end of the night phase 450 million cells were pelleted (2,000 *g* , 5 min,15 °C) and suspended in 30 mL TAP + 1 mM PMSF. From these suspensions aliquots (10 mL) were placed in standard glass Petri-dishes and either kept in the dark or illuminated for the indicated times with WL+UV (188 µmol photons $m^{-2}s^{-1}$; T8 Reptisun® 10.0UVB, ZooMed Laboratories). Deciliation was induced by rapid addition of 10 mL acetate buffer (40 mM Na-acetate, pH 4.5; 8% (w/v) sucrose; 1 mM $CaCl_2$). The pH was neutralized after 90 sec of agitation with 0.5 M NaOH. Cell bodies were separated (1,100 *g* , 5 min, 4 °C), the cilia were pelleted from the supernatant (12,250 *g* , 15 min, 4 °C) and suspended in TAP. Protein precipitation and solubilization was done as described above.

2.5 Isolation of extracellular vesicles

Six days old cultures (4.8 or 9.6 L) were concentrated prior to onset of illumination by centrifugation, and suspended in 400 or 800 mL fresh TAP supplemented with 1 mM PMSF under dim red safety light. Aliquots (200 mL) were transferred into glass Petri-dishes (20 cm diameter) and either kept in the dark or illuminated for the indicated times with WL + UV (188 µmol photons m^{-2} s^{-1}; 16 °C). When different strains were compared whenever possible identical total cell numbers were used per Petri-dish (7.5 billion/200 mL). Cells were pelleted (6,000 *g* , 10 min, 16 °C) and the supernatants were cleared further (11,000 *g* , 10 min, 16 °C) prior to ultracentrifugation (100,000 *g* ; 2 h, 4°C). The pellets were suspended in 3 mL TAP, washed to remove non vesicle-bound proteins via a second round of ultracentrifugation (200,000 *g* , 1h, 4°C) and finally suspended in an equal volume of 4x SDS-PAGE sample buffer supplemented with 6 mM tributylphosphine (TBP).

2.6 Indirect immunofluorescence, differential interference microscopy, eyespot area, cilia length and swimming speed determinations

For indirect immunofluorescence analysis cells at the end of the night phase from 40 mL culture were pelleted, suspended in PBS, allowed to settle on poly-L-lysine (0.1%) coated multiwall slides for 10 min and fixed for 4 min with formaldehyde (3.7%). Slides were dipped in PBS, submerged into -20 °C ethanol for 10 min, air-dried (30 min RT), rehydrated with blocking buffer [6% (w/v) fish gelatin, 1% BSA (w/v), 0.05% Tween20 (v/v) in PBS], and incubated overnight at 4 °C with the primary antibodies in blocking buffer (anti-ChR1 1:50; anti-acetylated tubulin 1:500). Slides were washed for 5 min in PBS, 4x in blocking buffer, stained with anti-rabbit Alexa-Fluor 488 and anti-mouse Alexa-Fluor 555 (1:1000 in blocking buffer), washed 2x in PBS, 2x in blocking buffer, and mounted (SlowFade Diamond Antifade, ThermoFisher Scientific). Images were acquired with an Axio Imager.M2 equipped with an ApoTome.2 module using a 63x objective (1.4 NA oil immersion, Plan Apochromat) as stacks of multiple optical sections. Maximum intensity projections were calculated with the ZEN blue software (all Carl Zeiss AG). Differential interference microscopy and eyespot measurements were carried out with unfixed cells as described (Wolfram et al., 2023). Cilia length measurements were done with Lugol fixed cells using phase contrast microscopy (Eclipse 800 microscope, 100x objective, 1.4 NA oil immersion, Plan Apochromat, Nikon). Pictures were captured with a DS-Qi1 cooled CCD camera driven by NIS-Elements BR 3.1 software (Nikon). Length measurements were conducted on one cilium per cell with the same software.

To determine the swimming speed, the cell suspension (1 x 10^5 cells/ml in TAP) was dark-adapted (1 h) prior to the measurement. Cells were imaged in a fluorescence cuvette with 1 mm gap and four polished sides under an Olympus IX73 inverted microscope using 20x objective and a dark-field Olympus (IX-ADUCD) condenser. The cell movements were recorded by a high-speed Zyla sCMOS camera (Andor, Oxford Instruments) using the software Solis I. The velocity of single cells was analyzed with the plugin TrackMate (Tinevez et al, 2017) of ImageJ2. Each cell was segmented in every recorded frame and its movement path was reconstructed as a track by allocating it an identity over all recorded frames. In the plugin, the filters for duration and displacement of tracks were set for 4 s and 80 µm.

2.7 Whole mount electron microscopy

EV pellets were suspended in sterile-filtrated PBS and mixed 1:1 with 4% formaldehyde in PBS. Aliquots (5 µl) were applied onto UV-irradiated 300-mesh formvar/carbon-coated grids, allowed to absorb for 10 min and further processed according to Baur et al. (2023) except that 4% uranyl acetate was used for staining. Further, incubation with methyl cellulose-uranyl acetate was omitted. Formaldehyde (4%) treated grids served as controls. Samples were examined with an EM10 electron microscope (Zeiss) equipped with a SC1000 Orius^{TM}CCD-camera controlled by the DigitalMicrograph^{TM}software 3.1 (GATAN).

2.8 RNA isolation and messenger RNA quantification

Culture growth, total RNA isolation and RT-qPCR was done as described (Wolfram et al., 2023). Primers for *CEP1* , *Hy5* , and*α-Tυβυλιν* (Table S2) were designed using NCBI Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye et al., 2012). Relative gene expression was analyzed according to Livak & Schmittgen (2001) with *α-Tυβυλιν* as housekeeping gene for normalization.

2.9 Data analysis and image processing

Biological replicates refer to independent cultures inoculated into different flasks from a starter flask. Immunoblot quantification, statistical analyses and image processing was done as described (Wolfram et al., 2023). EV size distributions with manual adjustments of the seeds and boundaries were determined using the TEM ExosomeAnalyzer (Kotrbová et al., 2019).

3 RESULTS

3.1 ChR1 degradation involves the endocytosis pathway

The ChR1 sequence contains typical signals for clathrin-mediated endocytosis (CME) and lysosomal targeting (Fig. S3). We thus analyzed the effect of Pitstop-2, a widely used inhibitor of CME and clathrin-independent endocytosis (CIE; Dutta et al., 2012), on light-induced ChR1 degradation (Fig.1A-B). Pitstop-2 incubation

of cells at the end of the night-phase led to a shift of the ChR1 monomer band by ˜ 2 - 10 kDa upon illumination. Hereafter we call these forms modified ChR1 ($ChR1_{mod}$). Additionally, ChR1-containing HMMCs were formed. These responses occurred also in dark kept cells, but substantially less pronounced (Fig. 1A). Identical effects on ChR1 were described for TEMPOL-induced inhibition of light-induced ChR1 degradation. Further, absence of all signals in ΔChR1 strains demonstrates signal specificity (Wolfram et al., 2023; Fig. 5E-J). Quantification of the total ChR1 signals revealed that light-induced degradation was inhibited by Pitstop-2 (Fig. 1B). Such shifts in the apparent M_r indicate PTMs like e.g. SUMOylation and mono-, multi-mono- or poly-ubiquitination, which are important for transport, localization, activity and degradation of membrane proteins (Foot et al., 2016; Sundvall, 2020). Notably, application of PR-619, a broad-spectrum inhibitor of deubiquitinating enzymes (DUBs), which also inhibits SUMO-specific proteases (Kitagawa & Takiya, 2020), had similar effects on light-induced ChR1 degradation as Pitstop-2 (Fig. 1C-E).

The actin-related protein 2/3 (Arp2/3) complex is involved in endocytosis of membrane proteins in *Chlamydomonas* (Bigge et al., 2023). To further support the above described findings, we used CK-666, an Arp2/3 inhibitor. CK-666 reduced light-induced ChR1 degradation in comparison to its inactive analog CK-689 and the DMSO control, but did not completely prevented it (Fig. 1F-G). Additional presence of PR-619 completely inhibited degradation and induced again the accumulation of $ChR1_{mod}$ forms (Fig. 1H-I). Their abundance is albeit somewhat reduced compared to the cell-wall less strain with the same PR-619 concentration, probably due to a reduced accessibility of PR-619 in strains with a cell-wall. In summary, these data indicate that light-induced ChR1 degradation is accompanied by its modification and possibly complex formations. Preventing endocytosis (Pitstop-2 and CK-666) and inhibiting DUBs and/or SUMO-specific proteases (PR-619) led to an increased accumulation of these forms in the light.

3.2 At least two cysteine proteases are involved in ChR1 endocytosis and degradation

In order to identify potential proteases involved in light-induced ChR1 degradation we screened the eyespot proteome of *Chlamydomonas*.Most identified proteases were related to the chloroplast and only three to the secretory pathway (Schmidt et al., 2006; Eitzinger et al., 2015). One is the hatching enzyme sporangine and therefore was excluded. The second (Cre12.g508500) is a metalloprotease with highest mRNA expression levels in the night phase, and thus is also a less likely candidate. The third is a cysteine endopeptidase (Cre09.g407700; CEP1). We thus first applied two cell-permeable inhibitors of cysteine proteases: E-64d, a broad-spectrum cysteine protease inhibitor, and PD 150606, which inhibits calpains. Several members of these calcium-dependent, non-lysosomal cysteine proteases are present in *Chlamydomonas*(Zhao et al., 2012). E-64d at the highest possible concentration without disturbing the cell viability had no significant effect (Fig. S4). In contrast, PD 150606 induced again accumulation of $ChR1_{mod}$ forms similar to those observed in Pitstop-2 and PR-619 treated cells (Fig. 2A). Signal quantification revealed complete inhibition of light-induced ChR1 degradation, whereas its inactive analog PD 145305 had no effect (Fig. 2B-C). Analysis under non-reducing conditions uncovered that PD 150606 treatment induced independent of illumination the formation of ChR1-containing complexes with M_r´s well above those of the HMMCs, which were not able to enter even a 6% PDA-SDS-PAGE gel (Fig. S5A-B). Coomassie staining of these samples revealed no significant differences. Thus, unspecific effects like general protein cross-linking are unlikely. A similar, but much less pronounced, effect was observed in illuminated samples for the DUB inhibitor PR-619 (Fig. S5C-D). Notably, calpains are involved in the regulation of CME and PD 150606 led to a reduced formation of endocytic vesicles in mammalian cells (Rudinskiy et al., 2009; Voronina et al., 2015). This might explain the here observed similar effects of these inhibitors on ChR1 degradation.

CEP1, however, is a papain-type C1A family member and not a calpain. To elucidate if it is involved in ChR1 degradation we analyzed three independent *CEP1* knockout strains. All of them have the insertion in the first exon which, if residual expression should occur, will lead to a likely non-functional severely truncated CEP1 (Fig. S2). All strains exhibited a nearly complete inhibition of the light-induced ChR1 degradation without an increased generation of $ChR1_{mod}$forms (Fig. 3A-B). In parallel, we observed a significantly increased total ChR1 level and eyespot area (Fig. 3C-D), whereas the growth and pigment content were unaffected (Fig. 3G-H). Notably, light-induced degradation of ChR2, the second transmembrane eyespot-localized receptor for

phototaxis, was not significantly affected, although its level was also increased (Fig. 3E-F). This surprising specificity for ChR1 makes an unspecific effect on endocytosis or general protein degradation by CEP1 deletion unlikely. To further rule out potential unspecific effects we also analyzed ChR1 degradation in two mutant strains of cysteine proteases not related to the eyespot: FAP246, an axonemal protein with papain-like and EF-hand domains involved in motility control of the cilia (Cai et al., 2021), and the calpain CAL3. In mutant strains of both, ChR1 degradation was unaffected (Fig. S6A-B). In summary, these data indicate that at least two different cysteine proteases are involved: CEP1 and a yet unidentified calpain, which probably acts indirectly via a regulatory role in the formation of endocytic vesicles. Further, these and data which will be described later support the idea that the $ChR1_{mod}$ forms and HMMCs are related to ChR1 sorting and incorporation into endocytic vesicles.

3.3 Expression of CEP1 is increased by low light and regulated by PHOT, SPA1 and cyclic nucleotides

To analyze if light affects the *CEP1* mRNA levels, cells were either grown at 60 µmol m^{-2} s^{-1} of WL for five days and then shifted at the beginning of the light phase to the indicated intensities or were grown over the whole period under these WL intensities. A significant increase in the mRNA occurs in both experimental setups at 60 µmol $m^{-2}s^{-1}$ WL compared to the dark samples. Higher intensities did not induce a further increase (Fig. 4A-B). In contrast, the mRNA levels of the transcription factor *Hy5,* which acts in conjunction with an upstream E3 ubiquitin ligase complex as one central regulator of light signaling also in *Chlamydomonas* (Gabilly et al., 2019; Lämmermann et al., 2020), further rise with increasing intensities (Fig. 4C-D). Saturation for *CEP1* mRNA light induction thus occurs at already relatively low WL intensities. This behavior was seen in another widely used laboratory strain (Fig. S7), indicating that this is a typical response of *Chlamydomonas* . It is noteworthy that in high-light-adapted cells, *CEP1* mRNA levels were already increased in the dark and did not increase further in the light phase, while HY5 expression also increased in these cells in the light (Fig. 4B-D).

The photoreceptor PHOT, a COP1-SPA1 E3 ubiquitin ligase and cAMP were pinpointed as key components controlling the cellular ChR1 abundance (Wolfram et al., 2023). Genes involved in ChR1 degradation controlled by this system are still not known. We thus asked if *CEP1* expression might be under their control. In the ΔPHOT strain the *CEP1* mRNA level and its light regulation were strongly reduced compared to the parental strain (Fig. 4E). A residual light stimulation, however, still occurred, indicating involvement of an additional light-dependent regulatory element. In line with these observations, the *CEP1*mRNA levels were significantly increased both in the dark and in the light in the *spa1* mutant (Fig. 4F). Further, inhibition of phosphodiesterases by IBMX, which results in a rise of cAMP but not cGMP in *Chlamydomonas* (Boonyareth et al., 2009), induced a significant increase in the *CEP1* mRNA in the dark (Fig. 4G). Notably, IBMX reduces the ChR1 protein starting level by ˜ 40% under identical conditions (Wolfram et al., 2023). In summary, *CEP1* is thus one of the downstream targets important for ChR1 degradation regulated by these central components of the photoprotective pathways in*Chlamydomonas* .

3.4 ChR1 and its modified forms are present in cilia

Awasthi et al. (2016) reported a dynamic redistribution of ChR1 between the cilia and eyespot during transitions from light to dark and vice-versa, i.e. exactly when the greatest fluctuations in the ChR1 levels occur. We thus surmised that cilia may play a role in ChR1 homeostasis. Despite the presence of ciliary targeting motifs in the C-terminus of ChR1 (Fig. S3; Sharma et al., 2023), its localization in the cilia was not reported in the numerous previous ChR1 localization studies (e.g. Mittelmeier et al., 2011; Thompson et al., 2017). We thus first verified the presence of ChR1 in the cilia with independent methods. Indirect immunofluorescence clearly detected ChR1 in the cilia (Fig. 5C-D), besides the well documented localization in the eyespot, vesicular structures in the BB region and along the ciliary roots and microtubules (Fig. 5A-B). This result was confirmed by immunoblot analysis of cilia isolated from different strains, demonstrating the expected enrichment in the ciliary membrane/matrix fraction. No signals occurred in cilia and crude extracts of a ΔChR1 strain validating signal specificity (Figs. 5E-F, S8A; Wolfram et al., 2023). Based on an equal protein load the ChR1 concentration in the cilia was clearly below that of the crude extract and

its level also rapidly declined upon illumination (Fig. 5G-H). ChR2 was also present in isolated cilia and its concentration also declined in the light (Fig. S8B). The ciliary ChR levels thus responded to illumination comparable to those in whole cells (Wolfram et al., 2023).

Next, we checked if the $ChR1_{mod}$ forms and HMMCs also occur in cilia. We thus isolated cilia and cell bodies from dark-kept or illuminated cells and of cells treated with TEMPOL or Pitstop-2 prior to illumination, as the latter two treatments massively induced the accumulation of these ChR1 forms in the light. $ChR1_{mod}$and HMMCs were detected in both, the cell bodies and the cilia fraction (Figs. 5J, S9). Additionally, the cilia from TEMPOL and Pitstop-2 treated cells differed in their total protein composition from the controls. Both exhibited comparable changes in the relative abundance of several proteins. However, also in the cilia of untreated cultures with an identical ciliary protein pattern, a proportion of the ChR1 exhibited a small but clear M_r shift (Fig. 5I). Thus, also the $ChR1_{mod}$ forms as well as the HMMCs can enter or are formed in the cilia. To get hints about the potential role(s) of cilia in ChR1 homeostasis we next used mutants with defects in ciliogenesis.

3.5 Light-induced ChR1 degradation is increased in mutants with defects in ciliogenesis

In several mutants affected in IFT components and the involved motor proteins, the ChR1 transport to the eyespot and cilia is restricted and ChR1-containing vesicles accumulate in the cells subapical region and close to the BBs (Awasthi et al., 2016). Decreased cellular ChR1 levels were thus expected and we surmised that also the light-induced degradation might be affected. We thus analyzed selected IFT and motor protein mutants for both parameters. Deletion of the IFT particle component IFT88 results in cells lacking cilia and ChR1 vesicle accumulation in the anterior region of the cell and close to the BBs (Awasthi et al., 2016; Pazour et al., 2000). We observed a reduced ChR1 starting level and an increased light-induced degradation rate in the ΔIFT88 mutant (Fig. 6A-C). Identical effects were observed in motor protein mutants defective either in retrograde (ΔLC8, which lacks this component of axonemal and cytoplasmic dyneins; Fig. 6F-G) or anterograde IFT (Δ*Kin2,* a kinesin-2 null mutant and its temperature-sensitive point mutant *Fla10-1* ; Fig. 6H-K). These mutants also do not form cilia and lead to ChR1 vesicle accumulation in the anterior region of the cell (Awasthi et al., 2016). In contrast, ChR1 levels and its degradation were not significantly impaired in the strain *bbs4-1*(Fig. 6D-E). IFT and cilia are normal in this strain, which is mutated in an IFT cargo adaptor needed for cilia export of selected signaling proteins (Lechtreck et al., 2009). ChR1 levels and degradation were also not significantly affected in strain Δ*FAP246* (Fig. S6A), which have motile cilia with a defect in the C1b projection of the central apparatus (Cai et al, 2021).

Based on these data we suspected that not only a functional IFT but also the presence of cilia might have an impact on the cellular homeostasis of ChR1. We thus additionally analyzed ciliogenetic mutants with one or no cilia, but not directly affected in IFT or motor proteins (Fig. 7). The *uni1* mutant, which possess only one cilium associated with the *trans* BB (Sale & Dutcher, 2023), exhibited a clear reduction in the ChR1 level and a slightly enhanced light-induced degradation compared to the parental strain. Both parameters were even more affected in two different *bld* mutants lacking both cilia (*bld2* and*bld7;* Fig. 7A-B). Notably, in two independent cilia-possessing suppressor mutants of *bld7* this effect was reverted (Fig. 7C-E). In parallel, the eyespot size increased significantly from 0.546 +/- 0.009 μm^2 in strain CC-2906 to 1.195 +/- 0.034 μm^2 in the suppressor mutant CC-2908 (n: 102 cells; unpaired t-test: $p < 0.0001$).

In many cilia-less mutants the organization of cytoskeletal elements is affected. For instance, *bld2* cells are defective in ε-tubulin, lack BBs and have disorganized microtubular roots (Dutcher et al., 2002; Ehler et al., 1995). We thus additionally analyzed ChR1 degradation in wild type cells pre-incubated with colchicine (CLC) for a short time period. Cells treated with CLC are not able to form cilia after deciliation, but possess a normal cytoskeleton (Rosenbaum et al., 1969). The ChR1 degradation rate in these cells was somewhat faster and also the final level in the light was slightly lower than in the identically treated controls without deciliation (Fig. 7F). Since the ChR1 starting levels were not significantly affected by this treatment (Fig. S10), these effects can most likely be attributed to the lack of cilia and not to unspecific CLC side effects. In summary, these data indicate that beside an undisturbed vesicle transport also the presence of cilia has an impact on the ChR1 turnover.

3.6 Inhibition of SUMOylation leads to a decreased ciliary level of ChR1

ChR1 contains a C-terminal SUMOylation motif and was among the proteins precipitated with an SUMO2 antibody (Fig. S3; Sharma et al., 2023). We thus used 2-D08, a cell permeable inhibitor of transfer of SUMO from the E2 thioester to its targets (Kim et al., 2014) and analyzed its effect on degradation and ciliary localization of ChR1. In crude extracts no significant effects on the total ChR1 levels or its light-induced degradation were evident (Fig. 8A-B). However, an average reduction of the ChR1 level by 42% was resolved in cilia isolated from 2-D08 treated dark-kept cells. In the cell bodies the level decreased only by 14%. This effect was also evident in cilia regenerated in the presence of 2-D08 after deciliation (Fig. 8C). In these cilia additionally ChR1 degradation products were resolved and their general protein pattern differed in the relative abundance of a few proteins when compared to the control. These data are in accordance with previous observations that disturbing SUMOylation affects the ciliary protein composition. The activity of the small GTPase ARL13b, which is known in vertebrates for its role in ciliary import and export of specific subsets of membrane and membrane-associated proteins, is affected by SUMOylation. The *Chlamydomonas arl13* mutant possess normal cilia, but is impeded in BBSome-dependent protein transport and phototaxis (Li et al., 2012; Dai et al., 2022). However, as in the *bbs4-1* mutant, neither the ChR1 levels nor the light-induced degradation were significantly affected in the *arl13* mutant (Fig. S11). Besides ARL13 additional members of these key molecular switches are known to be involved in ciliary targeting (Li & Hu, 2011). For example, in vertebrate systems the small GTPase ARF4 binds to the ciliary targeting motif VXPX and is involved in the formation of rhodopsin transport vesicles at the TGN. Further, ARF4 takes part in the retrograde transport from endosomes to the TGN (Deretic et al., 2005; Mazelova et al., 2009; Li & Hu, 2011; Nakai et al., 2013; Sung & Leroux, 2013; Deretic et al., 2021).

3.7 Deletion of the small GTPase ARL11 results in a decreased cellular ChR1 level

The *Chlamydomonas* orthologue of the human ARF4 is ARL11 (Sharma et al., 2023). These authors reported that ARL11 physically interacts with the ChR1 C-terminus and suggested that it might aid in ChR1 transport to the cilia and eyespot. To get first *in vivo* evidence for this suggestion, we generated two independent *arl11* deletion mutants: *arl11* -A has the insertion at the end of switch II and *arl11* -B at the end of the first G-Box (Fig. S2). Both strains, if residual expression should occur, will thus express only a non-functional protein. In both mutants the ChR1 levels were significantly reduced to a similar level, whereas its light-induced degradation rate was normal (Fig. 9A-C). This reduction was accompanied by a significant decrease in the eyespot area, indicating that the ChR1 targeting to the eyespot is affected (Fig. 9D-E). Further, although the swimming speed and cilia length of both *arl11* mutants were normal, the ChR1 level in the cilia was even more reduced than that in the cell bodies (Fig. 9F-G). Thus, our data support the suggestion that ARL11 plays an important role in ChR1 transport and targeting, although it is not solely responsible due to the residual ChR1 levels still present in the cilia.

3.8 $ChR1_{mod}$ and HMMCs are present in extracellular vesicles

Cryo-electron tomography revealed close to the eyespot uni- and bi-lamellar EVs (Engel et al., 2015). Böhm & Kreimer (2021) suggested that they may be related to ChR degradation and may represent exosomes released by fusion of multi-vesicular bodies (MVBs) from the endosomal pathway with the PM. To confirm this suggestion, we isolated EVs and analyzed them for the presence of ChR1. We detected $ChR1_{mod}$ forms and HMMCs in EVs from eight different strains. Notably, the unmodified ChR1 was never present (Figs. 10,S12). Further, in ΔCEP1 EVs these forms were enriched compared to those from the parental strain CC-3403, independent from the presence of a broad-spectrum protease inhibitor during their secretion and purification (Fig. 10B). Time-course analyses resolved that illumination led to both, a gradual increase in their abundance and the apparent M_r shift in these strains (Fig. 10C-D). Additionally, the proportion of ChR1 degradation products was lower in the EVs from ΔCEP1. Between strains the ChR1 turnover appears to differ, since the detectable $ChR1_{mod}$ amounts in EVs of other strains were already slightly higher in the dark and decreased somewhat faster upon illumination (Fig. S12A). However, compared to the cellular ChR1 level, the amounts secreted via the EVs appear to be extremely low. The total EV protein yield from a billion cells varied between 10 to 26 µg and based on the Coomassie staining ChR1 represents only a minor

portion.

Chlamydomonas also secretes EVs at the ciliary base, tip and along the whole cilia length (Vinay & Belleannée, 2022). As we observed a higher ChR1 turnover in cilia lacking strains, we surmised that the majority of the $ChR1_{mod}$ in EVs might be secreted at the ciliary base and/or the PM rather than along the cilia. We thus isolated EVs from CC-2906, a *bld7* mutant, and its cilia-possessing suppressor mutant CC-2908 to see whether they differ with respect to their $ChR1_{mod}$ pattern or other characteristics. Electron microscopy showed the typical appearance of negatively stained EVs for both but pinpointed clear differences in the size distributions (Fig. 11A-K). Although both fall in the typical size range of small EVs (below 150 nm; Vinay & Belleannée, 2022), those from the ciliated CC-2908 exhibited a broader size distribution, with a peak at 25 – 30 nm and a large proportion (68%) above 35 nm. The EVs of the *bld* strain CC-2906 peaked at 20 – 25 nm and 71 % of the population was below 35 nm (Fig. 11K). Further, the number was higher in the EV preparation of CC-2906 (Fig. 11L). Additionally, their protein composition differed. Whereas a complex pattern was resolved for EVs from ciliated strains, that of the cilia lacking strains CC-2906 and *bld-2* revealed enrichment of fewer proteins and were very similar (Figs. 10C-D,11M). The $ChR1_{mod}$ forms were, however, present in EVs of both strains. Also, no significant differences were observed in time-course experiments except that the modified ChRs were detectable for a longer time and their fraction appeared larger in the EVs of the cilia-less strain, which coincides with the higher degradation rates in *bld* -strains (Figs. 7C-D, S12B).

4 DISCUSSION

Chlamydomonas capability to perceive and process light signals for its orientation depends on the light-gated Ca^{2+}- and H^+-conducting photoreceptors ChR1 and ChR2 and their precise positioning in the eyespot (Sineshchekov et al., 2002; Greiner et al., 2017; Baidukova et al., 2022; Kreimer et al., 2023). Despite this pivotal role, our understanding of their homeostasis is still in its infancy. In this study, we therefore investigated aspects of light-induced degradation and targeting of ChR1 in more detail.

We established here that ChR1 internalization occurs via endocytosis and that light stimulates this process and the formation of modified ChR1 forms by using two mechanistically different inhibitors, Pitstop-2 and CK-666. Both affect CME and CIE and interfere with endosome formation also in *Chlamydomonas* (Bigge et al., 2023; Dutta et al, 2012; Chakrabarti et al., 2021; Papalazarou & Machesky, 2021). Thus, ChR1 internalization cannot be assigned unequivocally to one of these pathways. However, the presence of YXXØ motifs which target proteins to clathrin-coated pit regions favors CME. One YXXØ motif is within the distance from the last TMD typical for fast lysosomal targeting, and is followed by an acidic cluster with predicted phosphorylation motifs. Such clusters often act as endosome to TGN retrieval signals (Bonifacino & Traub, 2003; Moore et al., 2007, Chen et al., 2011; Arora & Van Damme, 2021). In congruency, we observed inhibited ChR1 degradation upon blocking endocytosis, whereas classical proteasome inhibitors have no effect. Involvement of combined intramembrane proteolysis and the proteasome in ChR1 degradation is thus unlikely. Notably, the motif for fast lysosomal targeting and the preceding dileucine-based signal are missing in ChR2 (Fig. S3), which partially explains the differences to ChR1 in its light-induced turnover (Fig. 3B,E; Wolfram et al., 2023).

Several further observations listed and discussed below support ChR1 turnover via the endosomal pathway and complement our understanding of its targeting.

(i) EVs contain ChR1 and its abundance therein dynamically changes with illumination. They are in the typical size-range of exosomes and cryo-electron tomography demonstrated uni- and bilamellar EVs close to the eyespot (Colombo et al., 2014; Engel et al., 2015; Vinay & Belleannée, 2022). However, ChRs are also present in the cilia, which secret EVs via their base, tip and along their entire length (Figs. 5C-H,S8; Awasthi et al., 2016; Vinay & Belleannée, 2022). Ciliary EVs have a unique protein composition, being among others enriched in ESCRT proteins, small GTPases and ubiquitinated proteins (Long et al., 2016). Our data demonstrate that ChR1-containing EVs are mainly secreted via the PM and/or the ciliary base. Notably, the EVs contain only $ChR1_{mod}$ and HMMCs. This underlines our suggestion that these forms are specifically

related to ChR1 sorting and degradation (Wolfram et al., 2023). In cells, these ChR1 forms accumulate in the light when endocytosis or DUBs and cysteine proteases are blocked (Figs. 1,2; Sharma et al., 2023). Ubiquitination and SUMOylation of membrane proteins are important signals for their targeting, endocytosis and degradation. Removal of these PTMs prior to the final degradation is required also for membrane proteins (Foot et al., 2016; Schwihla & Korbei, 2020; Sundvall, 2020). Thus, when protein synthesis is blocked, inhibiting proteases with specificity towards these PTMs leads to accumulation of the modified proteins. In parallel, lack of supply of the modifiers through recycling prevents target tagging and thereby additionally stabilizes the target proteins (Rossio et al., 2021; Snyder & Silva, 2021). However, also non-proteinaceous modification of membrane proteins can facilitate the formation of SDS-stable receptor aggregates (Feig et al, 2007).

(ii) Calpain inhibition lowers the secretion of EVs and their specific inhibitor PD 150606 reduces formation of endocytic vacuoles (Voronina et al., 2015; Catalano & O'Driscoll, 2019). Upon PD 150606 treatment we here observed a blocked ChR1 degradation and accumulation of HMMCs. Calpains are non-lysosomal, Ca^{2+}-activated cysteine proteases present also in *Chlamydomonas* (Zhao et al., 2012). Calpain and local Ca^{2+} signaling both regulate endomembrane trafficking (Burgoyne & Clague, 2003; Rudinskiy et al., 2009; Himschoot et al., 2017). Thus, involvement of a calpain-like activity in ChR1 turnover would allow direct coupling of photoreceptor internalization in the eyespot region to the actual light intensity via Ca^{2+}-dependent signaling initiated by ChR activation. The association of ChR1 with a voltage-gated Ca^{2+}-channel reported by Sanyal et al. (2023) might be of importance here. Additionally, ChR1 phosphorylation is regulated by a Ca^{2+}-based feedback loop (Bohm et al., 2019). Phosphorylation is also a signal for endocytosis of membrane proteins, which can either be independent of or work together with ubiquitination to drive sorting (Bonifacino & Traub, 2003; Arora & Van Damme, 2021). Notably, inhibiting ChR1 hyper-phosphorylation with TEMPOL also blocks its degradation and induces $ChR1_{mod}$ and HMMC formation (Fig. 5J, Bohm et al., 2019; Wolfram et al., 2023). Thus, hyper-phosphorylation cannot be responsible for the observed M_r shifts of ChR1. Resolving the nature of the PTM(s) and the HMMC composition is subject of our ongoing analyses.

(iii) CEP1 is the first molecularly unveiled protease essentially involved in ChR1 degradation. It belongs to the papain-type C1A family, members of which are synthesized as inactive precursors in the ER and then usually transported to vacuoles, lysosomes or are secreted. They are activated by cleaving off an inhibitory N-terminal pro-peptide via limited intra- or inter-molecular proteolysis (Martinez et al., 2012). CEP1 lacks an ER retention signal and proteomic approaches detected it in lysosome-related organelles, the soluble secretome and an eyespot fraction (Eitzinger et al., 2015; Luxmi et al., 2018; Long et al., 2023). Its central role for ChR1 homeostasis is pinpointed by the nearly complete loss of light-induced ChR1 degradation and nearly doubled ChR1 levels in the knockout lines. Notably, ChR2 degradation was not significantly affected, making possible general effects on membrane protein internalization and degradation due to CEP1 deletion unlikely (Fig. 3). The same surprising specificity in the control of ChR degradation was observed in ΔPHOT strains. Wolfram et al. (2023) demonstrated that the abundance of ChR1, but not of ChR2, is regulated partially by a PHOT-controlled signaling cascade involving a COP1-SPA1 E3 ubiquitin ligase and a light-independent pathway via cAMP. Now we add CEP1 as an additional downstream component to these cascade(s) as its mRNA level depends not only on a functional PHOT and SPA1, but also doubles upon increased cAMP levels in the dark (Fig. 4, see Fig. 12A for a schematic model). Previously, a purely light stimulated increase of CEP1 was noticed in a proteomic study of the interplay of light and oxygen in the ROS response of *Chlamydomonas* (Barth et al., 2014). The low light saturation of the *CEP1* mRNA increase in our study underlines further the importance of a tightly regulated ChR1 level for the general light acclimation response and that transcriptional regulation of photoprotection in *Chlamydomonas* starts already during the dark-to-light transition (Redekop et al., 2022; Wolfram et al., 2023).

(iv) Our study also shows that disrupting either central IFT components, the involved motor proteins or cilia formation all resulted in reduced ChR1 levels and increased degradation rates. Affecting intracellular vesicle transport - irrespective whether e.g. from Golgi towards the PM or during retrieval from endosomes - induces accumulation of ChR1-containing vesicles in the BB region and reduced eyespot targeting. This

was earlier demonstrated by ChR1 immunolocalization in similar mutants (Awasthi et al., 2016). To avoid possible cellular disturbance by the mis-targeted and undelivered photoreceptor, ChR1 degradation rates apparently must increase under such conditions. Additionally, whenever cilia formation is affected, vesicle fusion and endocytic sites close to the ciliary base, which are known from ciliated protists and vertebrate cells (Molla-Herman et al., 2010; Ghossoub et al, 2011; Benmerah, 2013), might also be hindered. This again could result in the necessity of increased ChR1 degradation. Further, cilia are a reservoir for membrane receptors in many systems. They can travel in and out of the cilia and the total ciliary membrane is shed in [?] 6h by *Chlamydomonas* . Continuous resupply comes from both the cytoplasm and the PM via different mechanisms such as diffusion after delivery near the ciliary base, endocytosis and vesicle transport in the ciliary lumen (Dentler, 2013; Bigge et al., 2023; Ruba et al., 2023). Ciliary involvement as a short-term reservoir prior to endocytosis and degradation or eyespot targeting is thus conceivable for ChR1. Further, the ciliary targeting motif VXPX is crucial for the *in vitro* interaction of ChR1 with ARL11 (Sharma et al., 2023). We present here the first *in vivo* demonstration of the functional relevance of ARL11 for ChR1 transport and targeting to the eyespot and cilia. As its vertebrate counterpart ARF4 (Nakai et al., 2013), ARL11 might additionally be involved in ChR1 retrieval from endosomes. Its relative proportion hereby can, however, only be minor as the ChR1 degradation was normal in these mutants. Further, residual ciliary ChR1 amounts in the ARL11 mutants indicate that additional components must be involved in targeting (Fig. 9). As blocking SUMOylation reduces the ChR1 level and its stability in the cilia without affecting that in the cell bodies or its general degradation (Fig. 8), our data also foster the suggestion of Sharma et al. (2023) that SUMO is involved in ChR1 trafficking. In vertebrates SUMOylation of ARL13b promotes cilia targeting of rhodopsin and other sensory receptors, although receptor SUMOylation is necessary but not always sufficient for ciliary import (Li et al., 2012; McIntyre et al., 2015). Albeit deletion of ARL13 in *Chlamydomonas* impedes the BBSome-mediated transport of selected proteins and thereby also phototaxis (Dai et al., 2022), our data with *arl13* and *bbs4-1* showed that ChR1 transport does not involve these components. The schematic model in Figure 12B summarizes our current view of ChR1 internalization. An exciting future task will be the identification of further necessary targeting components. This and deciphering the basis of the ChR1 M_r shifts and why ChR1 is also transported into the cilia will allow to develop a more detailed molecular view on ChR1 homeostasis.

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REFERENCES

Arora D, Van Damme D. (2021). Motif-based endomembrane trafficking Plant Physiol 186: 221–238, https://doi.org/10.1093/plphys/kiab077

Awasthi M, Ranjan P, Sharma K, Veetil SK, Kateriya S (2016). The trafficking of bacterial type rhodopsins into the *Chlamydomonas* eyespot and fagella is IFT mediated. Sci Rep 6:34646. https://doi.org/10.1038/srep34646

Baidukova O, Oppermann J, Kelterborn S, Lahore RGF, Schumacher D, Evers H, Kamrani YY, Hegemann P (2022). Gating and ion selectivity of channelrhodopsins are critical for photo-activated orientation of *Chlamydomonas* as shown by in vivo point mutation. Nat Commun 13, 7253. https://doi.org/10.1038/s41467-022-35018-6

Barth J, Bergner SV, Jaeger D, Niehues A, Schulze S, Scholz M, Fufezan C (2014). The interplay of light and oxygen in the reactive oxygen stress response of *Chlamydomonas reinhardtii* dissected by quantitative mass spectrometry. Mol Cell Proteomics: 969-89. https://doi.org/10.1074/mcp.M113.032771

Baur R, Karl F, Böttcher-Loschinski R, Stoll A, Völkl S, Gießl A, Flamann C, Bruns H,Schlötzer-Schrehardt U, Böttcher M, Schewe DM, Fischer T, Jitschin R, Mackensen A, Mougiakakos D (2023). Accumulation of T-cell-suppressive PD-L1high extracellular vesicles is associated with GvHD and might impact GvL efficacy. J Immunother Cancer 11:e006362. https://doi.org/10.1136/jitc-2022-006362

Benmerah A (2013). The ciliary pocket. Curr Op Cell Biol 25: 78-84 https://doi.org/10.1016/j.ceb.2012.10.011

Bigge BM, Rosenthal NE, Avasthi P (2023). Initial ciliary assembly in *Chlamydomonas* requires Arp2/3 complex–dependent endocytosis. Mol Biol Cell 34:4; https://doi.org/10.1091/mbc.E22-09-0443

Blom N, Sicheritz-Pontén T, Gupta R, Gammeltoft S, Brunak S. (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics 4:1633-49, https://doi.org/10.1002/pmic.200300771

Böhm M, Kreimer G (2021). Orient in the world with a single eye: the green algal eyespot and phototaxis. In: FM Cánovas, U Lüttge, MC Risueño, H Pretzsch (Eds.). Prog Bot 82: 259–304

Böhm M, Boness D, Fantisch E, Erhard H, Frauenholz J, Kowalzyk Z, Marcinkowski N, Kateriya S, Hegemann P, Kreimer G (2019). Channelrhodopsin-1 phosphorylation changes with the phototactic behavior and responds to physiological stimuli in *Chlamydomonas* . Plant Cell 31: 886-910, https://doi.org/10.1105/tpc.18.00936

Bonifacino JS, Traub LM (2003). Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 72: 395-447

Boonyareth M, Saranak J, Pinthong D, Sanvarinda Y, Foster KW (2009). Roles of cyclic AMP in regulation of phototaxis in *Chlamydomonas reinhardtii* . Biologia 64: 1058-1065

Burgoyne RD, Clague MJ (2003). Calcium and calmodulin in membrane fusion. Biochim Biophys Acta Mol Cell Res 1641: 137-143, https://doi.org/10.1016/S0167-4889(03)00089-2

Cai K, Zhao Y, Zhao L, Phan N, Hou Y, Cheng X, Witman GB, Nicastro D (2021). Structural organization of the C1b projection within the ciliary central apparatus. J Cell Sci 134: jcs254227. https://doi.org/10.1242/jcs.254227

Catalano M, O'Driscoll L (2019). Inhibiting extracellular vesicles formation and release: a review of EV inhibitors. J Extracell Vesicles 9: 1703244, https://doi.org/10.1080/20013078.2019.1703244

Chakrabarti R, Lee M, Higgs HN (2021). Multiple roles for actin in secretory and endocytic pathways. Curr Biol 31: R603-R618, https://doi.org/10.1016/j.cub.2021.03.038

Chen X, Irani NG, Friml J (2011). Clathrin-mediated endocytosis: the gateway into plant cells. Curr Op Plant Biol 14: 674-682, https://doi.org/10.1016/j.pbi.2011.08.006

Colombo M, Raposo G, Thery C. (2014). Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol 30: 255–289

Concepcion F, Mendez A, Chen J (2002). The carboxyl-terminal domain is essential for rhodopsin transport in rod photoreceptors. Vision Res 42: 417-426

Dai J, Zhang G, Alkhofash RA, Mekonnen B, Saravanan S, Xue B, Fan Z-C, Betleja E, Cole DG, Liu P, Lechtreck K (2022). Loss of ARL13 impedes BBSome-dependent cargo export from *Chlamydomonas* cilia. J Cell Biol 221: e202201050. https://doi.org/10.1083/jcb.202201050

Dentler W. (2013). A role for the membrane in regulating *Chlamydomonas* flagellar length. PLoS One. 2013;8(1):e53366. https://doi.org/10.1371/journal.pone.0053366

Deretic D, Williams AH, Ransom N, Morel V, Hargrave PA, Arendt A (2005). Rhodopsin c terminus, the site of mutations causing retinal disease, regulates trafficking by binding to ADP-ribosylation factor 4 (ARF4). Proc Natl Acad Sci USA 102: 3301-3306

Deretic D, Lorentzen E, Fresquez T (2021). The ins and outs of the Arf4-based ciliary membrane-targeting complex. Small GTPases 12: 1-12, https://doi.org/10.1080/21541248.2019.1616355

Dutcher SK, Morrissette NS, Preble AM, Rackley C, Stanga J (2002). ε-Tubulin is an essential component of the centriole. Mol Biol Cell 13: 3859-3869

Dutta D, Williamson CD, Cole NB, Donaldson JG (2012). Pitstop 2 is a potent inhibitor of clathrin-independent endocytosis. PLoS One 7:e45799. https://doi.org/10.1371/journal.pone.0045799

Ehler LL, Holmes JA, Dutcher SK (1995). Loss of spatial control of the mitotic spindle apparatus in a *Chlamydomonas reinhardtii* mutant strain lacking basal bodies. Genetics 141: 945-960

Eitzinger N, Wagner V, Weisheit W, Geimer S, Boness D, Kreimer G, Mittag M (2015). Proteomic analysis of a fraction with intact eyespots of*Chlamydomonas reinhardtii* and assignment of protein methylation. Front Plant Sci 6:1085. https://doi.org/10.3389/fpls.2015.01085

Engel BD, Schaffer M, Kuhn Cuellar L, Villa E, Plitzko JM, Baumeister W (2015) Native architecture of the *Chlamydomonas* chloroplast revealed by in situ cryo-electron tomography. eLife 4:e04889. https://doi.org/10.7554/eLife.04889

Erickson E, Wakao S, Niyogi KK (2015). Light stress and photoprotection in *Chlamydomonas reinhardtii* . Plant J 82: 449–465

Feig C, Tchikov V, Schütze S, Peter ME (2007). Palmitoylation of CD95 facilitates formation of SDS-stable receptor aggregates that initiate apoptosis signaling. EMBO J 26: 221-231

Finst RJ, Kim PJ, Quarmby LM (1998). Genetics of the deflagellation pathway in *Chlamydomonas* . Genetics 149: 927-936

Foot N, Henshall T, Kumar S (2017). Ubiquitination and the regulation of membrane proteins. Physiol Rev 97: 253-281

Gabilly ST, Baker CR, Wakao S, Crisanto T, Guan K, Guiet E, Guadagno CR, Niyogi KK (2019). Regulation of photoprotection gene expression in*Chlamydomonas* by a putative E3 ubiquitin ligase complex and a homolog of CONSTANS. Proc Natl Acad Sci USA 116: 17556-17562

Ghossoub R, Molla-Herman A, Bastin P, Benmerah A (2011). The ciliary pocket: a once-forgotten membrane domain at the base of cilia. Biol Cell. 103:131-44. https://doi.org/10.1042/BC20100128

Govorunova EG, Sineshchekov OA (2023). Channelrhodopsins: From phototaxis to optogenetics. Biochemistry (Moscow) 88: 1555-1570

Greiner A, Kelterborn S, Evers H, Kreimer G, Sizova I, Hegemann P (2017). Targeting of photoreceptor genes in *Chlamydomonas reinhardtii* via zinc-finger nucleases and CRISPR/Cas9. Plant Cell 29: 2498-2518, https://doi.org/10.1105/tpc.17.00659

Harris EH (2008). *Chlamydomonas* in the laboratory. In EH Harris (ed), The *Chlamydomonas* Sourcebook (2^{nd}edition)., Introduction to *Chlamydomonas* and its laboratory use. Vol. 1 (pp. 241-302). Academic Press

Himschoot E, Pleskot R, Van Damme D, Vanneste S (2017). The ins and outs of Ca^{2+} in plant endomembrane trafficking. Curr Op Plant Biol 40: 131-137, https://doi.org/10.1016/j.pbi.2017.09.003

Holmes JA, Dutcher SK (1989). Cellular asymmetry in *Chlamydomonas*reinhardtii. J Cell Sci 94: 273–285

Kato HE, Zhang F, Yizhar O, Ramakrishnan C, Nishizawa T, Hirata K, Ito J, Aita Y, Tsukazaki T, Hayashi S, Hegemann P, Maturana AD, Ishitani R, Deiseroth K, Nureki O (2012). Crystal structure of the channelrhodopsin light-gated cation-channel. Nature 482: 369-375

Keady BT, Le YZ, Pazour GJ (2011) IFT20 is required for opsin trafficking and photoreceptor outer segment development. Mol Biol Cell 22: https://doi.org/10.1091%2Fmbc.E10-09-0792

Kelterborn S, Boehning F, Sizova I, Baidukova O, Evers H, Hegemann P (2022). Gene editing in green alga *Chlamydomonas reinhardtii* via CRISPR-Cas9 ribonucleoproteins. In MD Zurbriggen (ed) Plant Synthetic Biology. Methods in Molecular Biology, Vol 2379. https://doi.org/10.1007/978-1-0716-1791-5_3, Humana

Khanna H, Hurd TW, Concepcion L, Shu X, Parapuram SK, He S, Akimoto M, Wright AF, Margolis B, Williams DS, Swaroo A (2005). RPGR-ORF15, which is mutated in retinitis pigmentosa, associates with SMC1, SMC3, and microtubule transport proteins. J Biol Chem 280: 33580 –33587

Kim YS, Keyser SG, Schneekloth Jr. JS (2014). Synthesis of 2',3',4'-trihydroxyflavone (2-D08), an inhibitor of protein sumoylation. Bioorg Med Chem Lett 24: 1094-1097

Kitagawa T, Takiya S (2020). Regulation of genes for ubiquitination and SUMO-specific protease involved in larval development of the silkworm,*Bombyx mori* . Develop Growth Differ 62: 438-449

Kotrbova A, Štěpka K, Maška M, Pálenik JJ, Ilkovics L, Klemová D, Kravec M, Hubatka F, Dave Z, Hampl A, Bryja V, Matula P, Pospíchalová V (2019). TEM ExosomeAnalyzer: a computer-assisted software tool for quantitative evaluation of extracellular vesicles in transmission electron microscopy images. J Extracell Vesicles 21:1560808. https://doi.org/10.1080/20013078.2018.1560808

Kreimer G, Wakabayashi K, Hegemann P, Dieckmann C (2023). The eyespot and behavioral light responses. In SK Dutcher (Ed), The Chlamydomonas Source Book (3^{rd} edition), Cell motility and behavior. Vol 3 (pp 391-419). Elsevier

Kong S, Du X, Peng C, Wu Y, Li H, Jin X, Hou L, Deng K, Xu T, Tao W (2013). Dlic1 deficiency impairs ciliogenesis of photoreceptors by destabilizing dynein. Cell Res 23: 835–850

Lämmermann N, Wulf D, Chang KS, Wichmann J, Jang J, Jin ES, Bräutigam A, Wobbe L, Kruse O (2020). Ubiquitin ligase component LRS1 and transcription factor CrHy5 act as a light switch for photoprotection in*Chlamydomonas* . bioRxiv https://doi.org/10.1101/2020.02.10.942334

Lechtreck K-F, Johnson EC, Sakai T, Cochran D, Ballif BA, Rush J, Pazour GJ, Ikebe M, Witman GB (2009). The *Chlamydomonas reinhardtii*BBSome is an IFT cargo required for export of specific signaling proteins from flagella. J Cell Biol 187: 1117–1132, https://doi.org/10.1083/jcb.200909183

Li Y, Hu J (2011). Small GTPases and cilia. Protein & Cell 2: 13-25, https://doi.org/10.1007/s13238-011-1004-7

Li Y, Zhang Q, Wei Q, Zhang Y, Ling K, Hu J (2012). SUMOylation of the small GTPase ARL-13 promotes ciliary targeting of sensory receptors. J Cell Biol 199: 589–598

Li X, Patena W, Fauser F, Jinkerson RE, Saroussi S, Meyer MT, Ivanova N, Robertson JM, Yue R, Zhang R, Vilarrasa-Blasi J, Wittkopp TM, Ramundo S, Blum SR, Goh A, Laudon M, Srikumar T, Lefebvre PA, Grossman AR, Jonikas MC (2019). A genome-wide algal mutant library and functional screen identifies genes required for eukaryotic photosynthesis. Nat Genet 51: 627-635, https://doi.org/10.1038/s41588-019-0370-6

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2[?][?]C(T) method. Methods 25: 402-408

Long H, Zhang F, Xu N, Liu G, Diener DR, Rosenbaum JL, Huang K (2016). Comparative analysis of ciliary membranes and ectosomes. Curr Biol 26: 3327-3335, https://doi.org/10.1016/j.cub.2016.09.055

Long H, Fang J, Ye L, Zhang B, Hui C, Deng X, Merchant SS, Huang K (2023). Structural and functional regulation of *Chlamydomonas*lysosome-related organelles during environmental changes. Plant Physiol 192: 927–944, https://doi.org/10.1093/plphys/kiad189

Luxmi R, Blaby-Haas C, Kumar D, Rauniyar N, King SM, Mains RE, Eipper BA (2018). Proteases shape the *Chlamydomonas* secretome: Comparison to classical neuropeptide processing machinery. Proteomes 6:36, https://doi.org/10.3390/proteomes6040036

Martinez M, Cambra I, Gonzalez-Melendi P, Santamaria ME, Diaz I. (2012). C1A cysteine-proteases and their inhibitors in plants. Physiol Plantarum 145: 85-94 https://doi.org/10.1111/j.1399-3054.2012.01569.x

Matsuura K, Lefebvre PA, Kamiya R, Hirono M (2002). Kinesin-II is not essential for mitosis and cell growth in *Chlamydomonas* . Cell Motil Cytoskel 52:195-201

Mazelova J, Astuto-Gribble L, Inoue H, Tam BM, Schonteich E, Prekeris R, Moritz OL, Randazzo PA, Deretic D (2009). Ciliary targeting motif VxPx directs assembly of a trafficking module through Arf4. EMBO J 28: 183-192

McIntyre JC, Joiner AM, Zhang L, Iniguez-Lluhi J, Martens JR (2015). SUMOylation regulates ciliary localization of olfactory signaling proteins. J Cell Sci 128: 1934–1945. https://doi.org/10.1242/jcs.164673

Mittag M, Kiaulehn S, Johnson C (2005). The circadian clock in*Chlamydomonas reinhardtii* . What is it for? Plant Physiol 137: 399-409

Mittelmeier TM, Dieckmann C, Boyd JS, Lamb MR (2011). Asymmetric properties of the *Chlamydomonas* reinhardtii cytoskeleton direct rhodopsin photoreceptor localization. J Cell Biol 193: 741–753

Molla-Herman A, Ghossoub R, Blisnick T, Meunier A, Serres C, Silbermann F, Emmerson C, Romeo K, Bourdoncle P, Schmitt A, Saunier S, Spassky N, Bastin P, Benmerah A (2010). The ciliary pocket: an endocytic membrane domain at the base of primary and motile cilia. J Cell Sci 123: 1785–1795, doi: https://doi.org/10.1242/jcs.059519

Moore CAC, Milano SK, Benovic JL (2007). Regulation of receptor trafficking by GRKs and arrestins. Annu Rev Physiol 69: 451-482

Nakai W, Kondo Y, Saitoh A, Naito T, Nakayama K, Shin H-W (2013). ARF1 and ARF4 regulate recycling endosomal morphology and retrograde transport from endosomes to the Golgi apparatus. Mol Biol Cell 24:16, 2570-2581

Papalazarou V, Machesky LM (2021). The cell pushes back: The Arp2/3 complex is a key orchestrator of cellular responses to environmental forces. Curr Opp Cell Biol 68: 37-44, https://doi.org/10.1016/j.ceb.2020.08.012

Pazour GJ, Dickert BL, Vucica Y, Seeley ES, Rosenbaum JL, Witman GB, Cole DG (2000). Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene Tg737, are required for assembly of cilia and flagella. J Cell Biol 151: 709–718, https://doi.org/10.1083/jcb.151.3.709

Pazour GJ, Agrin N, Leszyk J, Witman GB (2005). Proteomic analysis of a eukaryotic cilium. J Cell Biol 170: 103-113

Petroutsos D, Busch A, Janssen I, Trompelt K, Bergner SV, Weinl S, Holtkamp M, Karst U, Kudla J, Hippler M (2011). The chloroplast calcium sensor CAS is required for photoacclimation in *Chlamydomonas reinhardtii* . Plant Cell 23: 2950-2963

Petroutsos D, Tokutsu R, Maruyama S, Flori S, Greiner A, Magneschi L, Cusant L, Kottke T, Mittag M, Hegemann P, Finazzi G, Minagawa J (2016). A blue-light photoreceptor mediates the feedback regulation of photosynthesis. Nature 537: 563-566

Piasecki BP, Silflow CD (2009). The UNI1 and UNI2 genes function in the transition of triplet to doublet microtubules between the centriole and cilium in *Chlamydomonas* . Mol Biol Cell 20: 368-378

Redekop P, Sanz-Luque E, Yuan Y, Villain G, Petroutsos D, Grossman AR (2022). Transcriptional regulation of photoprotection in dark-to-light transition – More than just a matter of excess light energy. Sci. Adv.8, eabn1832

Rosenbaum JL, Moulder JE, Ringo DL (1969). Flagellar elongation and shortening in Chlamydomonas: The use of cycloheximide and colchicine to study the synthesis and assembly of flagellar proteins. J Cell Biol 41: 600-619

Rossio V, Paulo JA, Chick J, Brasher B, Gygi SP, King RW (2021). Proteomics of broad deubiquitylase inhibition unmasks redundant enzyme function to reveal substrates and assess enzyme specificity. Cell Chem Biol 28: 487-502

Ruba A, Tingey M, Luo W, Yu J, Evangelou A, Higgins R, Khim S, Yang W (2023). The ciliary lumen accommodates passive diffusion and vesicle-assisted trafficking in cytoplasm–ciliary transport. Mol Biol Cell 34: ar59 1-19, https://doi.org/10.1091/mbc.E22-10-0452

Rudinskiy N, Grishchuk Y, Vaslin A, Puyal J, Delacourte A, Hirling H, Clarke PGH, Luthi-Carter R (2009). Calpain hydrolysis of α- and β2-adaptins decreases clathrin-dependent endocytosis and may promote neurodegeneration. J Biol Chem 284: 12447-12458

Ruiz-Sola MÁ, Flori S, Yuan Y, Villain G, Sanz-Luque E, Redekop P, Tokutsu R, Kueken A, Tsichla A, Kepesidis G, Allorent G, Arend M,Iacono F, Finazzi G, Hippler M, Nikoloski Z, Minagawa J, Grossman AR, Petroutsos D (2023). Light-independent regulation of algal photoprotection by CO_2 availability. Nat Commun 14: 1977, https://doi.org/10.1038/s41467-023-37800-6

Sale WS, Dutcher SK (2023). Landmark contributions of*Chlamydomonas* to understanding cilia. In SK Dutcher (Ed), The Chlamydomonas Source Book (3rd edition), Cell motility and behavior. Vol 3 (pp 1-34). Elsevier

Sanyal SK, Awasthi M, Ranjan P, Sharma S, Pandey GK, Kateriya S (2023). Characterization of *Chlamydomonas* voltage-gated calcium channel and its interaction with photoreceptor support VG-GC modulated photobehavioral response in the green alga. Int J Biol Macromol 245:125492, https://doi.org/10.1016/j.ijbiomac.2023.125492

Schmidt M, Gessner G, Luff M, Heiland I, Wagner V, Kaminski M, Geimer S, Eitzinger N, Reißenweber T, Voytsekh O, Fiedler M, Mittag M, Kreimer G (2006). Proteomic analysis of the eyespot of *Chlamydomonas reinhardtii* provides novel insights into its components and tactic movements. Plant Cell 18: 1908-1930

Schwihla M, Korbei B (2020). The Beginning of the end: Initial Steps in the degradation of plasma membrane proteins. Front Plant Sci 11: 680, doi:10.3389/fpls.2020.00680

Sharma K, Sizowa I, Sanyal SK, Pandey GK, Hegemann P, Kateriya S (2023). Deciphering the role of cytoplasmic domain of channelrhodopsin in modulation of the interactome and SUMOylome of *Chlamydomonas reinhardtii* . Int J Biol Macromol 243:125135, https://doi.org/10.1016/j.ijbiomac.2023.125135

Sineshchekov OA, Jung KH, Spudich JL (2002). Two rhodopsins mediate phototaxis to low- and high-intensity ligth in *Chlamydomonas reinhardtii* . Proc Natl Acad Sci USA 99: 8689-8694

Snyder NA, Silva GM (2021). Deubiquitinating enzymes (DUBs): Regulation, homeostasis, and oxidative stress response. J Biol Chem 297: https://doi.org/10.1016/j.jbc.2021.101077

Sundvall M (2020). Role of ubiquitin and SUMO in intracellular trafficking. Curr Issues Mol Biol 35: 99-108

Sung C-H, Leroux MR (2013). The roles of evolutionary conserved functional modules in cilia-related trafficking. Nature Cell Biol 15: 1387-1397

Tai AW, Chuang JZ, Bode C, Wolfrum U, Sung CH (1999). Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1. Cell 97: 877-887

Thompson MD, Mittelmeier TM, Dieckmann CL (2017). Chlamydomonas: the eyespot. In: Hippler M (ed) Chlamydomonas: molecular genetics and physiology. Microbiology monographs, Vol 30 (pp 257–281). Springer

Tinevez J-Y, Perry N, Schindelin J., Hoopes GM, Reynolds GD, Laplantine E, Bednarek SY, Shorte SL, Eliceiri KW (2017). TrackMate: An open and extensible platform for single particle tracking. Methods 115: 80–90

Tokutsu R, Fujimura-Kamada K, Matsuo T, Yamasaki T, Minagawa J (2019). The CONSTANS flowering complex controls the protective response of photosynthesis in the green alga Chlamydomonas. Nat Commun 10: 4099, https://doi.org/10.1038/s41467-019-11989-x

Trippens J, Greiner A, Schellwat J, Neukam M, Rottmann T, Lu Y, Kateriya S, Hegemann P, Kreimer G (2012). Phototropin influence on eyespot development and regulation of phototactic behavior in*Chlamydomonas reinhardtii* . Plant Cell 24: 4687-4702

Trippens J, Reißenweber T, Kreimer G (2017). The chloroplast calcium sensor protein CAS affects phototactic behaviour in *Chlamydomonas reinhardtii* (Chlorophyceae) at low light intensities. Phycologia 56: 261-270, https://doi.org/10.2216/16-67.1

Trivedi D, Colin E, Louie CM, Williams DS (2012). Live-cell imaging evidence for the ciliary transport of rod photoreceptor opsin by heterotrimeric kinesin-2. J Neurosci 32: 10587-10593

Vierock J, Hegemann P (2023). Sensory photoreceptors in*Chlamydomonas* . In AR Grossmann, F-A Wollman (Eds), The Chlamydomonas Source Book (3rd edition), Organellar and metabolic processes. Vol 2 (pp 205-222). Elsevier

Vinay L, Belleannée C (2022). EV duty vehicles: Features and functions of ciliary extracellular vesicles. Front Genet 13:916233, https://doi.org/10.3389/fgene.2022.916233

Voronina S, Collier D, Chvanov M, Middlehurst B, Beckett AJ, Prior IA, Criddle DN, Begg M, Mikoshiba K, Sutton R, Tepikin AV (2015). The role of Ca^{2+} influx in endocytic vacuole formation in pancreatic acinar cells. Biochem J 465: 405–412. https://doi.org/10.1042/BJ20140398

Wagner V, Ullmann K, Mollwo A, Kaminski M, Mittag M, Kreimer G (2008). The phosphoproteome of a *Chlamydomonas reinhardtii* eyespot fraction includes key proteins of the light signaling pathway. Plant Physiol 146: 323-324, https://doi.org/10.1104/pp.107.109645

Walther Z, Vashishtha M, Hall JL (1994). The *Chlamydomonas* FLA10 gene encodes a novel kinesin-homologous protein. J Cell Biol 126:175-188

Wang J, Deretic D (2014). Molecular complexes that direct rhodopsin transport to primary cilia Prog Retin Eye Res. 38: 10.1016/j.preteyeres.2013.08.004.

Williams DS (2002). Transport to the photoreceptor outer segment by myosin VIIa and kinesin II. Vision Res 42: 455-462, https://doi.org/10.1016/S0042-6989(01)00228-0

Witman G (1986) Isolation of *Chlamydomonas* flagella and flagellar axonemes. Meth Enzymol 134: 280-290

Wolfram M, Greif A, Sizova I, Baidukova O, Hegemann P, Kreimer G (2023). Multifactoial in vivo regulation of the photoreceptor channelrhodopsin-1 abundance. Plant Cell Environ 46: 2778–2793 https://doi.org/10.1111/pce.14657

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL (2012). Primer-Blast: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13 134:

https://doi.org/10.1186/1471-2105-13-134

Zhao J (2007). Sumoylation regulates diverse biological processes. Cell Mol Life Sci 64: 3017–3033, https://doi.org/10.1007/s00018-007-7137-4

Zhao S, Liang Z, Demko V, Wilson R, Johansen W, Olsen OA, Shalchian-Tabrizi K (2012). Massive expansion of the calpain gene family in unicellular eukaryotes. BMC Evol Biol. 12:193, https://doi.org/10.1186/1471-2148-12-193

Zones JM, Blaby IK, Merchant SS, Umen JG (2015) High-resolution profiling of a synchronized diurnal transcriptome from *Chlamydomonas reinhardtii* reveals continuous cell and metabolic differentiation. Plant Cell 27: 2743-2769

FIGURE LEGENDS

Figure 1: Inhibition of endocytosis and deubiquitinylating enzymes (DUBs) reduces light-induced ChR1-degradation.

(A, B) The endocytosis inhibitor Pitstop-2 impedes light-induced ChR1 degradation and induces ChR1 modifications and formation of ChR1-containing HMMCs. (A) CC-125 cells were pre-incubated at the end of the night phase for 2 h in the dark with CHX and 15 μM Pitstop-2 or the solvent DMSO. The cells were then illuminated for the indicated times with WL + UV (81 μmol $m^{-2}s^{-1}$) or kept further in the dark. MetOH-Chloroform precipitated proteins were dissolved in reducing sample buffer, separated by SDS-PAGE and analyzed by immunoblotting with anti-ChR1 as described. Representative immunoblots (anti-ChR1) of three independent experiments are shown. Equal protein load is demonstrated by Coomassie Brilliant Blue (Coomassie) staining of the lower part of the blots. -: ChR1 monomer; * indicates the region of the shifted ChR1 monomer band (˜ 10 kDa; $ChR1_{mod}$) and HMMCs indicate the position of the ChR1-containing high M_r complexes. (B) Quantification of immunoblot analysis shown in (A). Data plotted are the mean ± SEM ($n = 3$) and given as percentage of the total ChR1 content of the corresponding dark control. The differences between the samples treated with Pitstop-2 and DMSO in the light are significant (unpaired t-test; p -values: < 0.00025).

(C - E) PR-619, a broad spectrum DUB inhibitor, induces formation of $ChR1_{mod}$ forms and ChR1-containg HMMCs in the light and reduces ChR1 degradation. At the end of the night phase CW15 cells were pre-incubated (3 h) in the dark with CHX and 10 μM PR-619 or the solvent DMSO before illumination with WL + UV (81 μmol m^{-2} s^{-1}) for the indicated times. Protein extraction and further processing were as described. (C) A typical immunoblot analysis of whole cell extracts of three independent biological experiments is shown. * marks the $ChR1_{mod}$region and arrows indicate that of the HMMCs quantified in (E). (D) Quantification of the immunoblot analysis shown in (C). Data are given as percentage of the total ChR1 content of the corresponding dark samples (mean ± SEM from three independent experiments). (E) Quantification of the relative proportions of the ChR1 monomer (including the bands marked by *) and HMMC bands marked in (C). For quantification, the sum of the monomers and HMMCs in each lane was set to 100%. Data are given as mean ± SEM of three independent experiments.

(F, G) Effects of the Arp2/3 complex inhibitor CK-666 on light-induced ChR1 degradation. (F) CC-125 cells were pre-incubated at the end of the night phase for 2 h in the dark with CHX and 250 μM CK-666 or, as controls, with the same concentration of the inactive analog CK-689 or the solvent DMSO. Illumination with WL + UV (81 μmol m^{-2} s^{-1}) and further processing was as described. A representative immunoblot of three independent experiments is shown. -: ChR1 monomer; * indicates the region of the shifted ChR1 monomer band (˜ 10 kDa; $ChR1_{mod}$) and HMMCs indicate the position of the ChR1-containing high M_r complexes. (G) Quantification of the immunoblot analysis shown in (F). Data are the mean ± SEM from three independent biological replicates.

(H, I) CC-125 cells were pre-incubated in the dark for 1 h with CHX and the DUB inhibitor PR-619 (10 μM) prior to addition of 250 μM CK-666, its inactive analog CK-689 or the solvent DMSO. Following an additional 2 h dark incubation the cultures were illuminated as above. -: ChR1 monomer; * indicates

the $ChR1_{mod}$ region and HMMCs indicate the position of the ChR1-containing high M_r complexes. (I) Quantification of the immunoblot analysis shown in (H). Data presented are the mean ± SEM from three independent biological replicates

Figure 2: The calpain inhibitor PD 150606 prevents light-induced ChR1 degradation. CW15 cells at the end of the night phase were incubated for 3 h in the dark in the presence of CHX either with PD 150606, its inactive analogue PD 145305 (both 60 µM) or the solvent EtOH prior to illumination for the indicated times with WL + UV (81 µmol m^{-2} s^{-1}). MetOH-Chloroform precipitated proteins were dissolved in reducing sample buffer, separated by SDS-PAGE and analyzed by immunoblotting. **(A)**Immunoblot analysis of the samples after separation by standard, reducing SDS-PAGE. A representative blot of six independent biological replicates is shown. Coomassie staining of the lower part of the blot serves as a loading control. * marks the region of the $ChR1_{mod}$ forms and arrows indicate the position of the ChR1 monomer and HMMCs quantified in (C). **(B)** Quantification of immunoblot analysis of CW15 cells treated as described above. Data are given as percentage of the total ChR1 content of the corresponding dark samples (mean ± SEM from six independent experiments). **(C)**Quantification of the relative proportions of the ChR1 monomer (including the $ChR1_{mod}$ region) and HMMCs marked in (A) by arrows. For quantification, the sum of the monomers and HMMCs in each lane was set to 100%. Data are the mean ± SEM from six independent biological experiments.

Figure 3: Effects of cysteine endopeptidase CEP1 knock-out on ChR1 and ChR2 starting content, degradation, eyespot size and growth. Three independent ΔCEP1 strains (ΔCEP1-A to C) along with the parental strain CC-3403 (wt) were pre-incubated in the dark with CHX and illuminated for the indicated times with WL + UV (81 µmol m^{-2} s^{-1}). Further processing and quantification of the ChRs was done as described. Light-induced ChR1**(A, B)** and ChR2 **(E)** degradation and normalized starting levels at the end of the night phase (**C:** ChR1;**F:** ChR2). Data plotted are the mean ± SEM from three independent biological replicates. **(D)** Box plot (whiskers min to max) of the eyespot area of the four strains. Different lower case letters indicate statistically significant differences (ANOVA analyses with Tukey´s multiple comparison post test, $p < 0.0001$;$n = 102$ to 113 cells grown under 60 µmol $m^{-2}s^{-1}$ WL). Cultures were grown and analyzed in parallel.**(G)** Growth curves of the ΔCEP1 strains and their parental strain CC-3403 (wt) in TAP medium under WL (60 µmol photons m^{-2} s^{-1}). **(H)** Total chlorophyll and carotenoid content of cells after five days of growth as in (G). Data in G and H are the mean ± SEM of three independent biological replicates. Differences are not significant.

Figure 4: *CEP1* mRNA levels respond to low intensity WL and are controlled by PHOT, the E3 ligase complex component SPA1 and cyclic nucleotides.

(A, B) Responses of *CEP1* mRNA levels to low intensity WL. (A) CC-125 cells were grown at 60 µmol $m^{-2}s^{-1}$ WL in a 14/10 h light/dark cycle for 5 days. mRNA levels were determined by RT-qPCR from samples harvested directly prior to onset of illumination (dark) and 2 h after illumination with the indicated light intensities. mRNA levels were normalized to*α-τυβυλιν* and are given relative to those of the dark sample (mean ± SD; *n:* three independent biological replicates). Different lower case letters mark significant differences (two-tailed t-test, p -values [?] 0.05). (B) Cells of strain CC-125 were grown at the indicated WL intensities, analyzed and normalized as described above. Different lower case letters mark significant differences (mean +- SD, n = three independent biological replicates; two-tailed t-test, p -value [?] 0.05).

(C, D) Responses of *HY5* mRNA levels to low intensity WL.*HY5* mRNA in CC-125 cells, in (C) grown as in (A) and (D) grown as in (B), was analyzed directly prior (dark) or two h after onset of illumination (light) as described above. Sample sizes and statistical treatments as in (A) and (B), significant differences to the corresponding dark levels are marked with different lower case letters.

(E) *CEP1* mRNA levels in the ΔPHOT and the parental (wt) strain were determined by RT-qPCR from cultures harvested directly prior (dark) and 2 h after onset of the light phase. mRNA levels were normalized to *α-τυβυλιν* and are given relative to those of the wt strain in the dark ± SD (three independent biological replicates). Different lower case letters: statistically significant differences (two-tailed t-test, p -values [?]

0.05).

(F) *CEP1* mRNA levels in the *spa1* mutant, which is defective in the E3 ubiquitin ligase component SPA1 (Tokutsu et al., 2019) and its corresponding parental strain (wt) were determined, normalized and plotted as in (E). Data are the mean +- SD of three independent experiments. Different lower case letters indicate statistically significant differences (two-tailed t-test,p -values [?] 0.05).

(G) Cells of strain CC-125 were grown as in (A). *CEP1*mRNA levels were determined directly prior to the onset of illumination in control cultures and cultures incubated for 21 h with 1 mM IBMX in the absence of CHX. Mean +- SD; n = three independent biological replicates; different lower case letters: statistically significant difference (two-tailed t-test; p -value: 0.004).

Figure 5: ChR1 and its modified forms are present in the cell body and cilia.

(A – D) Indirect immunolocalization of ChR1 (magenta) and acetylated tubulin (acTUB, green) in permeabilized cells (A, B) and detached cilia (C, D). Note that ChR1 in cilia can only be detected with longer exposure times. Images shown are maximum intensity projections of representative cells and cilia of strain CC-125 analyzed at the end of the night phase. White arrows indicate the position of the eyespot; DIC: differential interference contrast; scale bars: 10 µm.

(E – H) Cilia (C) were isolated from the indicated strains either at the end of the night phase under red safety light or after illumination by the pH shock method and analyzed along with crude extracts (CE) by SDS-PAGE as described. Coommassie Brilliant Blue (Coomassie) staining indicates a comparable protein load. (E) The ChR1 signal is detected in the CE and purified C of dark-kept cells of the parental strain (wt, CC-3403) but not in the ΔChR1 strain. Protein load: 8 µg. (F) Purified C of dark-kept CC-125 cells were sub-fractionated into the membrane-matrix (MM) and axonemal fraction (Ax) and analyzed by immunoblotting along with the CE. Protein load: 4 µg. (G, H) The ChR1 level in highly purified (G) and rapidly isolated cilia (H) decreases upon illumination. CC-3403 cells were illuminated either for 2 h (G) or the indicated times (H) with WL + UV (81 µmol $m^{-2}s^{-1}$) prior to immunoblot analyses. For comparison, also the CE is shown in (G). Protein load: 8 µg. Data plotted in (H) are the mean ± SEM of three independent biological replicates.

(I, J) $ChR1_{mod}$ forms are present in cilia. (I) Cells from strain CC-125 were harvested at the end of the night phase, pre-incubated for 1 h with CHX and the following fractions were isolated from dark-kept cells or illuminated cells (1 h; WL + UV; 188 µmol photons m^{-2} s^{-1}): crude extract (CE), cell bodies (CB), and cilia (C). Samples were separated by SDS-PAGE and analyzed by immunoblotting with anti-ChR1. Equal protein load is demonstrated by Coommassie Brilliant Blue (Coomassie) staining of the lower part of the blots. (J) CC-125 cells were treated as in (I) and, where indicated, additionally incubated with 100 mM TEMPOL. Fractions (8 µg) shown in I and J were separated on one gel and the immunoblots of the purified ciliary fractions were developed 7 – 8.5 x longer than the other fractions. * indicates the region of the $ChR1_{mod}$ forms and HMMCs indicate the position of the ChR1-containing high M_r complexes. Representative blots of two independent biological replicates are shown.

Figure 6: Light-induced ChR1 degradation and starting levels in mutants affected in components of the IFT machinery and the motor proteins dynein and kinesin-2

(A - C) ΔIFT88 cells were incubated along with the rescued strain CC-5687 at the end of the night phase for 1 h in the dark in the presence of CHX before being illuminated with WL + UV (81 µmol m^{-2} s^{-1}) for the indicated times. Protein extraction, further processing and ChR1 quantification were as described. (A) shows a representative immunoblot probed with anti-ChR1 and Coomassie Brilliant Blue (Coomassie) staining of the lower part of the blot to demonstrate equal protein load. (B) Quantification of the immunoblot analyses shown in (A). Data points are the mean ± SEM (n = three independent biological replicates). (C) Normalized ChR1 starting levels prior to the onset of illumination.

(D, E) Light-induced ChR1 degradation (D) and ChR1 starting levels (E) in cells just prior to illumination of the insertional mutant strain *bbs4-1* and the rescued strain CC-4373. Treatments and ChR1 quantification

were as described above. Data points are the mean ± SEM (n = four independent biological replicates).

(F, G) Cells of the strain ΔLC8 along with the rescued strain CC-3939 were treated as described in (A – C). (F) shows the light-induced degradation kinetics and (G) the ChR1 starting level normalized to CC-3939 just prior to illumination. Data plotted are the mean ± SEM of four independent biological replicates.

(H, I) Cells of the kinesin-2 null mutant strain ΔKin2 and strain CC-2929 (wildtype) cells were treated and analyzed as above. ΔKin2 lacks cilia completely (Matsuura et al., 2002). (H) shows the ChR1 degradation kinetics and (I) the normalized starting levels of the photoreceptor. Data points are the mean ± SEM (n = four independent biological replicates).

(J, K) Cells of the temperature-sensitive *Fla10-1* mutant of kinesin-2 (Wahlter et al., 1994) were grown at 15 °C and shifted for 20 h either to 33 °C or kept at 15 °C. Resorption of cilia at 33 °C was verified by phase contrast microscopy. At the end of the night phase both cultures were supplemented with CHX, pre-incubated for 1 h in the dark and the illuminated with WL + UV as above. The desired temperature was maintained during the illumination period. Data for the degradation kinetics (E) and the normalized starting levels at the end of the night phase (F) are the mean ± SEM of three independent biological replicates.

Figure 7: Light-induced ChR1 degradation and dark levels in strains affected in cilia formation.

Cells of the indicated strains were pre-incubated for 1 h in the dark with CHX and illuminated for the indicated times with WL + UV (81 μmol m^{-2} s^{-1}). Further processing and quantification of ChR1 was done as described.

(A, B) Light-induced ChR1 degradation of cells possessing only the *trans* cilium (*uni1*) and two cilia lacking (*bld*) strains (A) and their ChR1 starting levels (B) at the end of the night phase relative to strain CC-125 (wt). Data are the mean ± SEM of three independent biological replicates.

(C − E) Light-induced ChR1 degradation (C, D) and relative ChR1 starting levels (E) of two cilia lacking mutant strains (CC-2906, CC-2907) and their corresponding suppressor mutants (CC-2908, CC-2909). Data are the mean ± SEM of three independent biological replicates.

(F) Wild-type cells (CC-125) were pre-incubated at the end of the night phase in the dark for 1 h with CHX and, as indicated, with or without colchicine (CLC). Cells were then de-ciliated under red safety light by pH shock with acetate buffer. De-ciliation was verified by phase contrast microscopy (Fig. S10). The corresponding control cultures were treated as these cells, except that TAP was used instead of the acetate buffer. Cell bodies were pelleted, suspended in fresh TAP supplemented with CHX and CLC as indicated and incubated in the dark for 30 min. Cultures were then illuminated for the indicated times with WL + UV (81 μmol m^{-2} s^{-1}) and analyzed for their ChR1 content. Data are the mean ± SEM of five independent biological replicates.

Figure 8: Effects of the SUMOylation inhibitor 2-D08 on light-induced ChR1 degradation and its ciliary levels.

(A, B) CC-125 cells were harvested under red safety light at the end of the night phase, pre-incubated in the dark with fresh TAP with 2-D08 (50 μM) or the solvent DMSO for 6 h and illuminated for the indicated times with WL + UV (81 μmol $m^{-2}s^{-1}$). Additionally, CHX was added 1 h prior to illumination to the medium. Further processing and quantification of ChR1 was as described. Data points are the mean ± SEM of three independent biological replicates.

(C) CC-125 cells were harvested at the end of the night phase, suspended in fresh TAP without CHX supplemented with 2-D08 (50 μM) or DMSO and incubated for 6 h in the dark. Cells were then de-ciliated under red safety light by pH shock with acetate buffer. Successful deciliation was verified by phase contrast microscopy. After neutralization and centrifugation, the cell bodies were suspended in fresh TAP supplemented with 2-D08 or DMSO but without CHX. Cilia regeneration was allowed in the dark for 2 h prior to an additional de-ciliation. Samples (8 μg) of the crude extract (CE), cell bodies (CB), and purified cilia (C) were separated by SDS-PAGE and analyzed as described. Coommassie Brilliant Blue (Coomassie)

staining demonstrates equal protein load. All fractions were separated on one gel and the immunoblot of the purified cilia was developed 9.4 x longer than that of the other fractions. * indicates the region of the $ChR1_{mod}$ forms. Representative blots of two independent biological replicates are shown.

Figure 9: Effects of ARL11 knock-out on ChR1 starting levels, degradation, ciliary localization, cilia length, swimming speed and eyespot size.

(A – C) Two independent *arl11* mutant strains along with their parental strain CC-3403 were pre-incubated in the dark with CHX and illuminated for the indicated times with WL + UV (81 µmol m^{-2} s^{-1}). Further processing was done as described. (A, B) Light-induced ChR1 degradation and (C) normalized starting levels at the end of the night phase. Data plotted are the mean ± SEM from three independent biological replicates.**(D)** DIC images of the *arl11* mutant strains and their parental strain. Arrowhead: eyespot, scale bars: 5 µm. **(E)** Box plot (whiskers min to max) of the eyespot area. Cultures of the different strains were grown under 20 µmol $m^{-2}s^{-1}$ WL and analyzed in parallel. Different lower case letters indicate statistically significant differences (ANOVA analyses with Tukey´s multiple comparison post test, $p < 0.0001$;$n = 98$ to 108 cells). **(F)** Box plot (whiskers min to max) of the swimming speeds of cells from the indicated strains (*n:* 75 – 115). Scattered dot plot of the cilia length of the same strains at the beginning of the light phase (*n* : 80 – 97; line: mean). No statistically significant differences between the strains were resolved by ANOVA analyses with Tukey´s multiple comparison post test for both parameters. **(G)** Purified cilia and cell bodies of the two*arl11* mutants and CC-3403 were isolated from dark grown cells and analyzed by immunoblotting.

Figure 10: $ChR1_{mod}$, HMMCs and ChR1 degradation products are present in extracellular vesicles (EVs).

(A) Detection of $ChR1_{mod}$ in EVs and proof of signal specificity. EVs and CE were isolated from two different parental strains and their corresponding ΔChR1 strains at the end of the dark phase under red safety light. Aliquots of 8 µg were analyzed after separation by SDS-PAGE and immunoblotting with anti-ChR1. * indicate the region of the $ChR1_{mod}$ forms and HMMCs indicate the position of the ChR1-containing high M_r complexes. See (E) for a list of all strains analyzed with similar results.**(B)** $ChR1_{mod}$ forms are enriched in ΔCEP1-C EVs in comparison to its parental strain CC-3403. EVs were isolated from cells illuminated for 2 h with WL + UV (188 µmol photons m^{-2} s^{-1}) in the presence or absence of 1mM PMSF. For comparison also the CE of ΔCEP1-C was loaded. Protein load: 8 µg. **(C, D)** Time-course analyses of the appearance of the $ChR1_{mod}$ forms upon illumination with WL + UV (188 µmol photons m^{-2} s^{-1}) in EVs from the strains ΔCEP1-C (C) and its parental strain CC-3403 (D). Note the difference in protein load between C (6 µg) and D (8 µg). **(E)**List of all strains analyzed for the presence of ChR1 in EVs.

Figure 11: EVs from strains with and without cilia differ in size and protein composition .

Whole mount electron microscopy of fixed, negatively stained EVs from the ciliated strain CC-2908 **(A-E)** and strain CC-2906 with no cilia **(F-J).** The EVs exhibit the typical cup-shaped morphology of exosomes. No vesicles were visible in identically treated control grids with buffer. EVs were isolated from the supernatant of 5 billion cells transferred at the end of the night phase to an identical volume of fresh TAP medium after a two h incubation period in the dark. Scale bars: 200 nm (A – E; G – J) and 500 nm (F). **(K, L)** Size distribution profiles of the EVs. Images were analyzed with the TEM ExosomeAnalyzer (Kotrbová et al., 2019; settings: minimal vesicle size 10 nm and 5 nm bin size). **(M)** Protein patterns of EVs isolated from the indicated strains. *bld2* and CC-2906 cells lack cilia. Protein load based on equal cell number used for EV isolation. For comparison also 6 µg of crude extracts (CE) resolved on the same gel are shown.

Figure 12: Schematic model depicting the regulation of*CEP1* expression (A) and ChR1 internalization via the endocytic pathway.

(A) Excitation of the blue-light receptor PHOT inhibits an E3 ubiquitin ligase complex (SPA1, COP1, CUL4, DDB1, RBX1, ubiquitin-conjugating enzyme E2), thereby preventing poly-ubiquitination (U) of transcription factors like Hy5 and other yet unidentified factors (?). The transcription factor interacting with the *CEP1* gene is not yet known. In turn the transcription factors accumulate in the nucleus and

lead to increased transcription of *CEP1* and additional genes involved in ChR1 degradation. Independent from light, increased cAMP levels can lead to increased levels of the *CEP1*mRNA. CEP1 is transported to lysosomes and MVBs. **(B)** Increasing illumination leads to removal of ChR1 from the PM region of the eyespot apparatus (EA) and its accelerated endocytotic internalization, most likely via CME. Prior or during endocytosis ChR1 is modified and forms HMMCs. The nature of the modifications is not yet known. Our inhibitor studies point to ubiquitination and/or SUMOylation. The cysteine protease CEP1 is essential for ChR1 degradation in lysosomes (LY) and MVBs. Additionally, a minor fraction of the $ChR1_{mod}$forms and HMMCs are secreted via fusion of MVBs at the PM and/or the ciliary base in EVs. The small GTPase ARL11, which interacts with the ciliary targeting VXPX motif in the C-terminus of ChR1 (Sharma et al., 2023), is important for the formation of ChR1 transport vesicles at the TGN, and/or their transport and targeting to the cilia and eyespot. It might have also a function in retrograde transport from the early endosomes (EE) to the TGN. Further, SUMOylation is important for cilia targeting of ChR1, which - as the potential role of the cilia in ChR1 homeostasis - is not depicted here. See text for a more detailed discussion.

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