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1 **Interdomain interactions reveal the molecular evolution of**  
2 **the Orange Carotenoid Protein**

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12

13 **Abstract**

14 The photoactive Orange Carotenoid Protein (OCP) is a blue-light intensity sensor  
15 involved in cyanobacterial photoprotection. Three OCP families co-exist (OCPX, OCP1  
16 and OCP2) which originated from the fusion of ancestral domain genes. Here we report  
17 the first characterization of an OCPX and the evolutionary characterization of OCP  
18 paralogues focusing on the role of the linker connecting the domains. The addition of  
19 the linker with specific amino acids enabled the photocycle of the OCP ancestor. OCPX  
20 is the paralog closest to this ancestor. A second diversification gave rise to OCP1 and  
21 OCP2. OCPX and OCP2 present fast deactivation and weak antenna interaction. In  
22 OCP1, the OCP deactivation became slower and the interaction with the antenna  
23 became stronger requiring another protein to detach the OCP from the antenna and  
24 accelerate its deactivation. OCP2 lost the tendency to dimerize present in OCPX and  
25 OCP1 and the role of its linker is slightly different giving a less controlled  
26 photoactivation.

27

28

## 29 **Introduction**

30 The Orange Carotenoid Protein, the light intensity sensor involved in cyanobacterial  
31 photoprotection, is a modular photoactive protein composed of an N-terminal domain  
32 (NTD) and a C-terminal domain (CTD) connected by a long flexible linker loop<sup>1-3</sup>. One  
33 keto-carotenoid molecule (3-hydroxy equinenone) is shared between the two domains<sup>4</sup>.  
34 In darkness, the OCP remains in the inactive “orange form” (OCP<sup>O</sup>) and adopts a closed  
35 conformation stabilized by the carotenoid and by interactions between the two  
36 domains<sup>4</sup>. Under strong blue-green light, the OCP is photoactivated and converted into  
37 the red active form (OCP<sup>R</sup>)<sup>5</sup>. Upon light absorption, the H-bonds between the CTD and  
38 the carotenoid are broken and the carotenoid migrates (12Å) into the NTD<sup>6</sup> in about 10  
39 μs<sup>7</sup>. Then the inter-domain interactions are disrupted and the protein adopts an open  
40 configuration<sup>6,8,9</sup> with a higher hydrodynamic radius<sup>10,11</sup>. By interacting with the core of  
41 the cyanobacterial antenna, the phycobilisome (PBS), the NTD of OCP<sup>R</sup> induces the  
42 dissipation of the excitation energy as heat<sup>6,12-14</sup>. The OCP<sup>R</sup> slowly relaxes back to  
43 OCP<sup>O</sup> in darkness<sup>5</sup>. Another cyanobacterial protein, called Fluorescence Recovery  
44 Protein (FRP), accelerates this process<sup>15,16</sup> by increasing the probability of the proper  
45 configuration of domains and carotenoid in which they may interact to form OCP<sup>O</sup><sup>17</sup>.  
46 The main binding site of the FRP is in a CTD region that is exposed only in the red  
47 active form<sup>16,18,19,20</sup>. FRP binding also helps OCP detachment from PBSs<sup>13,21</sup>. Both FRP  
48 activities are essential to recover the full photosynthetic capacity under low light  
49 conditions.

50 Phylogenetic analysis of the OCP family has shown the existence of three different OCP  
51 clades: OCP1, OCP2 and OCPX<sup>22</sup>. All previous studies (except one) characterizing the

52 activity and photoactivation of the OCP were done using OCP1 proteins. Recently, the  
53 OCP2, from *Tolypothrix* sp. PCC 7601 (hereafter *Tolypothrix*), was characterized<sup>22,23</sup>.  
54 No OCPX was characterized in the past. In addition to the OCP genes, genes of  
55 homologues of OCP domains are present in many cyanobacterial genomes<sup>4,24,25,26</sup>. The  
56 existence of these homologues have suggested a modular model for the evolution of  
57 OCP<sup>24,23</sup>.

58 The OCP1 and OCP2 domains can be isolated independently as carotenoid proteins  
59 (holo-protein) or apo-proteins (without carotenoid), depending on the expression system  
60 <sup>27,23,6,25</sup>. Interaction between holo-CTD and apo-NTD results in the formation of a  
61 complex, which is spectrally similar to OCP<sup>O</sup> (hereafter, OCP-like complex)<sup>23,27,25,28</sup>.  
62 This complex can be photoactivated leading to efficient carotenoid translocation from the  
63 CTD to the NTD and complex disassembly<sup>23,25,27</sup>. Afterwards, reassembling of the OCP-  
64 like complex from holo-NTD and apo-CTD was only observed in the case of the OCP2  
65 domains<sup>23</sup>. This suggested that OCP2 is more primitive than OCP1 and hinted towards  
66 an evolutionary role of the linker<sup>23</sup>.

67 The OCP characteristics as a light-regulated modular protein raise the possibility of  
68 using the OCP as a photoswitch for an optogenetic toolbox, adding a new class of  
69 chromophore and a new type of triggering mechanism in which initially coupled sensor  
70 and effector modules reversibly separate upon light absorption. To this end, an  
71 understanding of the inter-domain interactions and of the role of the linker connecting  
72 the two domains during the photocycle is required for all three OCP types. In this work  
73 we did an evolutionary and functional study of the three OCP clades and report the first  
74 characterization of two OCPXs, from *Scytonema hofmanni* PCC 7110 (hereafter

75 *Scytonema*) and *Synechocystis* PCC 7509. Our results show the essential role of the  
76 linker and specifically of some of its amino acids, in the OCP photocycle. We propose a  
77 new evolutionary model for OCP in which a primitive OCP diversified into OCPX and the  
78 ancestor of the modern OCP2 and OCP1, which arose as the result of a second  
79 diversification.

80

## 81 **Results**

### 82 **OCP evolution and photocycle**

83 Most of the OCP-containing cyanobacteria strains have only OCP1 (139). A large  
84 number contain only OCPX (35). By contrast, most of strains containing OCP2 also  
85 harbor OCP1 or OCPX (15); OCP2 is found alone in only 5 strains. In order to better  
86 understand the evolutionary history of OCP, we re-analyzed the phylogenetic history of  
87 OCP. This time we rooted the tree using CTDH and NTF2 as outgroups (see Materials  
88 and Methods and Supplementary Fig. 1). The analysis of the rooted tree strongly  
89 suggests that there was a first diversification (Fig. 1A, I) that gave rise to an ancestral  
90 OCPX and to the common ancestor of OCP1 and OCP2. Diversification of this ancestor  
91 (Fig. 1A, II) led to the modern OCP1 and OCP2 proteins. Our model shows that the  
92 OCP2 clade is not evolutionarily older than OCP1 (as it was previously proposed<sup>22,23</sup>),  
93 instead the common OCP1 and OCP2 ancestor diverged along two different pathways  
94 that probably led to different functional specializations. In *Tolypothrix*, OCP2 is  
95 expressed only under stress conditions and in a mutant lacking chromatic adaptation  
96 while OCP1 is constitutive<sup>22</sup>. It is important to note that different subgroups may exist  
97 within the OCP1 clade, in particular a small subgroup containing marine *Synechococcus*  
98 strains seems to be the most divergent OCP1 (Supplementary Fig. 1). Boundaries  
99 between OCPX and the rest of the tree are unclear, and a small population seems to be  
100 in an intermediary position between OCPX and OCP2/OCP1 (Fig. 1A).

101 To study the different properties of OCP clades and understand their evolution, we  
102 overexpressed and isolated an OCP from each clade: OCP1 from *Synechocystis* sp.  
103 PCC 6803 (hereafter OCP1), OCP2 from *Tolypothrix* (hereafter OCP2) and OCPX from

104 *Scytonema* (hereafter OCPX). *Tolypothrix* OCP2 and *Scytonema* OCPX were chosen  
105 because their primary sequences are close to the OCP2 and OCPX consensus  
106 sequences, respectively (Supplementary Fig 2). Furthermore, we also partially studied  
107 the OCP2 and OCPX from *Synechocystis* PCC 7509 to confirm the characteristics of  
108 each clade. Overexpression was performed in a canthaxanthin producing *E. coli* strain  
109 since echinenone binding in *Tolypothrix* and *Scytonema* OCPs induces lower  
110 photoactivity (Supplementary Fig. 3). The spectrum of OCPX<sup>O</sup> showed less vibrational  
111 band resolution compared that of OCP1<sup>O</sup> and OCP2<sup>O</sup>. The OCPX<sup>R</sup> and OCP1<sup>R</sup> spectra  
112 were identical, with a maximum at 525 nm. The OCP2<sup>R</sup> spectrum was red shifted by 10  
113 nm (Fig. 2A). At 8°C, the accumulation of *Synechocystis* OCP1<sup>R</sup> was slightly faster than  
114 that of OCP2<sup>R</sup> and OCPX<sup>R</sup> (Fig. 2B). However, *Tolypothrix* OCP1<sup>R</sup> accumulated slower  
115 than *Synechocystis* OCP1<sup>R</sup> and *Tolypothrix* OCP2<sup>R</sup> (Fig. 1B), in agreement with  
116 previous observations<sup>22</sup>. This indicates that variations in photoactivation rates can exist  
117 between different OCP1s and not only between OCP1 and OCP2 (OCPX). The back  
118 conversion (OCP<sup>R</sup> to OCP<sup>O</sup>) of OCPX and OCP2 was faster than that of either OCP1,  
119 OCPX displaying the fastest conversion rate (Fig. 2C and Supplementary Fig 3C). As  
120 expected OCP1 back conversion was accelerated by the presence of *Synechocystis*  
121 FRP (Fig 2C). This FRP accelerated the back reaction of all the OCP1s tested in our  
122 laboratory including *Tolypothrix* OCP1 (Supplementary Fig 3). By contrast, FRP did not  
123 accelerate the back conversion of OCPX and OCP2 (Fig. 2C and<sup>22</sup>). Cyanobacteria  
124 strains containing only OCPX or OCP2 do not contain the *frp* gene<sup>22</sup>. OCPX and OCP2  
125 of *Synechocystis* sp. PCC 7509 presented the same photoactivation and recovery  
126 characteristics of the *Scytonema* OCPX and *Tolypothrix* OCP2 respectively



127 (Supplementary Fig 4).

128 Essential amino acids involved in the FRP-OCP interaction are 100% conserved in the  
129 three OCP families, in particular F299<sup>21</sup> and the negative charge of D220<sup>21</sup>  
130 (Supplementary Fig 2). However, other amino acids which are also thought to be  
131 involved in the OCP-FRP interaction<sup>16</sup> are not conserved: changes concern R229 to  
132 E(OCP2) or K(OCPX) and D262 to G(OCP2) or N(OCPX) (Supplementary Fig. 2). In  
133 the past, we demonstrated that simple mutations of R229 or D262 do not hinder the  
134 OCP-FRP interaction<sup>21</sup>. To further investigate the effect of these mutations on the OCP-  
135 FRP interaction, we created a *Synechocystis* OCP1 R229E/D262K double mutant. In  
136 this case, FRP was unable to accelerate OCP1 back conversion, suggesting that FRP  
137 cannot interact with OCP2 and OCPX due to these amino acid exchanges (Fig. 2C).  
138 Interestingly, these mutations also accelerated the back conversion even without FRP,  
139 making OCP1 more similar to OCP2 and OCPX.

140 Next, we studied the OCP-PBS interaction by measuring the PBS fluorescence  
141 quenching induced by the three different OCPs. *Synechocystis* PBS were used since it  
142 was previously demonstrated that these are the most suitable PBS for in vitro  
143 experiments<sup>29</sup>. The three photoactivated OCPs were able to induce large fluorescence  
144 quenching in isolated PBS at 1 M phosphate, a concentration which favors OCP-PBS  
145 interaction (Fig. 2D). By decreasing the concentration of phosphate from 1 M to 0.5 M  
146 differences between the OCPs became measurable: OCP1 induced the largest  
147 quenching and OCPX the lowest. This was due to weaker attachment of OCP2 and  
148 OCPX to PBS, indicated by a faster recovery of OCP-induced PBS fluorescence  
149 quenching (Fig. 2E). *In vivo*, in *Scytonema* cells containing only OCPX and lacking

150 FRP, strong blue-green light induced a large fluorescence quenching (Fig. 2F),  
151 indicating that OCPX binding to *Scytonema* PBSs in the cells can induce PBS  
152 fluorescence quenching. Furthermore, in these cells, OCPX was able to detach from  
153 PBS and allow fluorescence recovery in the absence of FRP (Fig. 2F). This was also  
154 observed in *Gloeobacter violaceus* cells which also contain only OCPXs and no FRP<sup>30</sup>.

### 155 **Oligomeric state of the different OCPs**

156 In a native gel, OCP1 and both OCPXs appeared as dimers while both OCP2s  
157 appeared as a monomer (Fig. 3A and Supplementary Fig 5). In size-exclusion  
158 chromatography experiments, the elution volume of OCP1 and both OCPXs exhibited a  
159 strong dependency on protein concentration indicating a tendency to dimerize (Fig. 3B  
160 and C and Supplementary Fig. 5). OCP1 and OCPX were in monomer-dimer  
161 equilibrium with an exchange rate faster than the experiment timescale, giving only one  
162 elution peak. Under our experimental conditions, no complete dimerization was  
163 observed even for the highest OCP concentrations (120  $\mu$ M). The change in the elution  
164 volume was larger for OCPX than OCP1, suggesting a higher dimerization propensity of  
165 OCPX. On the other hand, the elution peak of both OCP2s corresponding to its  
166 monomeric form was not affected by protein concentration confirming that the OCP2-  
167 monomer is stable (Fig. 3D and Supplementary Fig 5). Even though the three OCPs  
168 have similar molecular weights, OCP1 had a slightly larger elution volume than  
169 monomeric OCPX and OCP2 suggesting differences in monomer structure (Fig. 3D and  
170 Supplementary Fig 5).

171 It has previously been proposed that the hydrogen bond R27-D19 between two OCP1  
172 monomers stabilizes the OCP1 dimer. Indeed, the mutated R27L OCP1 remained as a

173 monomer at all concentrations (Fig. 3A and 3E). In OCP2, R27 which is required to  
174 stabilize the dimer, is replaced by S and this could explain the stability of OCP2  
175 monomer. However, R27 is also absent in OCPX, which forms dimers, implying that  
176 other amino acids are involved in monomer interactions in OCPX.

### 177 **Interdomain interaction in the different OCP clades**

178 We then studied the interaction between isolated OCP-CTD (CTD) and isolated OCP-  
179 NTD (NTD) from the three OCPs. We analyzed the binding affinity of different pairs of  
180 NTD-CTD by titrating a fixed canthaxanthin binding CTD (CAN-CTD) concentration with  
181 increasing amounts of apo-NTD. We measured the formation of OCP-like complexes  
182 (hereafter, OCP-like) (Supplementary Fig. 6). The resulting saturating curves showed  
183 that significantly more apo-NTD1 (more than 25  $\mu$ M) and apo-NTDX (22 $\mu$ M) than apo-  
184 NTD2 (5 $\mu$ M) were needed to form maximal concentrations of OCP-like complexes (Fig  
185 4A). Furthermore, the formation of OCP2-like was considerably faster than that of the  
186 OCPX-like and OCP1-like (Fig. 4B) and its photoactivation was the slowest while that of  
187 OCP1-like was the fastest (Fig. 4D, E and F). Taken together all these results suggest a  
188 stronger interaction of CTD2-NTD2 than CTDX-NTDX and NTD1-CTD1, the latter  
189 displaying the weakest interaction; CTDX-NTDX interaction is intermediary. However, at  
190 this stage we cannot discard that differences in carotenoid affinity between the different  
191 NTDs could also influence OCP-like formation<sup>31,32</sup>.

192 To study the role of the linker connecting the two OCP domains in OCP photoactivation  
193 we compared the photoactivation rate of the OCP-like complexes (lacking the linker)  
194 with that of the original OCPs. OCP1-like and OCPX-like complexes showed a faster  
195 photoactivation than their respective OCPs (Fig. 4D and F). Hence, the presence of the

196 linker slowed down photoactivation. By contrast, photoactivation of OCP2-like was  
197 slower than that of OCP2 showing that the presence of the linker accelerated the  
198 photoactivation in this case (Fig. 4E). In the photoactivated OCP and OCP-like  
199 complexes, the domains were fully separated and the carotenoid was in the NTD. While  
200 in the OCP<sup>R</sup>, the domains were still attached via the linker, in the photoactivated OCP-  
201 like, however, the domains were completely free without connections between them. In  
202 darkness, holo-NTD1 was not able to form again an OCP-like complex by interaction  
203 with apo-CTD1, even in the presence of FRP (Fig. 4C and<sup>23</sup>). OCPX-like complexes  
204 recovered only 10 % of their initial state and OCP2-like considerably re-assembled from  
205 holo-NTD2 and apo-CTD2 indicating that in this case the domains were reassembled in  
206 the proper orientation to allow the carotenoid to move towards the CTD (in agreement  
207 with previous report<sup>23</sup>). OCP2-like recovery was slower than that of OCP2 (Fig. 4C).  
208 Interestingly, when independently isolated holo-NTD2 was incubated with apo-CTD2, no  
209 formation of OCP2-like was observed (Supplementary Fig. 6C). These results suggest  
210 that upon photoactivation NTD2 and CTD2 remain somewhat interacting and/or that  
211 when the domains are isolated separately apo-CTD can adopt a conformation that  
212 hinders the uptake of the carotenoid (see <sup>31</sup>).

213

#### 214 **The role of the linker in photoactivity and recovery of OCP1**

215 The linker loop connecting the OCP domains is a 25-28 amino acid unstructured region  
216 with only a few residues conserved (Fig.1B and 1C). In order to study the role of the  
217 linker loop, highly conserved amino acids were mutated in *Synechocystis* OCP1:  
218 K167A, R171A, E191K, E174K, R185A, R185E, E174K-R185E, Δ174-179 and P175A-

219 P179A. All these mutated OCP1s were isolated from an echinenone (ECN) producing  
220 *E. coli*. In darkness, the OCP1-linker mutants (with the exception of those containing the  
221 E174K mutation) showed the same orange absorbance spectrum as WT OCP1  
222 (Supplementary Fig 7). The mutated OCP1s carrying the E174K mutation showed a  
223 decrease in the 470 nm peak and an increase at 550 nm, especially the OCP1 E174K-  
224 R185E mutant (Supplementary Fig. 7). Spectral deconvolution showed that this change  
225 was related to the accumulation of OCP<sup>R</sup> even in darkness (approximately 45% of  
226 OCP<sup>R</sup> in E174K-R185E).

227 After 5 min illumination with strong white light, all OCP1-linker mutants exhibited the  
228 typical spectrum of OCP<sup>R</sup> (Supplementary Fig. 7). All OCP1 mutants (except for K167A)  
229 accumulated the OCP<sup>R</sup> form faster than the WT OCP1 (Fig. 5A and Supplementary Fig.  
230 8). This affected the photoactivation light dependency: the mutations in the linker  
231 induced higher OCP<sup>R</sup> accumulation at low light intensities (Fig. 5C). The back-  
232 conversion kinetics to the orange state were slower in the OCP1 mutants than in WT  
233 OCP1 (Fig. 5B and Supplementary Fig. 8). E174K and R185A mutations largely  
234 affected the rate of recovery; OCP1 E174K-R185E and OCP1  $\Delta$ 174-179 presented the  
235 slowest kinetics. Furthermore, OCP<sup>R</sup> was more stable at all the temperatures tested in  
236 the linker mutants than in the WT OCP1 (Supplementary Fig. 8). The only exception  
237 was OCP1 K167A, which showed the opposite effect: a slower photoconversion and a  
238 faster recovery than WT OCP1.

239 To determine the activation energy of photoactivation and back conversion, the kinetics  
240 were measured at different temperatures and Arrhenius plots were generated (Fig. 5D,  
241 5E and Supplementary Fig. 8). The calculated activation energy ( $E_a$ ) of the back

242 conversion for WT OCP1, 37 kcal/mol (in agreement with the values previously  
243 published<sup>10</sup>), is statistically the same as in the mutants (Supplementary Fig. 8). By  
244 contrast, the pre-exponential factor which is related to the probability of a collision  
245 occurring in the proper orientation was different in mutants and WT. The Arrhenius plot  
246 of WT OCP1 photoactivation showed a positive convexity that can be explained by a  
247 decrease in activation energy of photoactivation at high temperatures (higher than  
248 33°C), possibly due to the destabilization of protein interactions at these high  
249 temperatures as previously suggested<sup>10,33</sup>. In the linker mutants (with exception of  
250 K167A) the break point of the convex plot is shifted toward lower temperatures (Fig. 5D,  
251 from 33°C (I) to 20°C (II)). This indicated that the temperature required to destabilize the  
252 OCP structure is lower for the linker mutants.

253

#### 254 **Role of the linker in the OCP1 interaction with phycobilisomes and FRP**

255 In order to study the role of the mutated amino acids on the OCP-PBS interaction, *in*  
256 *vitro* OCP induced PBS fluorescence quenching was assessed. Most of the linker-  
257 mutants induced similar amplitudes of PBS fluorescence quenching as OCP WT (Fig.  
258 6A). PBS fluorescence recovery was slower when the fluorescence quenching was  
259 induced by most of these OCP mutants due to a higher stability of their OCP<sup>R</sup> (Fig. 6B).  
260 By contrast, OCP P175A/P179A induced less fluorescence quenching and the recovery  
261 was faster than that of OCP WT despite its high OCP<sup>R</sup> stability (Fig 5), indicating that  
262 the interaction with PBS was affected in this mutant (Fig. 6A and B).

263 Deletion of 6 amino acids in the  $\Delta$ 174-179 OCP caused an extensive loss of OCP  
264 quenching activity in spite the fact that this mutation stabilized the OCP<sup>R</sup>. This could be

265 due to a partial opening of the domains although the protein is red upon illumination. It  
266 was recently demonstrated that the protein can become red even without opening<sup>7,34</sup>.  
267 Analysis of the hydrodynamic volume of photoactivated OCPs showed that the apparent  
268 Stoke's radius of the  $\Delta 174-179$  OCP was slightly smaller to that of the photoactivated  
269 WT OCP suggesting that although in the mutated photoactivated OCP the domains  
270 were separated, the complete opening of the protein was not fully reached  
271 (Supplementary Fig. 9). Nevertheless, we cannot discard that the lack of these specific  
272 amino acids also might have affected the OCP-PBS interaction.  
273 The addition of FRP to photoactivated OCPs accelerated the recovery in the WT OCP  
274 and all the OCP-linker mutants (compared Fig. 6D and E). Even the slowest recovery  
275 reactions (OCP E174K-R185E and OCP  $\Delta 174-179$ ) were accelerated by FRP addition.  
276 Furthermore, addition of FRP largely accelerated the recovery of PBS fluorescence in  
277 the PBSs quenched by all the OCP linker mutants (Fig. 6C).

278

### 279 **Role of the linker in the OCP2 and OCPX clades**

280 In OCP2 and OCPX the number of conserved residues in the linker is significantly lower  
281 than in OCP1 (Fig. 1C). In OCP2, E174 is not highly conserved in contrast to R185,  
282 which is highly conserved (Fig.1C). In OCPX, a higher diversity of amino acid  
283 composition is found in the linker region, where however E174 is highly conserved.  
284 Thus, to study whether the role of these amino acids in OCP2 and OCPX is similar to  
285 their role in OCP1 we created OCP2 R185E-CAN and OCPX E174K-CAN mutants for  
286 comparison with their respective WT OCP-CAN.

287 Both OCP2 and OCPX linker mutants showed a decreased rate of OCP<sup>R</sup> to OCP<sup>O</sup> back

288 conversion compared to their respective WTs (Supplementary Fig. 10). These results  
289 indicated that the mutations in the linker increased the stability of OCP<sup>R</sup> in OCP2 and  
290 OCPX as was the case for OCP1. Like in OCP1, mutation E174K slightly increased the  
291 rate of OCP<sup>R</sup> accumulation in OCPX (Supplementary Fig. 10) and decreases the light  
292 intensity required for complete photoconversion (Supplementary Fig. 11). On the other  
293 hand, the R185E mutation in OCP2 slightly decreased the rate of OCP<sup>R</sup> accumulation  
294 (Supplementary Fig. 10).

295

## 296 **Discussion**

297 According to the current evolutionary model, fusion of primitive *ctdh* and *hcp* genes  
298 gave rise to a primitive OCP<sup>23,24,22</sup>. We propose that the ancestral HCP was already  
299 able to interact with the PBS like the modern HCP4<sup>36</sup>. Addition of an appropriate linker  
300 and a CTD made the NTD-PBS interaction more stable, allowing for more efficient  
301 photoprotection<sup>6</sup>. We suppose that the primitive OCP already had the activities found in  
302 modern OCPs and that the diversification merely brought a more sophisticated  
303 regulation of OCP photoactivation and recovery.

304 The phylogenetic analysis of a rooted tree we present here shows that the primitive  
305 OCP diversified into OCPX and the ancestor of the modern OCP2s and OCP1s, which  
306 are the result of a second diversification. OCP1 and OCP2, which co-exist in many  
307 strains, seem to have diverged and acquired different specializations by maintaining or  
308 changing different properties present in their common ancestor which are still found in  
309 modern OCPX (Figure 1D). OCP2 is mostly present in strains which also constitutively  
310 express OCPX or OCP1. It is known that at least in *Tolypothrix*, OCP2 is expressed



311 only under stress conditions in which cells need more protection<sup>22</sup>. Thus, some specific  
312 properties of OCP2 might have an advantage for a better response to “permanent”  
313 stress conditions while OCP1 properties must be advantageous under low, medium and  
314 fluctuating light conditions.

315 The previous evolutionary model proposed that before the genes fused, an OCP-like  
316 complex existed formed by the heterodimer of a primitive NTD and CTD that shared a  
317 keto carotenoid. Based on *Tolypothrix* OCP2 results, it was originally proposed that this  
318 primitive OCP-like could photocycle even in the absence of the linker loop connecting  
319 the NTD and CTD<sup>22,23</sup>. However, here we have shown that this is probably not likely  
320 since the ancient OCPX-like complex cannot photocycle: after photoactivation, the  
321 isolated holo-NTDX and apo-CTDX were unable to reassemble to form the OCP-like  
322 complex. Thus, our results strongly suggest that the addition of the linker loop provided  
323 the primitive OCP with two new features: the possibility to photocycle by dark recovery  
324 into the inactive form and a more controlled photoactivation. The modern OCPX has  
325 kept these features, showing a fast dark recovery and a slightly negative effect of the  
326 linker on photoactivation. OCP2 kept the fast recovery present in its ancestor and in  
327 OCPX. In OCP1, two point mutations acquired during evolution in the CTD (in amino  
328 acids 229 and 262) made the recovery reaction considerably slower. This added one  
329 more point of regulation in the OCP photocycle which now required an exterior  
330 component, the FRP, in order to accelerate the recovery reaction.

331 The OCP1 clade conserved the slightly negative effect of the linker on photoactivation.  
332 This fine regulation allowed the accumulation of high concentrations of OCP<sup>R</sup> only at  
333 high light intensities, which is particularly important for OCP1 and OCPX since they are

334 expressed even under low light conditions<sup>12,22,30,37</sup>. In OCP2, the strong affinity between  
335 the domains renders photoactivation too slow for effective photoprotection in the  
336 absence of the linker which in this case, functions as an accelerator of photoactivation.  
337 Thus, the linker has opposite roles in OCP1 and OCP2 with respect to photoactivation.  
338 By contrast, it has the same accelerating role in the OCP<sup>R</sup> to OCP<sup>O</sup> conversion for all  
339 OCPs.

340 Specific linker amino acids are essential to the photocycle: E174K and R185E  
341 mutations greatly decreased the recovery rates in the three types of OCPs. The length  
342 of the linker is also important to facilitate the recovery to OCP<sup>O</sup> since the deletion of 6  
343 amino acids largely slowed down recovery. Thermodynamic analysis strongly  
344 suggested that the role of these amino-acids in the unstructured linker is to provide a  
345 specific environment, in which the OCP-domains are reoriented into the proper  
346 configuration allowing the inter-domain interaction that favors the formation of  
347 carotenoid tunnel and the recovery to OCP<sup>O</sup> (see Supplementary Fig 13). Interestingly,  
348 the linker area around the amino acids which most affected OCP recovery is the less  
349 flexible part of the linker according to MD simulations (Supplementary Fig. 12).

350 On the other hand, specific amino acids have only a minor effect on the OCP-PBS  
351 interaction. Only the simultaneous mutation of prolines 175 and 179 slightly decreased  
352 the strength of the OCP-PBS interaction. Most of the other linker mutations did not  
353 affect OCP-induced PBS fluorescence quenching. By contrast, the size of the linker was  
354 crucial. The deletion of 6 linker amino acids completely inhibited PBS fluorescence  
355 quenching strongly suggesting that a long linker is needed to allow a sufficiently wide  
356 separation of the domains allowing the NTD to be buried between two APC trimers in

357 the PBS core<sup>38</sup>.

358 The FRP was unable to induce recovery after photoactivation of OCP1-like complex  
359 indicating that the presence of the linker is essential for FRP activity. However, none of  
360 the mutated amino acids in the linker were shown to be essential for FRP interaction. It  
361 is possible that after the primary binding of FRP to the CTD, a high local concentration  
362 of NTD is required for its secondary binding to the OCP<sup>18,19,39</sup> and acceleration of back  
363 conversion. This high concentration is successfully achieved by connecting both  
364 domains through the linker.

365 A monomer to dimer transition could provide an additional regulation of the OCP  
366 function. Despite the fact that R27, which is essential for OCP1 dimerization, is not  
367 conserved in OCPX, OCPX is able to dimerize. This indicates that another interaction  
368 must stabilize the OCPX dimer. Further regulation by a dimer-to-monomer transition  
369 might not have been important for carrying out the OCP2 role under stress conditions,  
370 leading to its loss during evolution.

371 Taking all our results together, it is clear that the linker loop played an essential role  
372 during OCP evolution, along with inter-domain interaction, by allowing fine tuning of the  
373 OCP behavior and better photoprotection and functional divergence between the  
374 different families of OCPs. In addition, the understanding of the various molecular  
375 determinants controlling the OCP photoswitching could help in the design of optogenetic  
376 toolkit for synthetic biology.

377

## 378 **Materials and Methods**

379

380 **Construction of OCP, NTD and CTD Plasmids for expression in *E. coli* cells**

381 Construction of plasmid pCDF-OCPsynNtag carrying the *Synechocystis slr1963* gene  
382 with a His-tag encoding sequence in the 3'-terminus was described previously<sup>40</sup>. The  
383 point mutations were introduced by directed mutagenesis, using the pCDF-OCPsyn-  
384 Ntag plasmid as a template and mismatching primers (Eurofins) (Supplementary Table  
385 S1). The constructions of the pCDF-CTD1<sup>25</sup> and pCDF-NTD1<sup>6</sup> plasmids containing the  
386 sequences encoding the isolated *Synechocystis* CTD and NTD domains were described  
387 previously<sup>6,25</sup>.

388 Construction of pCDF-OCP2 and pCDF-OCP1 from *Tolypothrix*

389 The *Tolypothrix* sp. PCC 7601 *ocp1* and *ocp2* genes were amplified by PCR using  
390 genomic DNA of *Tolypothrix* sp. PCC7601 as template and the primers OCP1-pDuet (F  
391 and R) or OCP2-pDuet (F and R). The resulting PCR products were cloned into the  
392 BamHI and NotI site of pCDFDuet-1 creating the plasmids *pCDF-NtagOCP2* and *pCDF-*  
393 *NtagOCP1*. With this construction, a His-tag will be present in the N-terminus of the  
394 protein. The point mutation R185E was inserted in the OCP2 gene by direct  
395 mutagenesis (supplementary Table SI).

396 Construction of the *pCDF-NTD2 Ctag*

397 To construct the *pCDF-NTD2 Ctag* plasmid, the sequence coding the N-terminus His-  
398 tag in the *pCDF-NtagOCP2* plasmid was excised by mutagenesis using F-ocp2-BamHI  
399 and R-Duet to create the plasmid *pCDF-OCP2*. Then, a deletion of the nucleotides of  
400 the *ocp2* encoding the last 154 amino acids (CTD) and an addition of a sequence  
401 encoding a C-terminal His-tag were realized simultaneously by mutagenesis using the  
402 plasmid *pCDF-OCP2* as template and synthetic primers (F-DuetOCP2 Ctag and R-

403 NTD2 Ctag).

#### 404 Construction of the *pCDF-CTD2 Ctag*

405 To construct the *pCDF-CTD2 Ctag* plasmid, first, the nucleotides encoding for 6 His tag  
406 were added in the 3'-terminus of the *ocp2* gene by site-directed mutagenesis using the  
407 *pCDF-OCP2* and the synthetic primers (F-DuetOCP2 Ctag and R-DuetOCP2 Ctag).

408 Then, the nucleotides encoding the NTD (1-189) of the *ocp2* gene were deleted by site-  
409 directed mutagenesis using the plasmid *pCDF-OCP2 Ctag* as template and synthetic  
410 primers (F-DNTD2 and R-Duet).

#### 411 Construction of the *pCDF-NtagOCPx from Scytonema hofmanni PCC 7110*

412 The synthetic gene (Eurofins) of the OCPX from *Scytonema hofmanni* PCC7110 (DNA  
413 fragment from genome positions 2,712,095 to 2,713,054) was cloned into the BamHI  
414 and NotI site of pCDFDuet-1. With this construction, a His-tag will be present in the N-  
415 terminus of the protein. The point mutation E174K was inserted in the OCPX gene by  
416 directed mutagenesis (supplementary Table SI).

#### 417 Construction of the *pCDF-NTDX Ctag*

418 To construct the *pCDF-NTDX Ctag* plasmid, first, the N-terminal extension containing  
419 the His-tag initially present in the *pCDF-NtagOCPX* plasmid was excised by  
420 mutagenesis using F-OCPX-BamHI and R-Duet to create the plasmid *pCDF-OCPX*.

421 Then, a deletion of the last 154 amino acids (CTD) of the *OCPX* gene and an addition of  
422 C-terminal His-tagged were realized simultaneously by mutagenesis using the plasmid  
423 *pCDF-OCPX* as template and synthetic primers (F-DuetOCPX Ctag and R-NTDX Ctag).

#### 424 Construction of the *pCDF-CTDx Ctag*

425 To construct the *pCDF-CTDx Ctag* plasmid, the nucleotides encoding for 6 His tag were  
426 added in the 3'-terminus of the *ocpx* gene by site-directed mutagenesis using the *pCDF-*  
427 *OCPx* (previously described in this work) and the synthetic primers (F-DuetOCPx Ctag  
428 and R-DuetOCPx Ctag) to create the *pCDF-OCPx Ctag* plasmid. Then, the nucleotides  
429 encoding the N-terminal domain (1-189) of the *ocp2* gene were deleted by site-directed  
430 mutagenesis using the plasmid *pCDF-OCPx Ctag* as template and synthetic primers (F-  
431 NTDx and R-Duet).

#### 432 Construction of pCDF-OCP1 (linker mutants)

433 The specific punctual mutations in the linker region of pCDF-OCP1-Ntag were  
434 introduced by site-directed mutagenesis using the plasmid pCDF-OCP1-Ntag as  
435 template and the corresponding synthetic primers (Supplementary table 2A-B).

#### 436 Construction of the *pCDF-NtagOCPx* from *Synechocystis sp. PCC 7509*

437 The synthetic gene (Eurofins) of the OCPX from *Synechocystis sp. PCC 7509* (IMG ID :  
438 2517697059) was cloned into the BamHI and NotI site of pCDFDuet-1. With this  
439 construction, a His-tag will be present in the N-terminus of the protein.

#### 440 Construction of the *pCDF-NtagOCP2* from *Synechocystis sp. PCC 7509*

441 The synthetic gene (Eurofins) of the OCP2 from *Synechocystis sp. PCC 7509* (IMG ID:  
442 2517700424) was cloned into the BamHI and NotI site of pCDFDuet-1. With this  
443 construction, a His-tag will be present in the N-terminus of the protein.

#### 444 **Holoprotein production and purification in carotenoid-producing *E. coli***

445 BL21 (DE3) cells from Agilent Technologies were transformed simultaneously with three  
446 plasmids: (1) pACBETA, (2) pBAD-CrtW (for canthaxanthin production) or pBAD-CrtO

447 (for echinenone production), and (3) a pCDF-OCP plasmid (carrying either OCP1,  
448 OCP2, OCPX, the respective linker mutants, CTD1, NTD1, CTD2, NTD2, CTDX or  
449 NTDX). The construction of pACBETA and pBAD-CrtW(-CrtO) was described  
450 elsewhere<sup>40</sup>. The *crtBEIY* operon in pACBETA was constitutively expressed under the  
451 control of the *crtE* promoter, whereas the *crtW* and *crtO* gene were under the control of  
452 the arabinose-inducible promoter *araBAD* and the *OCP* genes were under the control of  
453 a T7 RNA polymerase promoter. The expression method used to obtain the holo-  
454 proteins was described previously<sup>40</sup>. OCP, NTD, CTD holo-proteins and apo-proteins  
455 expressed in *E. coli* were isolated as described elsewhere<sup>36,40</sup>. Briefly, cells were  
456 resuspended in lysis buffer (40 mM Tris pH 8/10% glycerol/300mM NaCl/1mM  
457 EDTA/1mM PMSF, 1mM caproic acid/1mM benzamidic acid/ 50  $\mu\text{g mL}^{-1}$  DNase) then  
458 broken in dim light using a French press. The membranes were pelleted, and the  
459 supernatant was loaded on a nickel affinity column (Ni-Probond resin, Invitrogen).  
460 Proteins were eluted with 250 mM imidazole and then dialyzed against 40 mM Tris-HCl  
461 pH 8. A final purification step was performed by hydrophobic interaction  
462 chromatography (HiTrap Phenyl HP column, GE Healthcare). Total OCP concentration  
463 was measured using the Bradford method and the carotenoid absorbance at 496 nm  
464 using  $A_{1\%}^{1\text{cm}}=2158$ . The construction of the *E. coli* strain overexpressing the  
465 *Synechocystis* FRP and the isolation of FRP was described in (Boulay et al, 2010)

#### 466 **Size Exclusion Chromatography**

467 Purified proteins were injected into a Superdex Increase 75 10/300 column (GE  
468 Healthcare) equilibrated with 40mM Tris-HCl pH 8, 150mM NaCl. The Akta FLPC  
469 system was equipped with a UV detector at 280 nm, 510nm and 560 nm and was set at

470 a flow rate of 0.8 mL/min. The Gel Filtration Calibration Kit LMW (GE Healthcare) was  
471 used to calibrate the column.

#### 472 **Absorbance measurements and kinetic analysis**

473 Absorbance spectra, kinetics of photoactivation (illumination with 5000  $\mu\text{mol photons m}^{-2}$   
474  $\text{s}^{-1}$  of white light) and dark recovery of the OCP were measured in a Specord S600  
475 spectrophotometer (Analyticjena) at 8, 12, 15, 19, 23, 27, 33 or 37 °C in 1 cm  
476 pathlength cuvette. Spectra were acquired from 250nm to 700nm for each time point.  
477 The percentage of OCP<sup>R</sup> formed was calculated from the absorbance changes at  
478 550nm and from spectral deconvolution. To study the effect of FRP, we added  
479 *Synechocystis FRP* (2.6  $\mu\text{M}$ ) just before turning off the light.

480 To study CTD-NTD complex formation, holo-CTDs (2.25  $\mu\text{M}$ ) were incubated with  
481 different concentrations of apo-NTD at 12°C for one hour in darkness. The percentage  
482 of OCP-like complexes was determined after spectral deconvolution (Supplementary  
483 Fig. 6).

#### 484 **Protein Native Gel Electrophoresis**

485 Nondenaturing gel electrophoresis was performed to determine the oligomeric state of  
486 OCPs. 10  $\mu\text{L}$  of purified proteins (10  $\mu\text{M}$ ) were applied to 15% native polyacrylamide  
487 gels (pH 8). Electrophoresis was carried out using 25 mM Tris/192 mM Gly buffer, pH 8.  
488 Bovine serum albumin (65 kD) and ovalbumin (45 kD) were used as marker proteins.

#### 489 **Isolation of PBS and Fluorescence measurements**

490 The purification of PBS from *Synechocystis* PCC 6803 was performed as previously  
491 described<sup>13</sup>. Fluorescence yield quenching was monitored using a pulse amplitude



492 modulated fluorimeter (101/102/103-PAM, Walz). Measurements were made in a 1 cm  
493 path length stirred cuvette. The PBS quenching induced by holo-OCPs was measured  
494 in 0.5 M or 1 M potassium phosphate buffer (pH=7.5) at 23°C in strong blue-green light  
495 ( $650 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). The PBS concentration used was 0.012  $\mu\text{M}$  and the ratio of  
496 carotenoid to PBS was 40:1. OCP samples were pre-illuminated with 5000  $\mu\text{mol}$   
497  $\text{photons m}^{-2} \text{s}^{-1}$  of white light.

### 498 **Phylogenetic analysis**

499 In order to build the OCP phylogenetic tree, we performed a BLAST<sup>41</sup> search, using as  
500 seed the *slr1963* protein sequence and an E-value cut-off of  $1 \times 10^{-4}$ , to retrieve protein  
501 sequences from IMG. For tree rooting, two outgroups (CTDH *all4940* and NTF2  
502 CP024957.1 *Streptomyces cavourensis*) were added independently giving the same  
503 result. Sequences obtained were then aligned using default ClustalOmega<sup>42</sup>. Different  
504 trees were constructed using different methods to confirm the result: Maximum  
505 likelihood (ML), Bayesian inference and Neighbor-Joining. ML analysis was done using  
506 PhyML 3.1<sup>43</sup> in order to build a phylogenetic tree, with the following parameters: LG  
507 substitution model, NNI topology search and aBayes algorithm for branch support.  
508 Bootstrapped Neighbor-Joining trees were constructed using the Kimura-2-parameter  
509 model and 1,000 bootstrap repeats were built to give statistical support. Bayesian  
510 analysis was performed using MrBayes 3.1.2<sup>44</sup> software. Metropolis-coupled Markov  
511 Chain Monte Carlo (MCMC) (using 4 chains) with 5,000,000 generations were run.  
512 Analysis of the distances between nodes was done using the biopython<sup>45</sup> library  
513 (Bio.Phylo module). Sequences logos were built with the online server Skyline<sup>46</sup>.

### 514 **Molecular dynamic simulation**

515 The Apo-OCP structure was modelled using the SWISS-MODEL<sup>47</sup> server, using the  
516 OCP structure (PDB: 3MG1) as template in order to model the missing part of the  
517 flexible linker. The simulation was performed using GROMACS<sup>48</sup> simulation package  
518 version 5.1.1. The coordinate and topology for the simulations were generated using the  
519 GROMACS pdb2gmx protocol using the OPLSAA/L force field. Solvation was obtained  
520 using the spc216 explicit water model. Simulations were carried out at constant  
521 temperature and pressure (300 K, 1 atm), in NaCl 150 mM for 100 ns.

## 522 **DATA AVAILABILITY**

523 The sequences of the OCPs isolated and studied in this article are accessible in the  
524 GenBank/EMBL data libraries (or the IMG database) under the following accession  
525 numbers: *slr1963* (OCP1 *Synechocystis*, IMG ID: 2514153952), Fdi2450 (OCP1  
526 *Tolypothrix*, IMG ID: 2501541336), Fdi7374 (OCP2 *Tolypothrix*, IMG ID: 2501546328)  
527 and WA1\_RS11680 (OCPX *Scytonema*, IMG ID: 2551963320). The protein sequences  
528 and their corresponding identifier for the IMG database used for the phylogenetic  
529 analysis can be found in the Supplementary Information (available as an .xls file in the  
530 online version of the article). The additional data that support the findings of this study  
531 are available from the corresponding author upon request.

532 The author responsible for correspondence and distribution of materials integral to the  
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534

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546

547 **AUTHOR CONTRIBUTIONS** F.M. performed all the OCP characterization experiments  
548 and constructed some mutants; A.W. constructed almost all the OCP mutants; D.K.  
549 conceived the project, designed and supervised almost all the experiments and  
550 analyzed data; the article was written by F.M. and D.K.

551

#### 552 **COMPETING INTERESTS**

553 The authors declare no competing financial or non-financial interests.

554

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690 **Figure legends**

691 **Figure 1 | OCP evolution and OCP linker. (a)** The phylogenetic tree from the Bayesian  
692 analysis of the OCP protein family was rooted using All4940 (*Anabaena* PCC 7120  
693 CTDH) as outgroup. Representative cyanobacteria are marked in the tree: 1  
694 (*Synechocystis* OCP1), 2 (*Arthrospira Maxima* OCP1), 3 (*Nostoc*. PCC 7120 OCP1), 4  
695 (*Tolypothrix* OCP1), 5 (*Tolypothrix* OCP2) and 6 (*Scytonema* OCPX). A complete tree  
696 with taxon names and branch support values is provide in Supplementary Fig. 1. **(b)**  
697 OCP1 from *Synechocystis* structure (PDB ID: 3MG1). NTD is colored in grey, CTD is  
698 colored in green and the linker is cyan. The linker amino acids targeted for mutational  
699 analysis are shown in red. **(c)** Sequence logos representation of multiple alignment of  
700 OCP subclades linker sequences. **(d)** Evolutionary model of the OCP. After gene fusion  
701 by addition of the linker OCP diverged (divergence point I) into OCPX and the ancestor  
702 of OCP1 and OCP2. The last diverged (divergence point II) into the modern OCP1 and  
703 OCP2

704

705 **Figure 2 | OCP 1, OCP2 and OCPX comparison. (a)** Absorbance spectra of OCP1<sup>O</sup>  
706 (green), OCP2<sup>O</sup> (blue), OCPX<sup>O</sup> (black), OCPx<sup>R</sup> (dashed black), OCP1<sup>R</sup> (dashed green)  
707 and OCP2<sup>R</sup> (dashed blue). The inlet shows the vibrionic structure of the OCP<sup>O</sup> spectra  
708 between 425 nm and 525 nm. **(b)** Kinetics of OCP photoactivation of *Synechocystis*  
709 OCP1 (green), OCP2 (blue), OCPX (black) and *Tolypothrix* OCP1 (red) at 8°C under  
710 5000 μE of white light illumination. **(c)** Kinetics of OCP deactivation of in the absence

711 (closed symbols) and presence (open symbols) of *Synechocystis* FRP (ratio 1 FRP: 1  
712 OCP) of *Synechocystis* OCP1 (green circle), OCP1-R229E-D262K (rose triangle),  
713 OCP2 (blue square) and OCPX (black triangle) at 8°C. **(d)** Kinetics of PBS fluorescence  
714 quenching under strong blue light ( $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) induced by pre-  
715 photoactivated OCPX (black triangle), OCP2 (blue square) and OCP1 (green circle) at  
716 phosphate 0.5 M (closed symbols) and at phosphate 1M (open symbols) at 23°C. **(e)**  
717 Kinetics of fluorescence recovery at 0.5 M phosphate. Same symbols than in (d). **(f)**  
718 OCP-related PBS fluorescence quenching induced by strong blue-green light ( $1000$   
719  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) measured in *Scytonema hofmanni* PCC 7110 cells (1 and 3: cells  
720 under dim blue light illumination of  $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ; 2: cells under high blue light  
721 of  $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). The curves (b-e) are an average of three independent  
722 experiments. The error bars represent the standard deviation of the data shown. The  
723 figures (a) and (f) show representative experiments, which were repeated 5 times with  
724 similar results.

725 **Figure 3 | OCP oligomeric state: Native gel and size exclusion chromatography.**

726 **(a)** Analysis of OCP oligomeric state by Coomassie-Blue stained Native PAGE: OCPX  
727 (10  $\mu\text{M}$ ), OCP2 (10  $\mu\text{M}$ ) and OCP1 (10  $\mu\text{M}$ ), two molecular markers (45 kDa and 66  
728 kDa) were added (lane 1 and 2). **(b to e)** Comparison of elution profiles in SEC at  
729 different concentrations: 120  $\mu\text{M}$  (blue), 80  $\mu\text{M}$  (orange), 40  $\mu\text{M}$  (red), 23  $\mu\text{M}$  (green), 4  
730  $\mu\text{M}$  (pink) and 2  $\mu\text{M}$  (sky blue) of holo-WT-OCP1<sup>o</sup> **(b)**, Holo-WT-OCPX<sup>o</sup> **(c)**, Holo-WT-  
731 OCP2<sup>o</sup> **(d)** and Holo-R27L-OCP1<sup>o</sup> **(e)**. Absorbance was measured at 510 nm. The  
732 figures (a-e) show representative experiments, which were repeated 3 times with similar  
733 results.

734 **Figure 4 | CTD-NTD interactions. (a)** Saturation curve of OCP-like formation from  
735 isolated CAN-CTD and apo-NTD. 2.25  $\mu\text{M}$  CAN-CTD was incubated with increasing  
736 concentrations of apo-NTD **(b)** Kinetics of OCP-like formation using CAN-CTD (2.25  
737  $\mu\text{M}$ ) and apo-NTD (18  $\mu\text{M}$ ) from OCP1 (green circle), OCP2 (blue square) and OCPX  
738 (red triangle). **(c)** Back-conversion from free domains to the OCP-like (CTD-NTD)  
739 complex at 23°C: CTD1-NTD1 (closed dark green circles), CTD1-NTD1 +  
740 *Synechocystis* FRP (open light green circles), CTD2-NTD2 (closed blue circles) and  
741 CTDx-NTDx (closed red circles), OCP2 (open orange diamond). **(d-f)** Comparison of  
742 photoactivation kinetics of full OCPs (closed symbols) and OCP-like complexes (open  
743 symbols) at 12°C under constant illumination of 1500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ : **(d)** OCP1  
744 and OCP1-like, **(e)** OCP2 and OCP2-like and **(f)** OCPX and OCPX-like. Inset in **(f)**:  
745 photoactivation kinetics of OCPX and OCPX-like normalized. The curves are an  
746 average of three independent experiments. The error bars represent the standard  
747 deviation of the data shown.

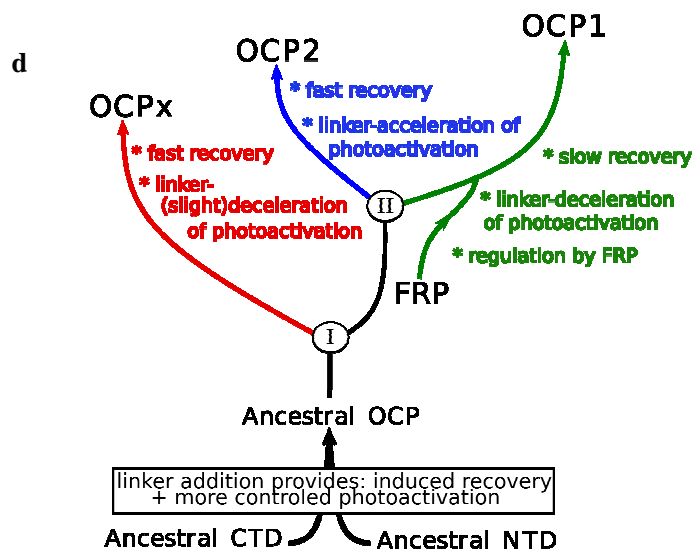
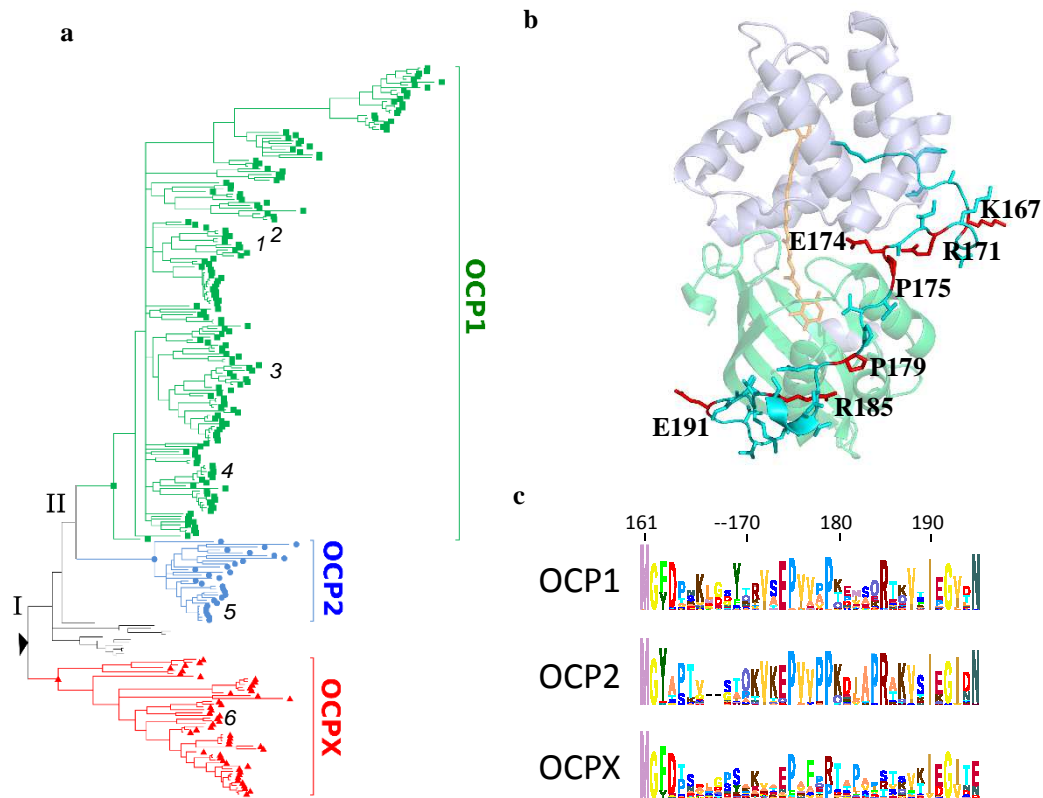
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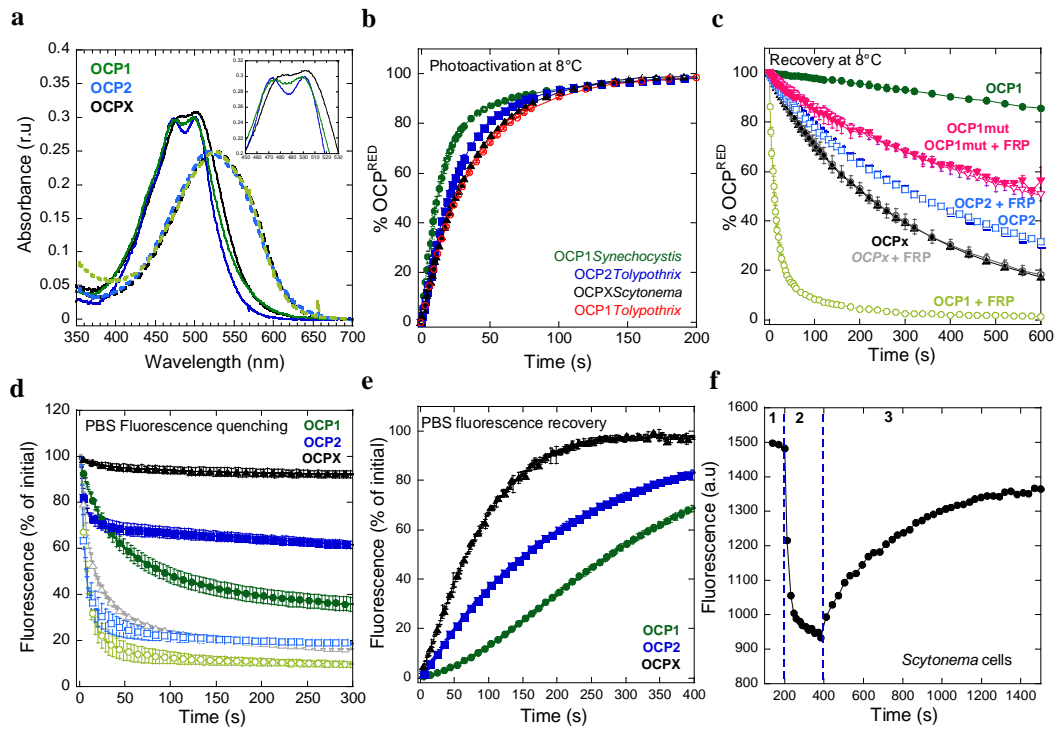
749 **Figure 5 | Photoactivity of OCP1 linker-mutants. (a)** Accumulation of  
750 photoactivated OCP<sup>R</sup> and **(b)** OCP<sup>R</sup> back-conversion to OCP<sup>O</sup> at 15°C of OCP WT  
751 (black closed circle), OCP E174K (fuchsia closed triangle), OCP E191K (orange closed  
752 square), OCP E174K-R185E (violet open triangle), OCP R185A (blue close diamond),  
753 OCP K167A (green open square) **(c)** Percentage of OCP<sup>R</sup> accumulated under different  
754 light intensities for WT OCP (black circles) and OCP-R185A (blue square) at 23°C. **(d)**  
755 Arrhenius plot of photoactivation of OCP WT (black), OCP E174K (red), OCP K167A  
756 (green), OCP R185A (sky blue) and OCP P175A-P179A (grey). Break point of the

757 convex curves is shown with dotted lines for the WT OCP (I) and the linker mutants (II)  
758 **(e)** Arrhenius plot of back-conversion  $OCP^R$  to  $OCP^O$  of OCP WT (black), OCP E174K-  
759 R185E (dark red), OCP R185A (sky blue), OCP K167A (green) and OCP R171A  
760 (orange). The curves are an average of three independent experiments. The error bars  
761 represent the standard deviation of the data shown.

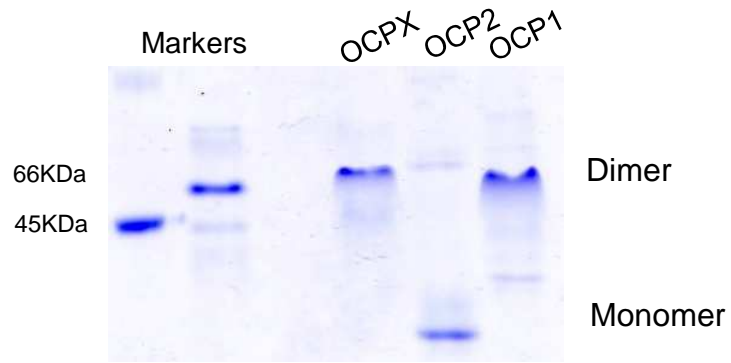
762 **Figure 6 | OCP1 linker mutants: interaction with PBS and FRP. (a)** Kinetics of PBS  
763 fluorescence quenching under strong blue light induced by previously photoactivated  
764 WT  $OCP^R$  (black),  $OCP^R$  E174K (red),  $OCP^R$  E174K-R185E (dark red),  $OCP^R$  R185A  
765 (sky blue),  $OCP^R$  R185E (blue),  $OCP^R$  K167A (green),  $OCP^R$  R171A (orange),  $OCP^R$   
766 P175A-P179A (grey) and  $OCP^R$   $\Delta$ 174-179 (pink). **(b and c)** Kinetics of dark recovery of  
767 PBS fluorescence in absence **(b)** or presence of *Synechocystis* FRP **(c)**. Back-  
768 conversion  $OCP^R$  to  $OCP^O$  at 8°C; in presence **(e)** or absence **(d)** of *Synechocystis* FRP  
769 (1 FRP per OCP) of  $OCP^R$  WT (black),  $OCP^R$  E174K (red),  $OCP^R$  E174K-R185E (dark  
770 red),  $OCP^R$  R185A (sky blue),  $OCP^R$  R185E (blue),  $OCP^R$  K167A (green),  $OCP^R$  R171A  
771 (orange),  $OCP^R$  P175A-P179A (grey) and  $OCP^R$   $\Delta$ 174-179 (pink). The curves are an  
772 average of three independent experiments. The error bars represent the standard  
773 deviation of the data shown.

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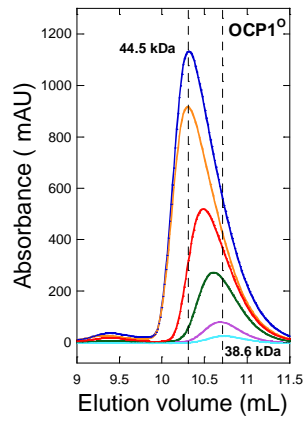




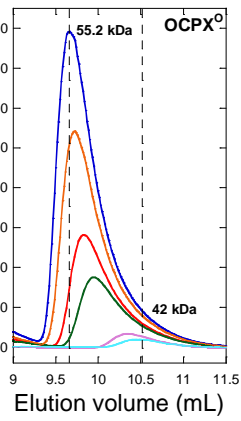
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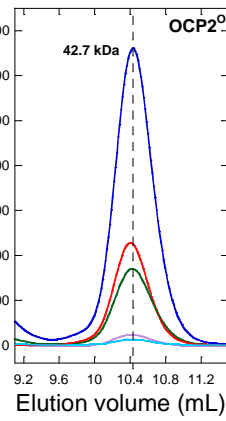
**b**



**c**



**d**



**e**

