Accessibility controls selective degradation of photosystem II subunits by FtsH protease

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The oxygen-evolving photosystem II (PSII) complex located in chloroplasts and cyanobacteria is sensitive to light-induced damage[1] which unless repaired causes reduction in photosynthetic capacity and growth. Although a potential target for crop improvement, the mechanism of PSII repair remains unclear. The D1 reaction center protein is the main target for photodamage[2], with repair involving the selective degradation of the damaged protein by FtsH protease[3]. How a single damaged PSII subunit is recognised for replacement is unknown. Here, we have tested dark stability of PSII subunits in strains of the cyanobacterium *Synechocystis* PCC 6803 blocked at specific stages of assembly. We have found that when D1, which is normally shielded by the CP43 subunit, becomes exposed in a photochemically active PSII complex lacking CP43, it is selectively degraded by FtsH even in the dark. Removal of the CP47 subunit, which increases accessibility of FtsH to the D2 subunit, induced dark degradation of D2 at a faster rate than that of D1. In contrast CP47 and CP43 are resistant to degradation in the dark. Our results indicate that protease accessibility induced by PSII disassembly is an important determinant in the selection of the D1 and D2 subunits to be degraded by FtsH.

The unusually high rate of synthesis and degradation, or turnover, of the D1 subunit of PSII, first observed over 40 years ago[4,5], reflects the selective replacement of D1 during the repair of PSII in response to light damage. In the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*), degradation of D1 is mediated by a specific membrane-bound FtsH2/FtsH3 protease complex[3]. How FtsH complexes differentiate between damaged and undamaged D1 subunits is unclear[6]. Given that the D1 protein is shielded in PSII by the PSII inner antenna, CP43, several small transmembrane PSII subunits as well as extrinsic proteins on the lumenal side of the complex, one possibility is that at least partial disassembly of PSII, possibly triggered by photodamage, facilitates contacts between FtsH and D1. If selective degradation of D1 is primarily driven by accessibility, which does not need to be caused just by photo-oxidative damage to D1, one interesting prediction is that undamaged D1 might be preferentially degraded in the dark in PSII complexes that have been mutated to improve access.

High resolution structures of cyanobacterial PSII have confirmed that the D1 and D2 reaction center subunits are shielded in the membrane by the intrinsic CP43 and CP47 subunits, and capped on the lumenal side of the membrane by three extrinsic subunits: PsbO, PsbU and PsbV (Fig. 1a)[7,8]. *Synechocystis* strains lacking the PsbO subunit still assemble oxygen-evolving PSII complexes but show a higher rate of D1 turnover than WT, possibly in response to an increase in the rate of photodamage to D1 due to perturbations on the donor side of PSII[9,10]. Enhanced D1 degradation in ΔPsbO might also be due to exposure of specific lumenal regions of D1 (normally hidden by PsbO and PsbU proteins, see Fig. 1) that are recognized by the lumenally exposed sequences of the FtsH complex. In the latter case the D1 protein in the ΔPsbO strain might still be degraded in the dark. To test this possibility, WT and the ΔPsbO strain were incubated in the dark in the absence and in the presence of the protein synthesis inhibitor lincomycin (LIN) to eliminate possible protein resynthesis. Subsequent

immunoblotting experiments confirmed that the major PSII proteins D1, D2, CP43 and CP47 were stable in the dark over a 4 h period, consistent with an inability of FtsH to access D1 and D2 in both WT and ΔPsbO unless the complex was further modified by photodamage (Fig. 1b)[10].

A *Synechocystis* mutant lacking the inner PSII antenna CP43 (ΔCP43 strain) also shows an extremely fast turnover of the D1 protein in the light[11] (Fig. S1). Like in ΔPsbO this turnover is dependent on the FtsH2/FtsH3 complex[11] and has been thought to relate to the fast light-induced damage occurring in the non-oxygen evolving PSII complex termed RC47, which lacks CP43 and associated low-molecular-mass (LMM) subunits but it is still photochemically active and able to transfer an electron from redox-active tyrosine Yz to bound plastoquinone Q_A[12]. However, based on the available structural models of cyanobacterial PSII[7,8], the absence of CP43 would necessarily expose the N-terminal stromal helix, first two trans-membrane helices and the interconnecting lumenal loop of D1, allowing interactions with other proteins in the membrane including the FtsH2/FtsH3 protease complex (Fig. 2a). To test whether FtsH could now degrade D1 in the absence of light-induced damage, immunoblotting experiments were performed on low-light grown cells transferred to the dark in the absence and presence of lincomycin. The data showed that levels of the CP47, D1 and D2 proteins were maintained in the absence of the inhibitor but in its presence the D1 protein was degraded to less than 20% of its initial level after 4 h of incubation (Fig.2b). The D2 protein was more stable but degraded to about 50% of its initial level while the CP47 antenna was not degraded at all. All three large PSII proteins were stable during the 4 h dark incubation in a derivative of the ΔCP43 mutant lacking the FtsH2/FtsH3 complex (ΔCP43/ΔFtsH2) confirming the crucial role of the FtsH2/FtsH3 complex in dark degradation (Fig. 2a). We also used native gel electrophoresis to check the assembly status of PSII proteins in ΔCP43 before and after dark incubation (Fig. 2c). At the beginning, all of D1 and

D2 were present in the RC47 complex, and only low amounts of CP47 were detected in an unassembled state. After the 4-hour dark treatment in the presence of inhibitor most of the D1 protein had been degraded while D2 and especially CP47 accumulated as unassembled proteins. A small amount of D2 and CP47 co-migrated in a region that could possibly correspond to a D2-CP47 degradation intermediate produced after D1 removal (Fig. 2c, vertical arrow). In contrast, in the absence of the FtsH2/FtsH3 complex, the RC47 complex found in the ΔCP43/ΔFtsH2 mutant remained intact.

Spectroscopic measurements have confirmed that the isolated RC47 complex is able to photoreduce Q_A and photo-oxidize tyrosine Yz with kinetics equivalent to that seen in non-oxygen-evolving PSII core complexes containing CP43 of WT[12]. Nevertheless, as the RC47 complex could be very sensitive to light-induced damage even during its growth under low light conditions, we pre-incubated the cells of the mutant in the dark for 14 hours to prevent possible light-induced damage to PSII before adding lincomycin (Fig. S2). After 4 hours of additional dark incubation the D1 protein was degraded to about 50% of its initial level. In summary, the data confirmed that the FtsH2/FtsH3 heterocomplex can degrade the D1 protein in the RC47 complex independent of light-induced damage. D2 is degraded slower, possibly after detachment of CP47 and LMM polypeptides PsbX and PsbY (Fig. 2a) while free CP47 is clearly much more resistant to proteolysis than D1 and D2.

It is still uncertain what triggers the detachment of CP43. Given that CP43 provides one of the amino-acid ligands to the Mn_4CaO_5 cluster, one possibility is that at least partial detachment of CP43 might be driven by light-induced destruction of the cluster, either as a primary effect of light-induced oxidative damage[13] or a secondary effect following damage elsewhere in PSII[13]. Detachment of some LMM PSII subunits like PsbJ may also change binding of CP43 and allow contact of D1 with FtsH. Another possibility is that light-induced oxidative damage to the polypeptide chain of D1 or bound co-factors weakens the interaction

of D1 with CP43. Light-induced damage to PSII could also cause peroxidation of the lipid belt located between the D1 protein and CP43 antenna[7,8] leading to destabilization of CP43 binding and opening the space for FtsH. Alternatively, a specific lipase activated by light-induced damage could attack the belt allowing access of FtsH to D1. We do not exclude the possibility that FtsH itself plays an active role in the partial disassembly of PSII during the initial phase of repair. Although the RC47 complex accumulates in the absence of FtsH2[11], detachment of CP43 in this case might be an effect of advanced light-induced damage such as the selective oxidation of Trp residues in CP43[14] which occurs in PSII when D1 cannot be rapidly replaced[15]. Detachment of CP43 most probably requires release of extrinsic lumenal proteins like PsbV. In this case lumenal parts of FtsH2/FtsH3 complex may provide temporary low-affinity binding sites to facilitate re-binding of extrinsic proteins to PSII during re-assembly.

If accessibility is important for selective degradation of D1, we reasoned that the removal of both CP43 and CP47 would now enhance degradation of D2 in addition to D1. To test this, we used a deletion strain lacking CP47 termed ΔCP47. In this strain PSII forms two RC complexes (RCa and RC*) containing D1, D2, PsbE, PsbF, PsbI and several additional proteins[16] while the second PSII antenna CP43 cannot attach to the complex and remains in an unassembled state[17]. According to the structural models of PSII the N-terminal tail of D2 as well as its N-terminal trans-membrane helices and the first lumenal loop are uncovered due to the absence of CP47 and PsbX (Fig. 3a, see also[16]). A pulse-chase experiment in cells exposed to light showed that the D2 protein was now turned-over at a rate exceeding that of the D1 protein, while unassembled CP43 was stable as observed for the CP47 antenna in the ΔCP43 strain (Fig. S1). When the cells of the mutant were incubated in the dark in the presence of lincomycin (Fig. 3b), the amount of the D2 protein decreased to about 50% of its initial content while the level of D1 (sum of mature D1 and iD1, a maturation intermediate[18])

decreased to about 70% of the initial level. Unlike the ΔCP43 mutant both proteins partly disappeared even in the absence of inhibitor suggesting that the absence of CP47 negatively affected the accumulation of D1 and D2 in the dark. When the FtsH2 protease was inactivated in the ΔCP47 strain, D1 and D2 were stabilized in the dark (Fig. 3a) again documenting the crucial role of the FtsH2/FtsH3 heterocomplex in degradation. 2D electrophoresis showed that the RCa complex containing D2, D1, cytochrome b-559, PsbI and the assembly factor Ycf48[16] disappeared slightly faster than the larger RC* complex which additionally contains the recently identified Ycf39-Hlip complex[16] (Fig. 3c). Overall, these data revealed that D2 degradation could also occur in the dark independently of light-induced damage in PSII RC assembly complexes.

Previous studies have often assumed that light-induced oxidative damage to D1 and D2 was required to trigger selective degradation[2]. The data presented here suggest that selective degradation of D1 and D2 can actually occur in the dark. This raises the new idea that undamaged D1 and D2 within PSII sub-complexes are already naturally triggered for proteolytic degradation by FtsH and, consequently, that unwanted degradation of undamaged D1 and D2 is prevented through the attachment of CP43 and CP47 antennae and LMM polypeptides. Given this, we suggest that damaged D1 is recognised and selectively degraded because of partial or complete detachment of CP43 from damaged PSII complexes which thereby directs the proteolytic machinery towards D1 rather than D2 degradation (for model see Fig. 4). Nevertheless, as the absence of CP43 may also affect binding of nearby LMM PSII subunits like PsbI and PsbJ, we cannot fully exclude that this modified binding may also contribute to the better accessibility of D1 to the protease. It would be logical for this accessibility to be regulated so that is synchronized with protein replacement but as yet the mechanistic details remain elusive. FtsH complexes play a key role in PSII repair in chloroplasts so it is likely that a similar situation applies to plants. In our model (Fig. 4), the

stimulatory effect of light on D1 and D2 degradation[2] is likely to be due to oxidative damage further destabilizing PSII structure to allow easier removal from the complex.

In contrast, we found that detached CP47 and CP43 are much more resistant to proteolytic degradation in the dark, possibly because their transmembrane helices are much more densely packed and stabilized by pigment binding, the N-terminal tail is inaccessible and accessory and LMM subunits bind on the periphery to prevent FtsH binding. Previous work on assembly mutants has reported that CP43 can accumulate to WT levels but that CP47 is much less stable and is a target of the FtsH2/FtsH3 complex[11]. Why detached CP47 is much more stable than pre-assembled CP47 is currently unclear and might reflect differences in the protein conformation depending on the content of bound pigments and auxiliary protein factors that help stabilize released CP47. Alternatively, pre-assembled CP47 might undergo more accurate quality control in the biogenesis membrane regions than in regions in which D1 and D2 are degraded.

Methods

Strains and Culture Conditions

The following previously described mutants of the glucose-tolerant strain of *Synechocystis* sp. PCC 6803, referred to here as wild-type (WT)[19], were used in the study: (i) the PsbO-less strain, ΔPsbO, with *psbO* gene inactivated by a spectinomycin (*spec*^{R}) resistance cassette[20], (ii) the CP43-less strain, ΔCP43, with *psbDIC* gene inactivated by a chloramphenicol (*Cm*^{R}) resistance cassette[21], FtsH2-less variant of CP43-less strain with *psbC* gene inactivated by an kanamycin resistance cassette and *ftsH2* gene inactivated by chloramphenicol resistance cassette[11], and (iii) the CP47-less strain, ΔCP47, with the *psbB* gene inactivated by an spectinomycin resistance cassette[22], and its FtsH2-less variant with *ftsH2* gene inactivated by

chloramphenicol resistance cassette. The latter ΔCP47/ΔFtsH2 double mutant was obtained by transforming the ΔCP47 cells by genomic DNA from *ftsH2*-less strain and selection for chloramphenicol resistance. The complete segregation of the mutant was confirmed by PCR. The strains were grown in BG-11 medium containing 5 mM glucose, solid media contained in addition 10 mM TES/NaOH, pH 8.2, 1.5 % agar and 0.3% sodium thiosulphate. 50-100 ml liquid cultures were shaken in 250 ml conical flasks at 29 °C with a surface irradiance of 10 µmol photons m^{-2} s^{-1} of white light due to light sensitivity of the *ftsH2* deletion strains. Cultures were analyzed in the exponential phase (OD_{750nm} in the range 0.6-0.8).

Before the dark incubation experiments the cells of each strain were divided into two aliquots, each placed into an Erlenmayer flask and stirred in the dark for four hours either in the absence or presence of lincomycin (LIN, 100 µg ml^{-1} final concentration)

Thylakoid preparation and protein analyses

Cyanobacterial membranes were prepared by breaking the cells using glass beads[23]. For analysis of protein complexes, isolated membranes were solubilized in 1% (w/w) dodecyl-β-D-maltoside (DM) and analyzed on 4-14% clear-native gel[24,23]. Individual components of protein complexes were resolved by incubating the gel strip from the first dimension in 2% SDS and 1% dithiothreitol for 30 min at room temperature and proteins were separated in the second dimension by SDS-electrophoresis in a denaturing 12-20% polyacrylamide gel containing 7 M urea[23]. Samples obtained for each strain were always analyzed on a single gel for direct comparison. One-dimensional SDS-PAGE for analysis of pulse-chase labeled proteins and for quantification of proteins in blots was carried out in the same 12-20% polyacrylamide gel containing 7 M urea. For autoradiography the gels were stained by Coomassie Blue, dried and exposed to Phosphorimager plate (GE Healthcare) overnight. The

intensity of the radioactively labeled bands was quantified by ImageQuant TL software (GE Healthcare). For immunoblotting, the gels were first stained with Sypro Orange (Stained gels) proteins from 1D or 2D gels were transferred onto a PVDF membrane and incubated with primary antibodies specific for D1, D2, CP43, CP47 and Ycf39[16] as well as with secondary antibody conjugated with horseradish peroxidase (Sigma-Aldrich, Germany). Samples with the same chlorophyll content (2 μg for 1D gels and 4 μg for 2D gel) were loaded onto the gel. For both 1D and 2D gels, bands of ATP synthase subunits α and β (AtpA/B) were used as the loading control and they are shown on the gels. In 1D blot the dilution series of the sample from the cells just before dark incubation (0h dark; 0.5, 1 and 2 μg of chlorophyll corresponding to 25, 50 and 100% of 0h dark sample) is also shown to document the response of the antibody. Three independent quantifications of proteins were performed and the values in the figures represent means of these measurements.

Models of PSII complexes based on the structure of *Thermosynechococcus elongatus* (PDB ID 4V62) were performed using PyMOL Molecular Graphics System. The D1 protein is shown in red, D2 in orange, CP47 in green, CP43 in pink, small PSII subunits in blue, PsbO in yellow, PsbU in blue-green and PsbV in dark violet.

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AuthorContributions

V.K. performed experiments performed and protein analyses under the supervision of J.K., J.K. designed the study, S.S. made model figures and P.N. and J.K. wrote the paper. All authors discussed the results and commented on the manuscript.

Competing financial interests
The authors declare no competing financial interests

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Figure legend

Figure 1. Model of the PSII core complex and complex lacking the lumenal subunits PsbO and PsbU (a) and the dark stability of PSII proteins in WT and PsbO-less mutant (b).

(a) Designation of proteins is described in Material and Methods, arrows designate N-terminal helices of D1 and D2, probable primary targets for the FtsH2/FtsH3 protease.

(b) Cells of the mutants were incubated in the dark in the presence (+LIN) and absence (-LIN) of lincomycin and their PSII protein content was assessed by immunoblotting. The values represent mean of three independent measurements of band intensities, SE did not exceed 8%.

Figure 2. Model of the RC47 assembly intermediate complex (a), degradation of PSII proteins in the ΔCP43 and ΔCP43/ΔFtsH2 mutant strains in the dark (b) and two dimensional analysis of membranes from the control and dark incubated mutant cells (c).

(a) Designation of proteins and helices as in Fig. 1.

(b) Cells of the mutants were incubated in the dark as in Fig.1, SE of three independent measurements did not exceed 10%.

(c) Proteins of the mutant cells were analyzed by 2D CN/SDS-PAGE, the gel stained by Sypro Orange (Gel stain) was electroblotted and used for immunodetection of PSII proteins.

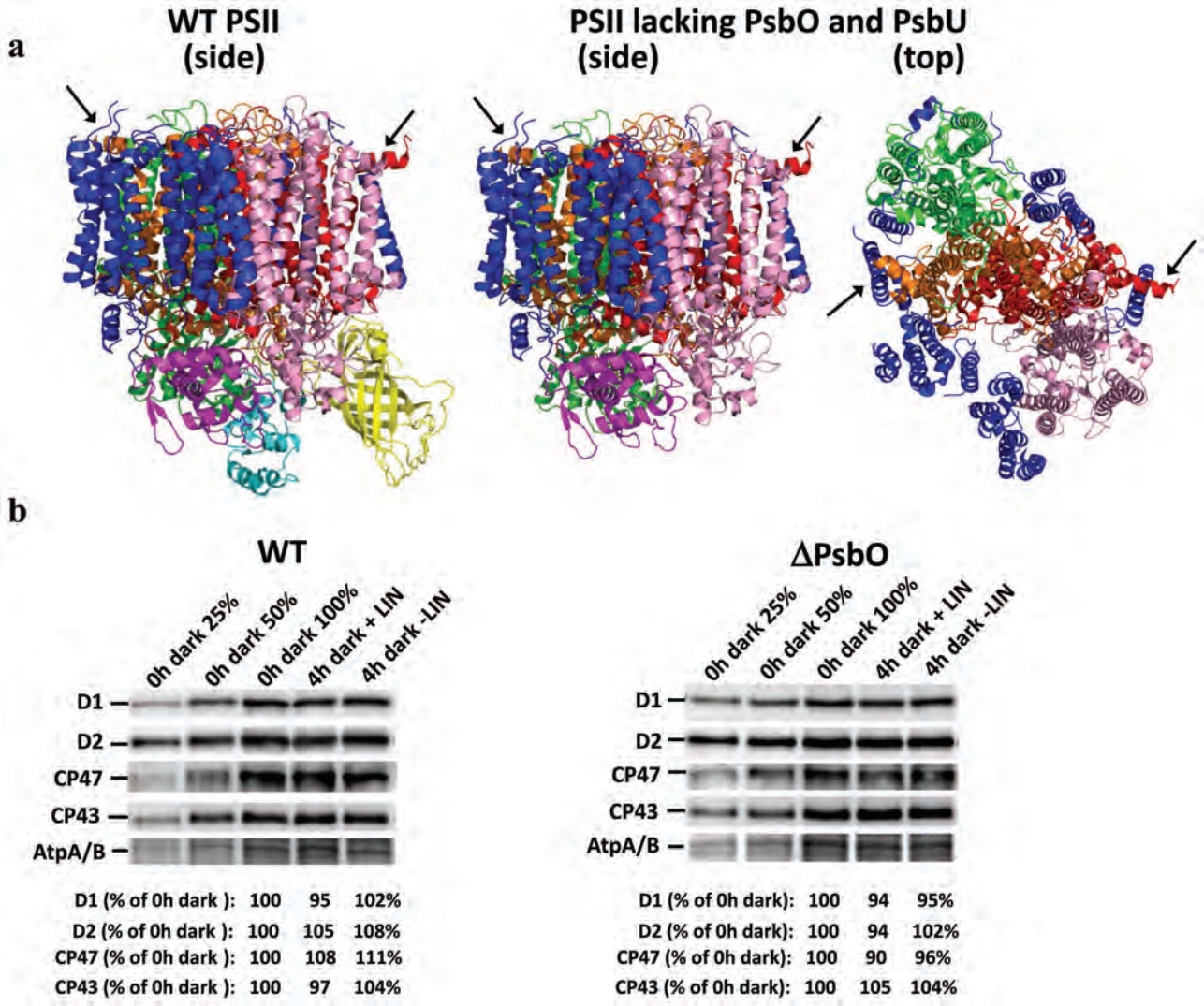
Figure 3. Model of the RCII assembly intermediate complex (a), degradation of the PSII proteins in the ΔCP47 and ΔCP47/ΔFtsH2 strain in the dark (b) and two dimensional analysis of membranes from the control and dark incubated mutant cells (c).

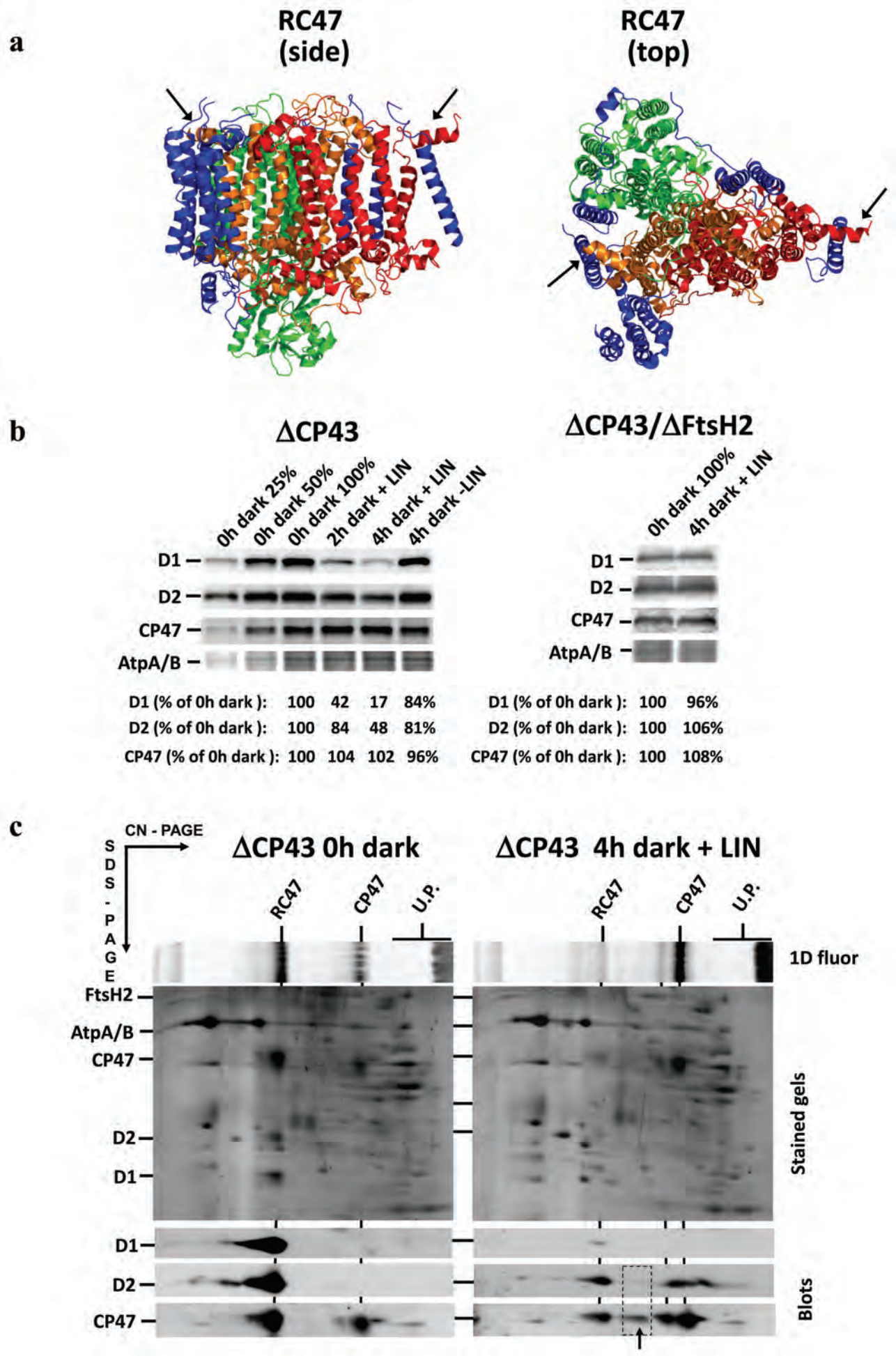
(a) Designation of proteins and helices as in Fig. 1.

(b) Cells of the mutants were incubated in the dark as in Fig.1, SE of three independent measurements did not exceed 10%.

(c) Proteins of the mutant cells were analyzed by 2D CN/SDS-PAGE, the gel stained by Sypro Orange (Gel stain) was electroblotted and used for immunodetection of PSII proteins.

Figure 4. Model of the selective degradation of the D1 and D2 proteins. PSII complexes are viewed perpendicular to the membrane plane with transmembrane helices shown in D1 (A) in red, D2 (D) in orange, CP47 (47) in green, CP43 (43) in pink and small subunits in blue. Only D1 and D2 in the assembly complexes lacking CP43 (RC47) or both CP43 and CP47 (RCII) can be approached by FtsH2/3 complex to initiate dark degradation. Photodamage to the monomeric PSII core complex (RCCII) and larger dimeric complexes (not shown) induces conformational changes to allow access of FtsH2/3 to damaged D1.


a
WT PSII
(side)
PSII lacking PsbO and PsbU
(side)
(top)
b
WT
ΔPsbO
0h dark 25%
0h dark 50%
0h dark 100%
4h dark + LIN
4h dark -LIN
D1
D2
CP47
CP43
AtpA/B
D1 (% of 0h dark): 100 95 102%
D2 (% of 0h dark): 100 105 108%
CP47 (% of 0h dark): 100 108 111%
CP43 (% of 0h dark): 100 97 104%
D1 (% of 0h dark): 100 94 95%
D2 (% of 0h dark): 100 94 102%
CP47 (% of 0h dark): 100 90 96%
CP43 (% of 0h dark): 100 105 104%


a
RC47
(side)
RC47
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b
ΔCP43
ΔCP43/ΔFtsH2
0h dark 25%
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2h dark + LIN
4h dark + LIN
4h dark -LIN
0h dark 100%
4h dark + LIN
D1
D2
CP47
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D1 (% of 0h dark): 100 42 17 84%
D2 (% of 0h dark): 100 84 48 81%
CP47 (% of 0h dark): 100 104 102 96%
D1 (% of 0h dark): 100 96%
D2 (% of 0h dark): 100 106%
CP47 (% of 0h dark): 100 108%
c
CN - PAGE
SDS-PAGE
ΔCP43 0h dark
ΔCP43 4h dark + LIN
RC47
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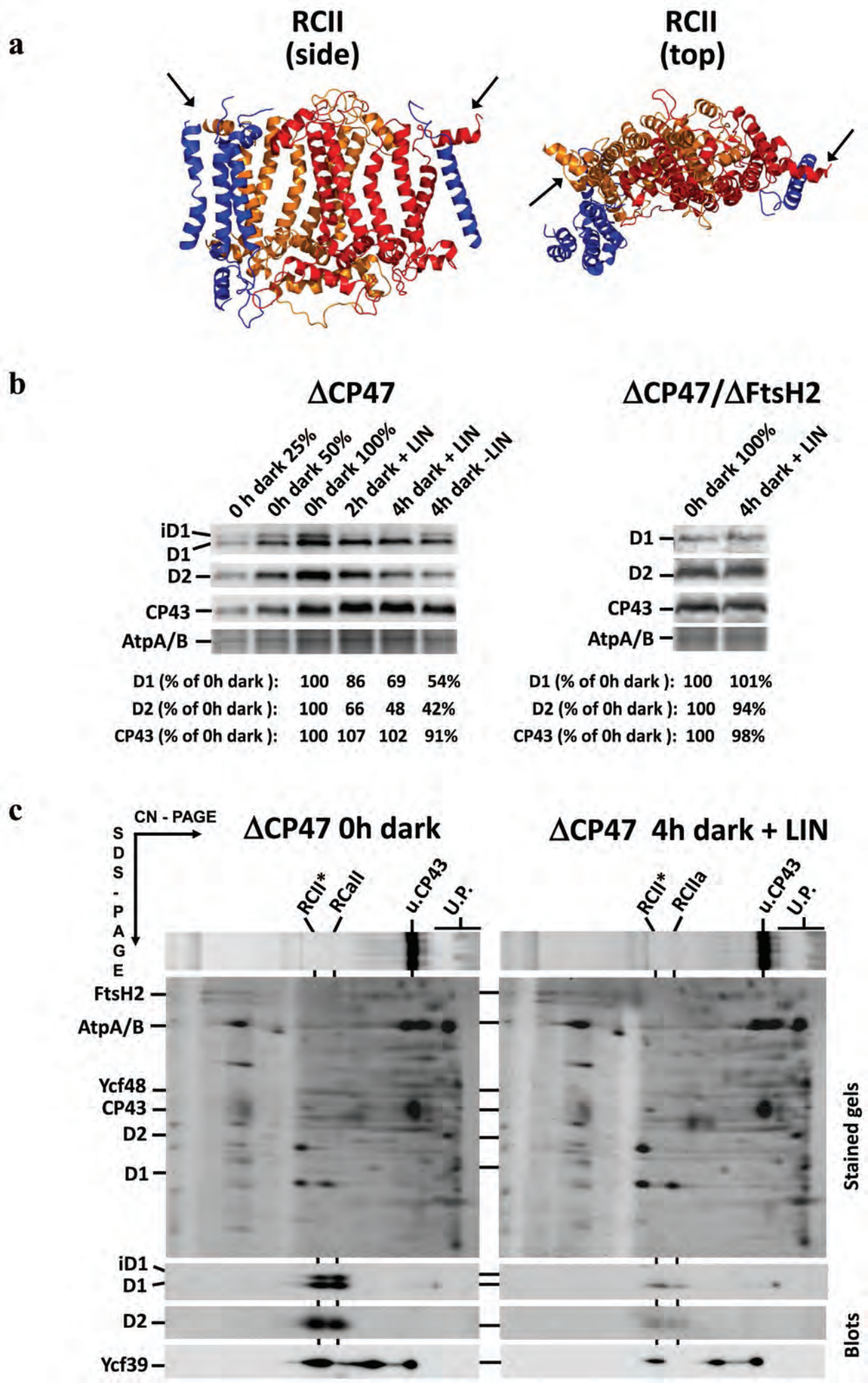
D1
Stained gels
D1
D2
CP47
Blots


a
RCII (side)
RCII (top)
b
ΔCP47
ΔCP47/ΔFtsH2
0 h dark 25%
0h dark 50%
0h dark 100%
2h dark + LIN
4h dark + LIN
4h dark -LIN
0h dark 100%
4h dark + LIN
iD1
D1
D2
CP43
AtpA/B
D1 (% of 0h dark): 100 86 69 54%
D2 (% of 0h dark): 100 66 48 42%
CP43 (% of 0h dark): 100 107 102 91%
D1 (% of 0h dark): 100 101%
D2 (% of 0h dark): 100 94%
CP43 (% of 0h dark): 100 98%
c
CN - PAGE
SDS-PAGE
ΔCP47 0h dark
ΔCP47 4h dark + LIN
RCII*
RCaII
RCIIa
u.CP43
U.P.
FtsH2
AtpA/B
Ycf48
CP43
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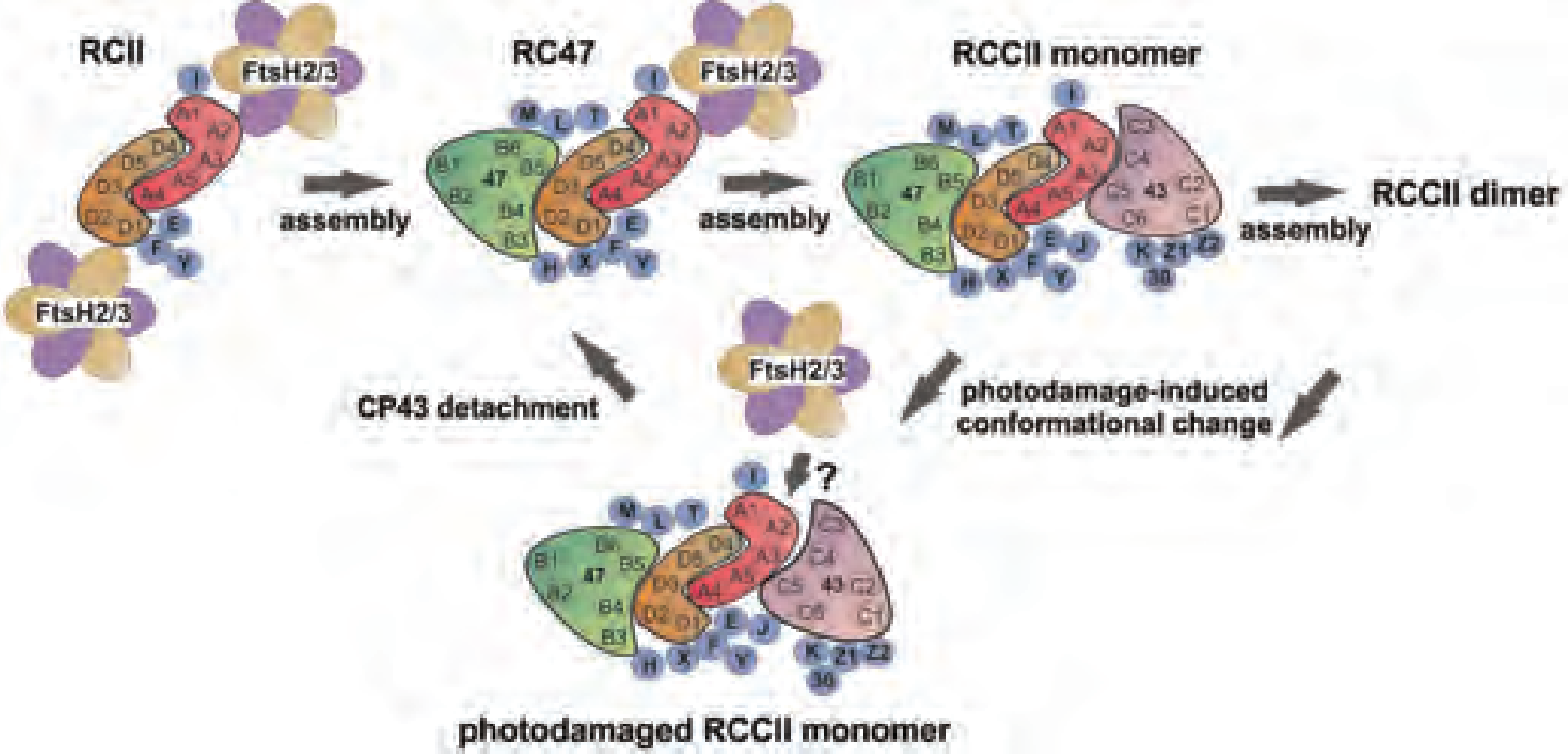
iD1
D1
D2
Ycf39
Stained gels
Blots


RCII
FtsH2/3
FtsH2/3
assembly
RC47
FtsH2/3
assembly
RCCII monomer
assembly
RCCII dimer
CP43 detachment
FtsH2/3
photodamage-induced
conformational change
?
photodamaged RCCII monomer