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Supplemental information

FtsH4 protease controls biogenesis of the PSII complex by dual regulation of high light-inducible proteins

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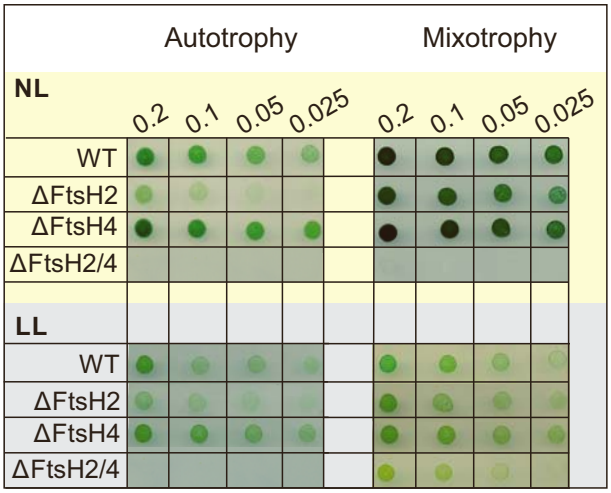
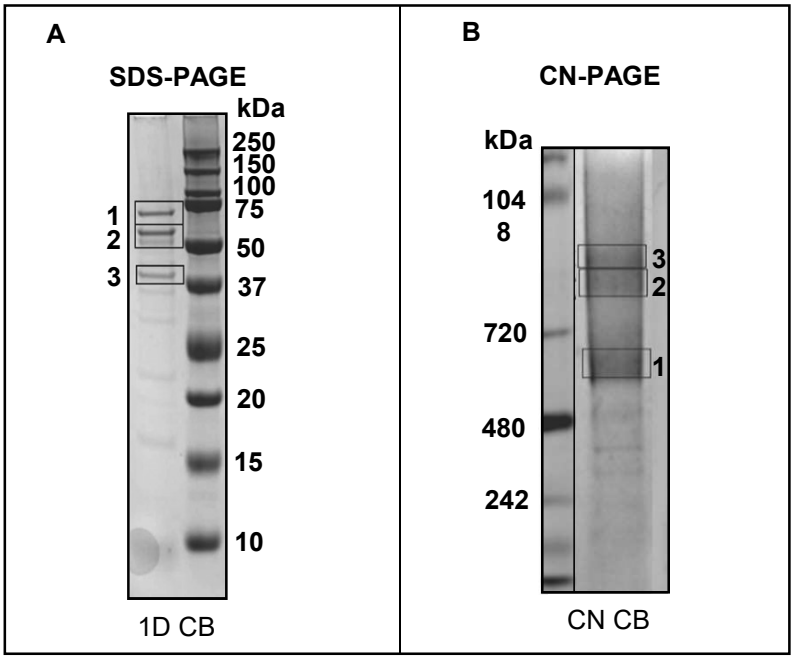
Supplementary Information

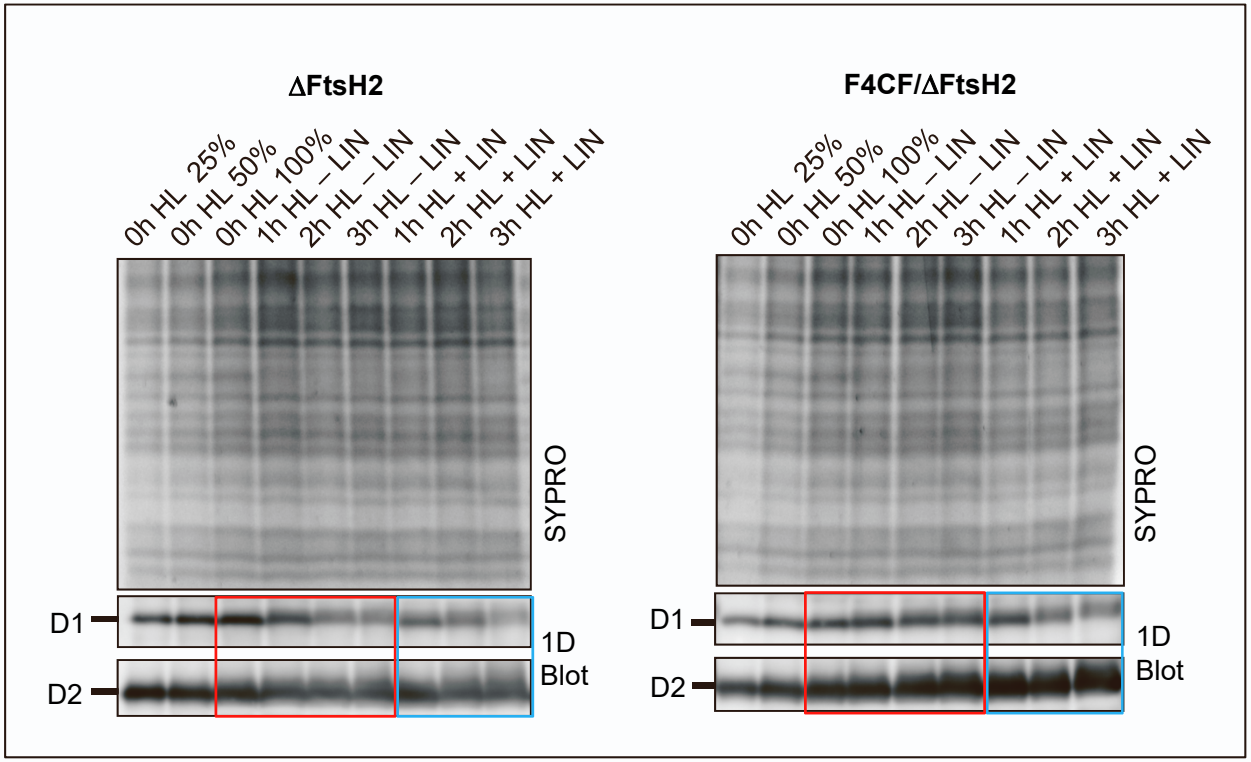
Supplementary Figures:



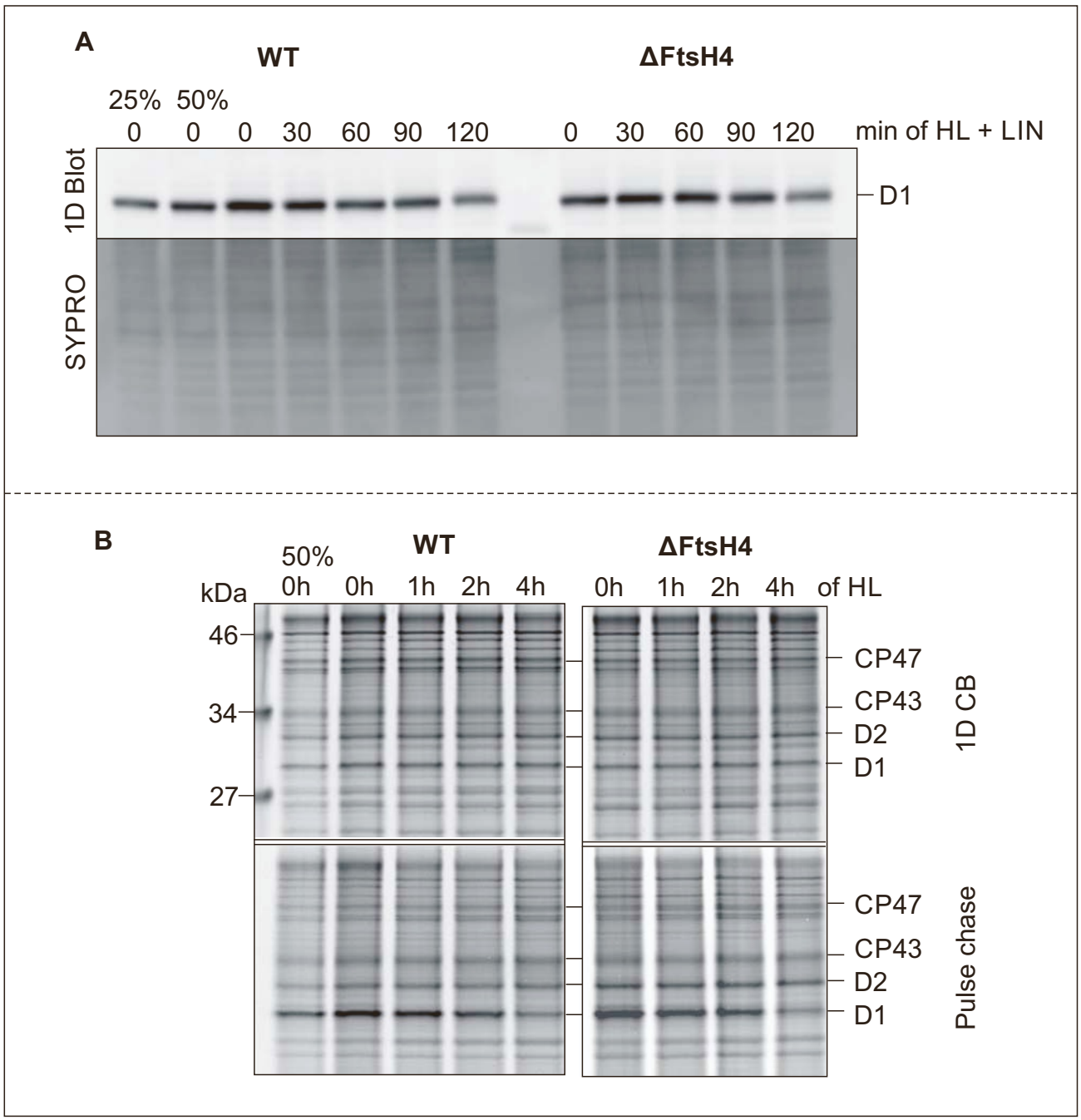
Supplementary Figure S1. Growth assay of WT, ΔFtsH2, ΔFtsH4, and ΔFtsH2/4 strains. Cells grown to exponential phase in liquid medium at normal light (NL, 40 µmol photons m^{-2} s^{-1}) were diluted to OD_{750nm} of 0.2, 0.1, 0.05, and 0.025, transferred on agar plates and exposed to NL or low light (LL, 5 µmol of photons m^{-2} s^{-1}) under autotrophic or mixotrophic conditions (5mM glucose supplement).



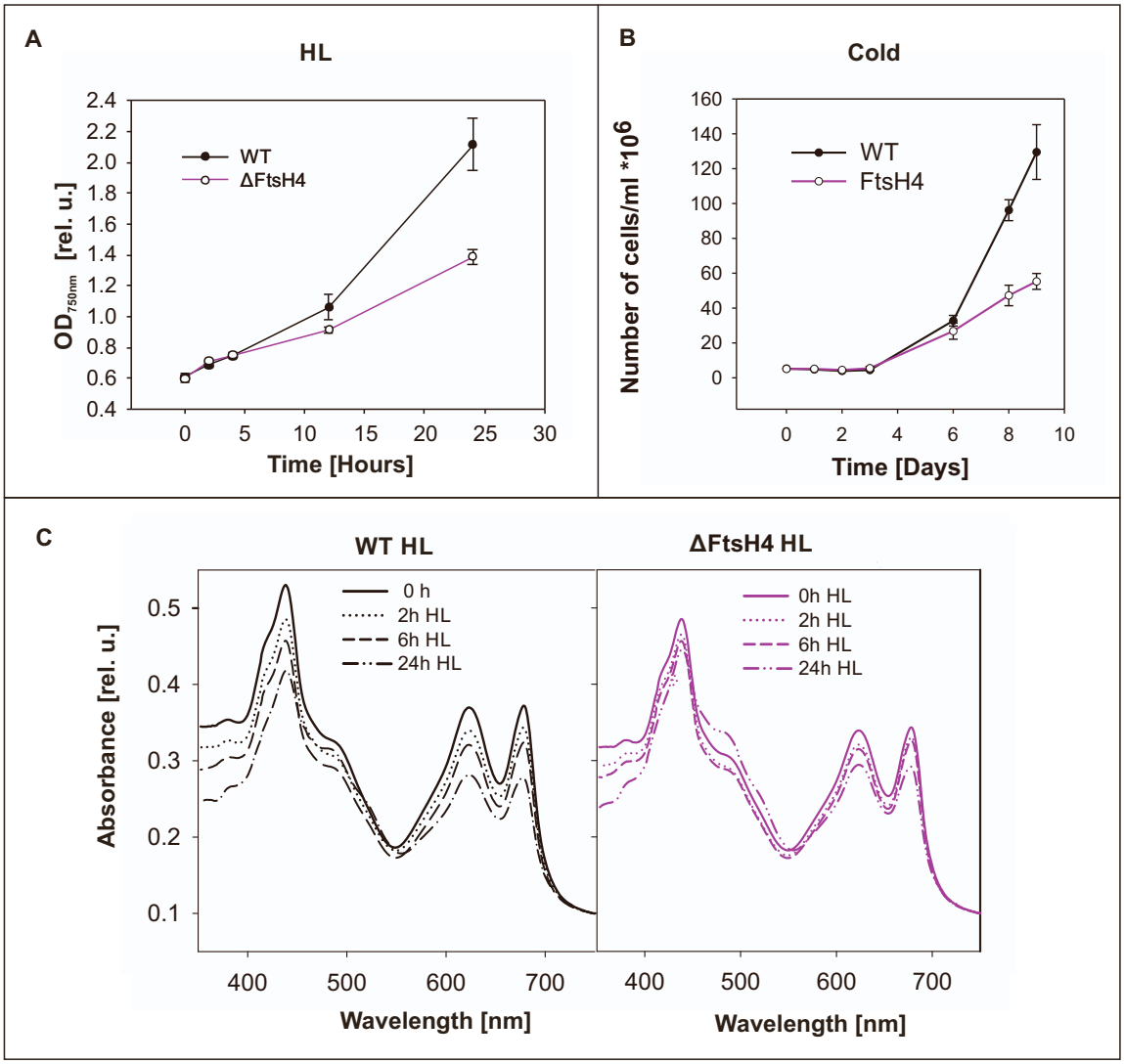
Supplementary Figure S2. In gel MS protein analysis of the F4CF/ΔFtsH2 pulldown. Pulldowns of FLAG-tagged FtsH4 isolated from the strain additionally lacking FtsH2 (F4CF/ΔFtsH2) were analyzed by 1D SDS-PAGE **(A)** or CN-PAGE **(B)**. Gels were stained by Coomassie Blue (CB). Protein bands in rectangles were cut out and analyzed by MS (MS results of SDS-PAGE or CN-PAGE are presented in Supplementary Table 2, 3 and Supplementary Dataset 2, respectively).



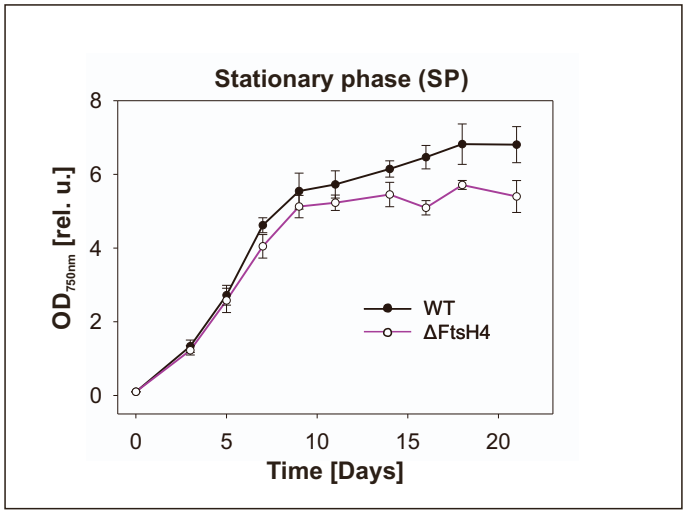
Supplementary Figure S3. The degradation of D1 and D2 proteins of photosystem II in the FtsH2-less (ΔFtsH2) and FtsH2-less strain overexpressing FLAG-tagged FtsH4 (F4CF/ΔFtsH2) under HL conditions. Cells were exposed to HL (500 µmol of photons m^{-2} s^{-1}) in the presence or absence of lincomycin (+LIN or –LIN, respectively) and their D1 and D2 protein content was assessed by immunoblotting using specific antibodies. Each sample contained 2 µg of Chl which corresponds to 100% in the dilution line. SYPRO Orange stained gel (SYPRO) is shown to document the equal loading.



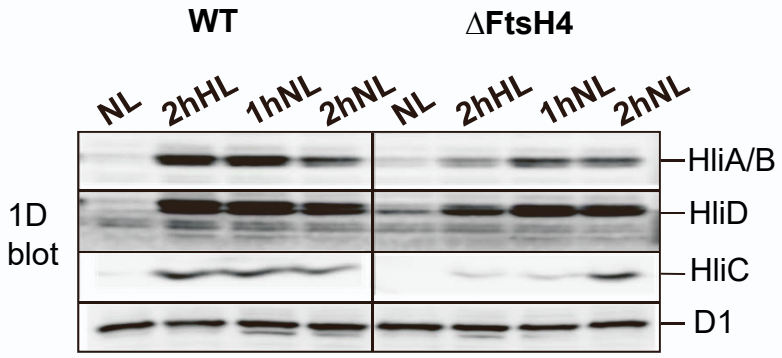
Supplementary Figure S4. Degradation of the D1 protein in WT and FtsH4-less strain (ΔFtsH4) under HL. A: Cells were exposed to HL (500 µmol of photons m^{-2} s^{-1}) in the presence of lincomycin (LIN) to prevent protein synthesis. Cell membranes were analyzed after 30, 60, 90, and 120 min of HL by immunoblotting using a specific antibody against D1. SYPRO Orange stained gel (SYPRO) is presented as a loading control. **B:** Degradation of D1 in HL monitored by radioactive pulse-chase labeling. Cells of both strains were subjected to 250 µmol of photons m^{-2} s^{-1} for 20 min in the presence of ^{35}[S] Met/Cys (0h). Then the cells were washed, supplemented with unlabeled Met/Cys, and subjected to HL. Membranes isolated from the labeled cells were analyzed by 1D SDS-PAGE. Each sample contained 2 µg of Chl which corresponds to 100% in the dilution line. The gel was stained with Coomassie Blue (CB) and, after drying, exposed in a phosphoimager overnight (Pulse chase).



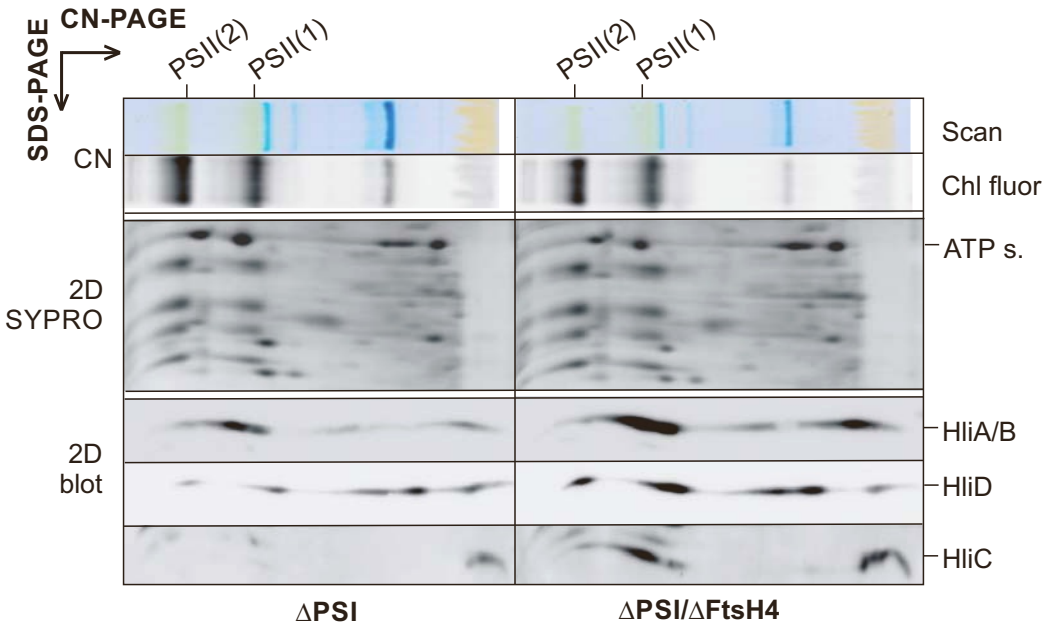
Supplementary Figure S5: Phenotype of WT and ΔFtsH4 under HL and cold stress conditions. A and B: Growth of WT and ΔFtsH4 cells during 24 h of the HL exposure (500 µmol of photons m^{-2} s^{-1}/28°C) monitored by OD_{750nm} measurement (A); and 9 days of cold stress (160 µmol of photons m^{-2} s^{-1}/18°C) monitored by cell counting using Multisizer 4, aperture 20 µm (B). Before the stress, cells were grown to exponential phase at normal light (40 µmol of photons m^{-2} s^{-1}/28°C) then diluted to OD_{750nm} 0.5 and exposed to the stress. Values are means of three biological replicates ± SD. **C**: Whole-cell absorption spectra of WT and ΔFtsH4 after 0, 2, 6, and 24 h of HL exposure.



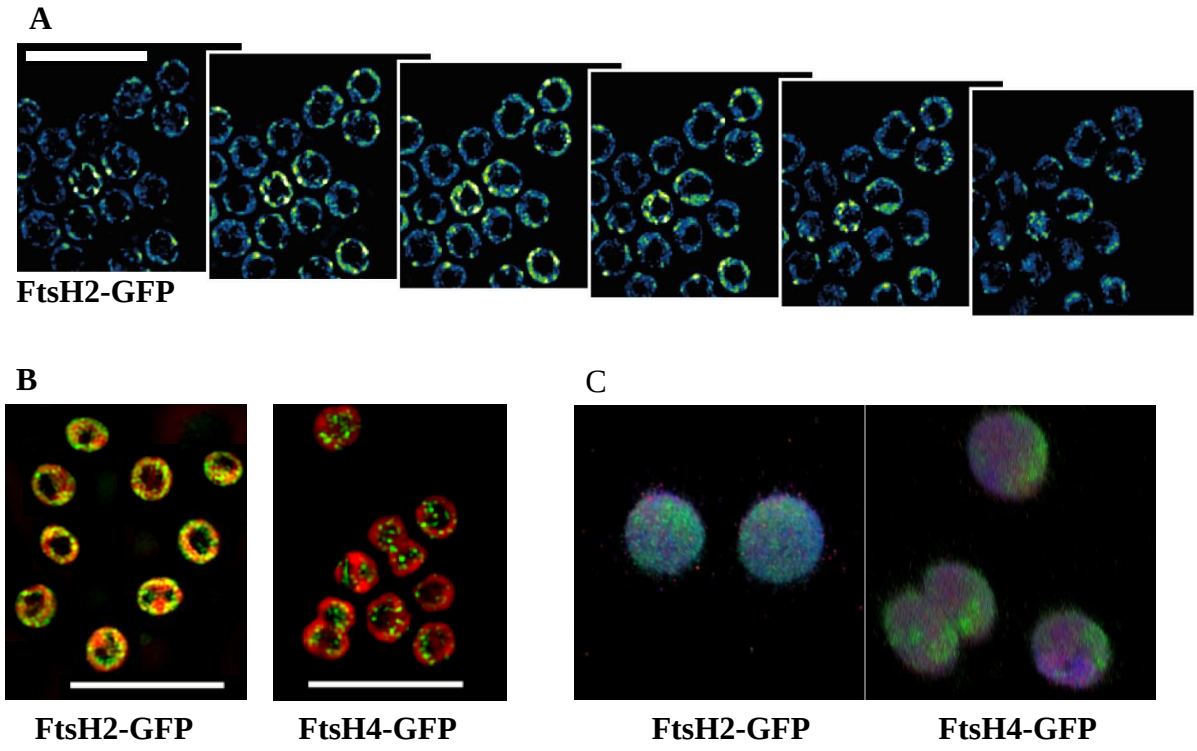
Supplementary Figure S6: Growth of WT and ΔFtsH4 until the stationary phase. WT and ΔFtsH4 cells in the exponential phase were diluted to OD_{750nm} 0.1 and grown without any other dilution for 21 days at NL. Both WT and ΔFtsH4 cells reached the stationary phase approximately on the 9th day. The final OD_{750nm} of the WT and ΔFtsH4 culture on 21st day of the growth was 6.8 or 5.4, respectively. Values are means of the four biological replicates ± SD.



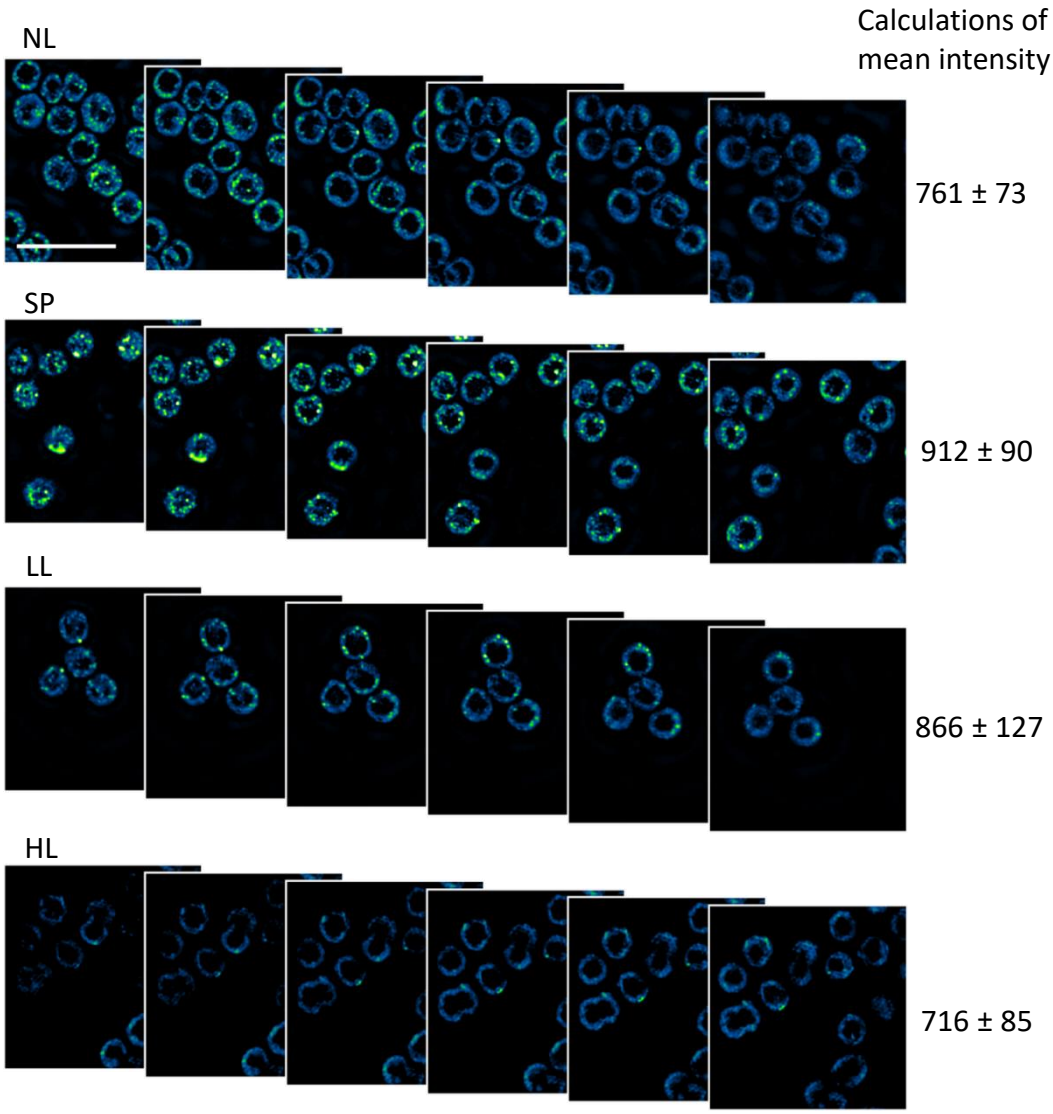
Supplementary Figure S7. Effect of *ftsH4* deletion on induction and accumulation of Hlips. Cells in exponential phase were grown at normal light (NL, 40 µmol of photons m^{-2} s^{-1}) and then exposed for 2 h to HL (500 µmol of photons m^{-2} s^{-1}, 2hHL) and then again shifted back to NL for 1 and 2 h (1hNL and 2hNL, respectively). Membranes isolated from the treated cells were analyzed by 1D SDS-PAGE/immunoblotting (1D blot). D1 signal was used as a loading control. Each loaded sample contained 1 µg of Chl. Antibodies specific for HliA/B, HliC, HliD, and D1 were used for immunoblotting.



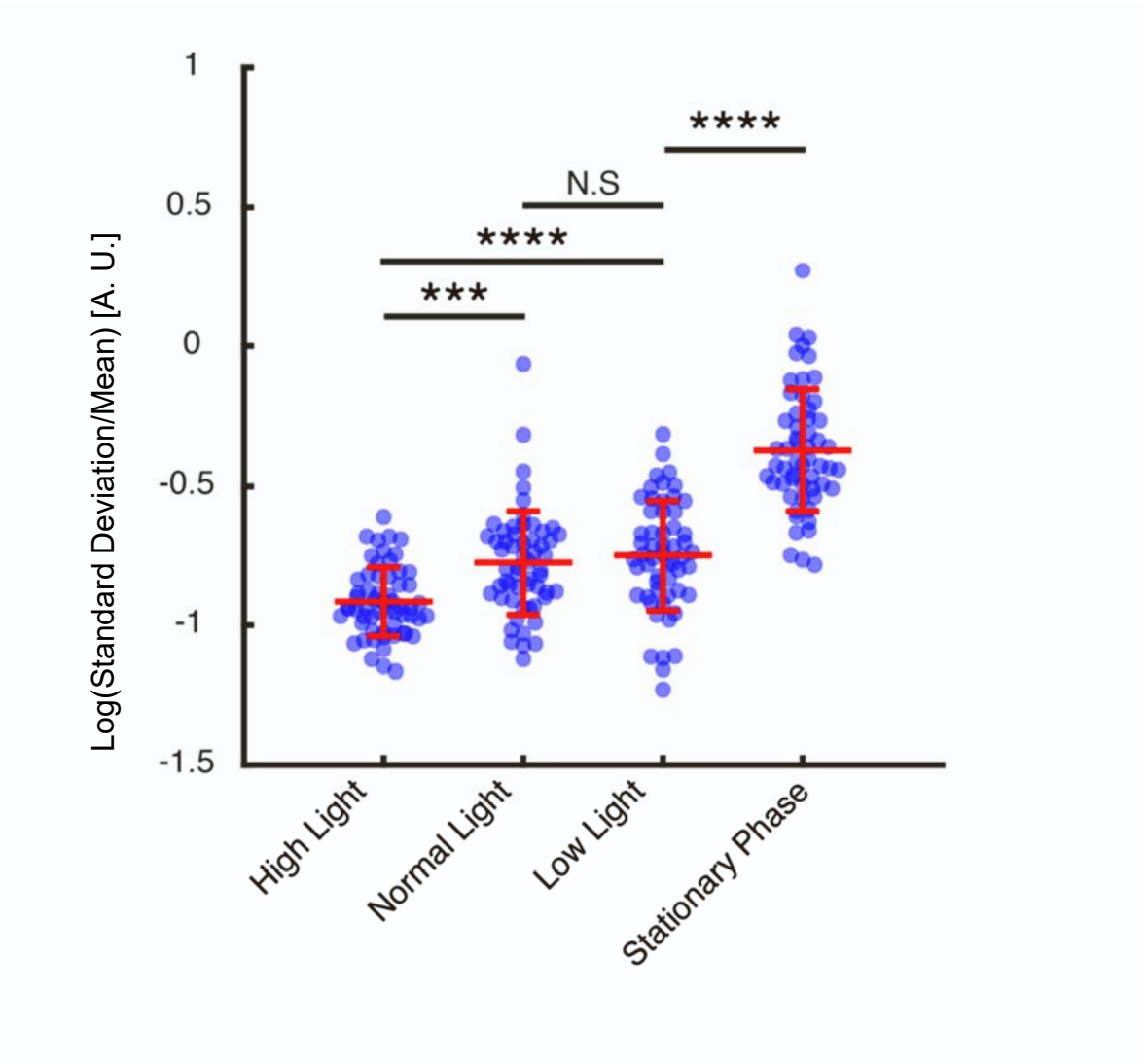
Supplementary Figure S8. Effect of *ftsH4* deletion on Hlips accumulation in PSI-less mutant. The membranes from PSI-less mutant (ΔPSI) and double mutant additionally lacking FtsH4 (ΔPSI/ΔFtsH4) grown heterotrophically at low light (5 mM glucose, 5 µmol of photons m^{-2} s^{-1}) were analyzed by 2D CN/SDS-PAGE. The CN gel was scanned (Scan) and its Chl autofluorescence detected (Chl fluor). The gel was stained by SYPRO Orange (SYPRO), electroblotted to PVDF membrane and the blot probed with antibodies specific for HliA/B, HliC, and HliD (2D blot). Each loaded sample contained 1.5 µg of Chl, the SYPRO stained gel documents the equal loading. Designation of complexes: PSII(1) and PSII(2): PSII monomer and dimer, respectively; ATP s.: α/β subunits of ATP synthase.



Supplementary Figure S9. Localization of FtsH2-GFP protein in *Synechocystis*. A: Axial slices used for Structured illumination microscopy (SIM) reconstructions of FtsH2-GFP (FtsH2 tagged with GFP). Descending axial slices are 125 nm apart and the scale bar is 5 µm. **B** and **C:** Comparison of FtsH2-GFP with FtsH4-GFP. **B**: 2D SIM, the overlap of the GFP signal (in green) with the chlorophyll auto-fluorescence (in red). The diffuse signal of FtsH2-GFP is present across the entire thylakoid membrane system occasionally with areas of higher intensity. Scale bar is 5 µm. **C:** 3D confocal microscopy. The overlap of the GFP signal with chlorophyll and phycobilisome auto-fluorescence in blue. Images were obtained using 8 2D slices with a thickness of 400 nm.



Supplementary Figure S10. Structured Illumination microscopy of FtsH4-GFP expressing cells grown under different conditions. FtsH4-GFP was imaged after acclimation to normal light (NL, 40 µmol photons m^{-2} s^{-1}), stationary phase (SP), low light (LL, 5 µmol of photons m^{-2} s^{-1}), and high light (HL, 500 µmol of photons m^{-2} s^{-1}). The images were placed on the same scale as at NL. The images are 125 nm apart axially and the scale bar represents 5 µm.



Supplementary Figure S11. Analysis of the FtsH4-GFP inhomogeneity in TM under different light conditions. The data was normalised against the mean and log-transformed. High light: 500 µmol of photons $m^{-2}s^{-1}$; Normal light: 40 µmol of photons $m^{-2}s^{-1}$; Low light: 5 µmol of photons $m^{-2}s^{-1}$. ****: P value < 5×10^{-5}; ***: $p < 5 \times 10^{-4}$; N.S.: not significant; n = 50 (50 cells represented by blue dots were analysed per each condition). P values and significance values were found with One-way ANOVA test with Tukey's Honestly Significant Difference Procedure. The error bars represent standard deviation of the calculated parameter Log(Standard Deviation/Mean).

Supplementary Tables:

Supplementary Table S1. Relative quantification of FtsH4 and core photosystem subunits in wild type *Synechocystis* thylakoid membranes.

Protein	**FtsH4**	**D1**	**D2**	**PsaA**	**PsaB**
Ratio to FtsH4	1.0	18.4	35.7	54.0	54.4
P value		0.0021	0.0034	0.0035	0.0025

Proteins extracted from thylakoid membranes were digested and the resultant proteolytic peptides analyzed by nanoLC-MS. The mass spectra were processed by searching against the *Synechocystis* sp. PCC 6803 reference proteome database using Byonic software. Relative quantification was performed by the label-free iBAQ method and expressed relative to FtsH4. P values were calculated using Student's t-test (paired, 2-sided) from 3 technical replicates (Supplementary Dataset 1).

Supplementary Table S2. Identification of major proteins in the F4CF/ΔFtsH2 FLAG pulldown resolved by SDS-PAGE.

	Protein	P value	Best score	Total Intensity	# of spectra	# of unique peptides	# of mod peptides	Coverage %	# AA's in protein
Band 1	FtsH4 (Sll1463)	1.3E-105	791	1047474290	88	31	4	61	628
Band 2	FtsH4 (Sll1463)	1.6E-93	829	1550259100	78	25	4	45	628
Band 3	FtsH4 (Sll1463)	5.1E-47	579	336645242	36	19	3	36	628

Protein bands 1, 2, and 3 (Supplementary Figure S2A) were excised and subjected to in gel digestion with trypsin. The proteolytic peptides were extracted and analyzed by nanoLC-MS and the mass spectra processed by searching against the *Synechocystis* sp. PCC 6803 reference proteome database using Byonic software. See Materials and Methods for further details. The search results are shown in the accompanying worksheets and have been filtered to remove common exogenous protein contaminants and identifications with P value > 0.05. The 'best score' refers to the highest scoring peptide spectrum match for the protein identification.

Supplementary Table S3: Relative quantification of the FtsH homologs and Sll1106 in bands obtained by separation of F4CF/ΔFtsH2 FLAG pulldown by CN-PAGE.

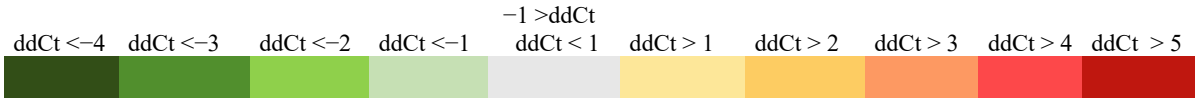
Protein	Percentage of FtsH4		
	Band 1	Band 2	Band 3
FtsH1/ Slr1390	0.08	0.02	0.04
FtsH3/Slr1604	0.17	0.22	0.13
FtsH4/Sll1463	100.00	100.00	100.00
Sll1106	9.77	8.23	8.98

Purified proteins were separated by CN-PAGE resulting in the three most abundant bands of approximate size 600, 820, and 900 kDa (Supplementary Fig. S2B). Band composition was analyzed by MS and the proportion of FtsH paralogs and Sll1106 was assessed by quantitative MS analysis using the sum of their iBAQ abundance scores. Relative quantification is expressed as percentage of FtsH4. For details see Supplementary Dataset 2.

Supplementary Table S4. Transcript levels of HL inducible genes in WT, ΔFtsH4, A3, and F4CF after short (30 min) and long (24 h) term HL exposure.

	WT NL	SD	ΔFtsH4 NL	SD	WT 30' HL	SD	ΔFtsH4 30' HL	SD	WT 24h HL	SD	ΔFtsH4 24h HL	SD
psbA2	0.00	0.15	0.08	0.02	1.94	0.31	1.95	0.25	0.95	0.31	0.96	0.36
psaA	0.00	0.23	0.42	0.12	-2.84	0.25	-1.25	0.53	-0.73	0.20	-0.41	0.05
ftsH2	0.00	0.08	-0.44	0.06	1.14	0.40	0.25	0.09	-2.42	0.42	-1.57	0.49
hliA	0.00	0.23	-0.33	0.04	3.86	0.69	2.36	1.36	-2.70	0.71	-1.24	0.69
hliB	0.00	0.1	-0.47	0.05	4.92	0.20	4.05	1.25	-0.18	0.49	0.92	0.52
hliC	0.00	0.09	-0.23	0.03	4.83	0.99	4.43	1.09	1.20	0.46	1.72	0.13
hliD	0.00	0.12	-0.24	0.02	1.56	0.23	1.15	0.64	-3.02	0.30	-15.27	0.56

	A3 NL	SD	F4CF NL	SD	A3 30' HL	SD	F4CF 30' HL	SD	A3 24h HL	SD	F4CF 24h HL	SD
psbA3	0.00	0.30	-0.70	0.07	1.77	0.35	2.62	0.03	2.01	0.34	1.77	0.08
psaA	0.00	0.07	-0.63	0.05	-2.18	0.01	-3.17	0.17	-0.07	0.09	-0.37	0.05
ftsH2	0.00	0.08	-0.99	0.13	0.80	0.41	2.18	1.02	-0.93	0.19	-0.51	0.04
hliA	0.00	0.30	-0.71	0.19	2.87	0.52	6.18	0.49	-0.41	0.16	-1.40	0.11
hliB	0.00	0.10	-0.02	0.20	5.71	0.42	7.23	0.66	1.18	0.07	1.43	0.09
hliC	0.00	0.15	-0.79	0.10	4.69	0.30	6.06	0.47	0.66	0.04	0.84	0.05
hliD	0.00	0.05	-0.63	0.05	-1.99	0.02	-1.21	0.03	0.21	0.10	0.07	0.02



RNA was isolated from WT, ΔFtsH4, A3, and F4CF cells grown under normal light (40 µmol of photons m^{-2} s^{-1}) and after 30 min or 24 h of HL (500 µmol of photons m^{-2} s^{-1}), respectively, according to Krynická et al. (2019). Expression of *hliA/B/C/D, psbA2, psaA*, and *ftsH2* transcripts was determined by RT PCR according to Krynická et al. (2014). Random primers were used for reverse transcription. Differential expression was presented using ΔΔCt model. *rnpB* was used as an internal control. ΔΔCt represents $\Delta Ct(control) - \Delta Ct(sample)$ which corresponds to $-\log_2 FC\ (control - sample)$. WT NL was a control for all ΔFtsH4 samples and HL treated WT samples and A3 NL was a control for all F4CF samples and HL treated A3 samples. Thus, ΔΔCt of the WT NL and A3 NL matches 0. Positive/negative ΔΔCt values correspond to more/less transcript compared to control. Means of 3 independent measurements ± SD are presented.

Supplementary Table S5. iBAQ quantification of major proteins in the F4CF pulldown.

Protein	iBAQ F4CF	% of FtsH4	Factor	P Value (T-test)
FtsH4/Sll1463	155.7	100.0	1.0	
Sll1106	39.8	25.5	3.9	7.90E-04
HofG/PilA1	10.1	6.5	15.4	8.50E-04
CurT/Slr0483	5.3	3.4	29.4	3.10E-03
PsaD	3.5	2.3	43.9	7.90E-02
CP47/PsbB	2.3	1.5	66.6	8.10E-01

Proteins extracted from the FtsH4-FLAG eluate by immuno-precipitation were digested and the resultant proteolytic peptides analyzed by nanoLC-MS. The mass spectra were processed by searching against the *Synechocystis* sp. PCC 6803 reference proteome database using Byonic software. Non-specifically purified proteins (FLAG eluate from WT membranes were used as a control, Supplementary Dataset 3) were filtered from the search. Table displays the top 5 proteins specifically co-purified with FtsH4 sorted by iBAQ score (Supplementary Dataset 3). They are expressed relative to FtsH4.

Supplementary Table S6. List of primers used in this study.

construct	primer sequence
F4CF	F: CGTATCCATATGGCCATCAAACCCCAACCC
	R: TTACCATCTAGATACCACTAGGGTGCCAGGAGC
Sll1106-FLAG	F: CTAGAGCATATGAGTGACTTAATTGTTATCGGC
	R: GAGGACGCTAGCTTCCGCCGCTGGGACGCCATC
ΔFtsH4	F: ATGGCCATCAAACCCCAACCCCAATGGC
	R: TTATACCACTAGGGTGCCAGGAGCTTG
FtsH4-His	F: GACGTCGCATGCTCCCGGCCG
	R: GAGGACGGCGCCTTAATGATGATGATGATGATGTACCACTAGGGTGCCAGGAGC
FtsH4-His D515N	F: GGCCCGTTGTAAATTATTGGCCGCGCCAG
	F: CTGGCGCGGCCAATAATTTACAACGGGCC
His-HliD	F: TAATAACATATGCACCATCACCATCACCATCACCATGGAAGTGAAGAACTACAACCG
	R: GCAATAAGCCAGATCTCTAGCGCAGTCCCAACCAGG