Thylakoid Localized Type 2 NAD(P)H Dehydrogenase NdbA Optimizes Light-Activated Heterotrophic Growth of Synechocystis sp. PCC 6803

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NdbA, one of the three type 2 NAD(P)H dehydrogenases (NDH-2) in Synechocystis sp. PCC 6803 (hereafter Synechocystis) was here localized to the thylakoid membrane (TM), unique for the three NDH-2s, and investigated with respect to photosynthetic and cellular redox metabolism. For this purpose, a deletion mutant (ΔndbA) and a complementation strain overexpressing NdbA (ΔndbA::ndbA) were constructed. It is demonstrated that NdbA is expressed at very low level in the wild-type (WT) Synechocystis under photoautotrophic (PA) growth whilst substantially higher expression occurs under light-activated heterotrophic growth (LAHG). The absence of NdbA resulted in non-optimal growth of Synechocystis under LAHG and concomitantly enhanced the expression of photoprotection-related flavodiiron proteins and carbon acquisition-related proteins as well as various transporters, but downregulated a few iron homeostasis-related proteins. NdbA overexpression, on the other hand, promoted photosynthetic pigment formation and functionality of photosystem I under LAHG conditions while distinct photoprotective and carbon concentrating proteins were downregulated. NdbA overexpression also exerted an effect on the expression of many signaling and gene regulation proteins. It is concluded that the amount and function of NdbA in the TM has a capacity to modulate the redox signaling of gene expression, but apparently has a major physiological role in maintaining iron homeostasis under LAHG conditions. LC-MS/MS data are available via ProteomeXchange with identifier PXD011671.

Keywords: Electron transport • Light-activated heterotrophic growth • NDH-2 dehydrogenase • Photosynthesis • Proteomics • Synechocystis • Thylakoid.

Introduction

Both the photosynthetic and respiratory electron transfer chains reside in the thylakoid membrane (TM) of oxygen evolving photosynthetic cyanobacteria, thus differing from the TM of photosynthetic eukaryotic organisms (for a review, see Vermaas 2001, Mullineaux 2014). Cyanobacteria additionally have a simpler respiratory electron transfer chain in the plasma membrane (PM). In respiration, ATP is produced by making use of the proton-motive force established when electrons derived from organic compounds are transferred via the electron transfer chain to terminal oxidases which donate electrons to molecular oxygen forming H₂O. One of the most important groups of proteins participating in bacterial-type respiration comprise the two types of pyridine nucleotide dehydrogenases, type 1 and type 2 NAD(P)H dehydrogenases (NDH-1 and NDH-2, respectively) (for a review, see Melo et al. 2004, Peltier et al. 2016). NDH-1 enzymes are complexes consisting of several subunits, and they pump protons across the membrane similar to the mitochondrial complex I (Strand et al. 2017). In cyanobacteria, NDH-1 complexes are localized in TM and are involved, besides respiration, in cyclic electron transfer and in C₃ uptake (for a review, see Batchchikova et al. 2011, Peltier et al. 2016). In contrast to NDH-1, NDH-2s are single polypeptides with molecular masses around 50 kDa and lack the ability for proton translocation across the membrane (Yagi 1991). NDH-2s typically have two GXGXXG motifs within the β-sheet–α-helix–β-sheet domains (Rossman fold) binding NAD(P)H and FAD or FMN (Wierenga et al. 1986). Nevertheless, the exact mechanism of NADH: quinone oxidoreduction by NDH-2s remains still unclear, yet hypothesis concerning the mechanism was recently proposed (Marreiros et al. 2017).

Even though all sequenced cyanobacterial species encode at least one NDH-2 enzyme in their genomes (Marreiros et al. 2016), the exact functional roles and locations of NDH-2s still remain elusive. When NDH-2 is the only enzyme capable of NADH oxidation in an organism, it is straightforward to address the main function for NDH-2 in respiratory chain-linked NADH turnover and eventual creation of the H⁺-gradient for ATP production (for a review, see Melo et al. 2004). Nevertheless, Synechocystis sp. PCC 6803 (hereafter Synechocystis) has several NADH oxidizing enzymes and research on their NDH-2s has rather pointed to a regulatory role than to the function in providing energy for metabolic needs (Howitt et al. 1999). The genome of Synechocystis possesses three genes encoding NDH-2s, ndbA (slr0851), ndbB (slr1743) and ndbC (slr1484) (Kaneko et al. 1996, Howitt et al. 1999). Recently, it was shown that NdbB is involved in vitamin K₁ biosynthesis (Fatih et al. 2015) and NdbC was assigned to have a significant role in regulation of carbon allocation between storage and the
biosynthesis pathways, being essential under heterotrophic growth conditions (Huokko et al. 2017).

To elucidate the role of NdbA, a deletion mutant and a corresponding complementation strain, that eventually overexpressed NdbA, were constructed followed by a thorough physiological and biochemical characterization of the strains, including the proteomic analyses. Here, we demonstrate that NdbA is located in the TM and its absence or overproduction does not exert any major effect on growth, photosynthetic capacity or expression of other proteins in respective strains under photoautotrophic (PA) conditions. Instead, the growth under light-activated heterotrophic growth (LAHG) conditions resulted in elevated level of NdbA in the TM of WT Synechocystis while the NdbA deletion strain demonstrated a growth retardation phenotype. NdbA overproduction, however, did not further enhance growth but maintained high photosynthetic pigmentation and oxidation capacity of photosystem I (PSI). Based on proteome analysis, the amount and function of NdbA in the TM has a capacity to modulate the redox signaling of gene expression, but apparently has a major physiological role in maintaining iron homeostasis under LAHG conditions.

**Results**

**Construction of the ndbA mutant strains and the ΔndbA::ndbA complementation strain**

To evaluate the role of NdbA in Synechocystis redox metabolism, we constructed deletion ΔndbA and complementation for ndbA deletion ΔndbA::ndbA strain. The segregation of ΔndbA strain was confirmed by PCR analysis (Supplementary Fig. S1A), and the absence of NdbA protein was shown by the Western blot analysis using isolated total proteins and specific antibody raised against NdbA (Agrisera) (Fig. 1A). The complementation strain for ndbA deletion, ΔndbA::ndbA, was obtained by transforming the ΔndbA mutant with a functional ndbA gene under a psbAII promoter (for details see Materials and Methods section). The correct insertion and the segregation were verified by PCR analysis (Supplementary Fig. S1B). The expression level of NdbA protein in the ΔndbA::ndbA strain, immunodetected from the total protein extract using α-NdbA, was remarkably higher than in WT under both PA and LAHG conditions (Figs. 1B, 3B).

**Growth of the ΔndbA mutant and the ΔndbA::ndbA strain under PA, photomixotrophic and LAHG conditions**

The growth of the WT, ΔndbA and ΔndbA::ndbA strains was monitored for several days under PA, photomixotrophic (MIXO) and LAHG conditions. No differences in growth rates were observed based on optical density (OD750) between the cells grown under either PA or MIXO conditions (Fig. 2A, B). In contrast, under LAHG conditions, after 2 d the ΔndbA growth decreased compared to WT, but the ΔndbA::ndbA strain continued to grow similar to WT (Fig. 2C). Based on these observations, we chose the PA and LAHG conditions to further characterize the function of NdbA protein in *Synechocystis* metabolism.

**Cellular localization of NdbA and expression under PA and LAHG conditions**

To specify the localization of NdbA in *Synechocystis* cell, the Western blotting with NdbA-specific antibody was applied to the protein fractions of TM and PM as well as to the soluble fraction (SF). Immunoblotting with α-NdbA clearly indicated that NdbA resided in TM while no signal was detected from the PM and SF (Fig. 3A). In addition, the expression level of NdbA under PA and LAHG conditions was estimated from the total protein extract, based on Western blot analysis. In WT, the amount of NdbA under LAHG conditions was clearly higher than under PA conditions while the complementation ΔndbA::ndbA strain showed distinct overexpression of NdbA under both the PA and LAHG conditions, yet with higher accumulation of NdbA under PA condition (Fig. 3B). This is in accordance with the use of light sensitive psbAII promoter for construction of the complementation strain but it is worth noting that clear activation of the promoter also occurred under LAHG conditions despite only short daily illumination periods (Fig. 3B).

**Ultrastructure of the ΔndbA mutant and the ΔndbA::ndbA strain under LAHG conditions**

The ultrastructure of WT, ΔndbA and ΔndbA::ndbA cells grown under LAHG condition was investigated with the transmission electron microscopy (TEM) (Fig. 4). Under these conditions in WT (Fig. 4A) and in the ΔndbA mutant (Fig. 4B) similar amount of thylakoids were present but the ΔndbA::ndbA strain (Fig. 4C) retained higher TM content. However, there was no significant difference in the cell size between the studied strains.

**Pigment phenotype and the room temperature whole cell absorption spectra under LAHG conditions**

After 4 d of growth under LAHG conditions the color phenotypes of WT culture, the ΔndbA culture and the ΔndbA::ndbA culture were different from each other. ΔndbA was paler green
compared to WT whereas the clearly greenest phenotype was expressed by \( \Delta \text{nbdA} \) (Fig. 5A). To further analyze the difference in pigment composition, the room temperature whole cell absorption spectra were recorded from strains grown for 4 d under LAHG conditions. Before measurements, the samples were normalized to the same OD\(_{750}\). No differences were observed in the spectra between \( \Delta \text{nbdA} \) and WT (Fig. 5B), whereas the spectra of the \( \Delta \text{nbdA}\_::\text{nbdA} \) strain demonstrated substantially higher peaks of chlorophyll \( \text{a} \) (Chl; peaks at 440 nm and 680 nm), phycobilins (peak at 626 nm) and carotenoids (peak at 480–490 nm) compared to WT and \( \Delta \text{nbdA} \).

### Differential protein expression in \( \Delta \text{nbdA} \) and \( \Delta \text{nbdA}\_::\text{nbdA} \) under PA and LAHG conditions

The consequences of the cellular amount of the NdbA protein on global protein expression in *Synechocystis* were investigated by label free mass spectrometry (MS) data dependent acquisition (DDA). The whole cell protein extracts from \( \Delta \text{nbdA} \), \( \Delta \text{nbdA}\_::\text{nbdA} \) and WT strains grown under PA and LAHG conditions were digested and analyzed, in untargeted manner, by liquid chromatography coupled to tandem mass spectrometry system (LC-MS/MS). The samples were injected in biological quadruplicates. In 12 independent mass spectrometry runs performed for PA and LAHG condition, we identified and then quantified (with at least 2 peptides) 2085 and 2136 proteins under PA and LAHG condition respectively (Supplementary Tables S1, S2). The results of relative quantification were further filtered with statistical significance cut-off set to \( P \)-value \(<0.05 \) and the fold change (FC) threshold of the practical significance set to \(-1.4 \leq \text{FC} \) and \( \text{FC} \geq 1.4 \).

Under PA conditions the deletion of NdbA resulted in 15 upregulated and downregulated proteins (Supplementary Table S3) whereas the overexpression of NdbA resulted in upregulation of 9 and in downregulation of 16 proteins compared to WT (Supplementary Table S4). With the same significance and practical thresholds, the deletion of NdbA under LAHG conditions resulted in upregulation of 44 and downregulation of 19 proteins (Supplementary Table S5) compared to WT, whereas the overexpression of NdbA resulted in upregulation of 223 and downregulation of 102 proteins compared to WT (Supplementary Table S6).

We first used the proteomics data to confirm the content of the NdbA protein in different strains under the PA and LAHG growth conditions. The NdbA protein was identified in WT grown under LAHG conditions and in \( \Delta \text{nbdA}\_::\text{nbdA} \) strain grown under PA and LAHG conditions. This is the first time...
Fig. 4 Transmission electron microscope (TEM) images of WT (A), ΔndbA (B) and ΔndbA::ndbA (C) cells grown under light-activated heterotrophic growth (LAHG) conditions. Cells were collected after 4 days. Figures are representatives of 100 cells photographed for every line.

Fig. 5 The color difference between WT, ΔndbA and ΔndbA::ndbA grown under light-activated heterotrophic growth (LAHG) conditions. (A) The color phenotype of WT, ΔndbA and ΔndbA::ndbA cultures grown under LAHG conditions for 4 days. (B) The whole cell absorption spectra at room temperature from WT (black line), ΔndbA (red line) and ΔndbA::ndbA (blue line) grown under LAHG conditions. OD_{750} was adjusted to 0.3 before recording the absorption spectra. Each curve is an average from four biological repetitions.
when NdbA is detected in WT by MS from unfractionated cell lysate. The NdbA abundance in WT grown under PA conditions remained under the detection level of mass spectrometer while it was distinct under LAHG conditions. At the same time, the $\Delta ndbA::ndbA$ overexpression strain showed elevated abundance of NdbA under PA, which also remained higher than that in WT under LAHG conditions (Fig. 6) in accordance to immunodetection results (Fig. 3B).

The global proteomes of the three strains grown under PA conditions demonstrated only minor differences. The inducible ABC-type HCO$_3^-$ transporter BCT1 (Omata et al. 1999), indicated by CmpB and CmpC, was found to be downregulated in both $\Delta ndbA$ and $\Delta ndbA::ndbA$ compared to WT (Supplementary Tables S3, S4). In addition, the amounts of Hik36 and Hik43 were higher in both mutant strains compared to WT. Transporters functioning in K uptake (KdpA, KdpB and KdpC) were strongly upregulated in $\Delta ndbA$ compared to WT, but this was not observed in the $\Delta ndbA::ndbA$ strain. The rest of proteins remained below the statistical and significance threshold.

WT, $\Delta ndbA$ and $\Delta ndbA::ndbA$ cells grown under LAHG conditions demonstrated distinct variations in protein expression, covering diverse functional groups as photosynthesis and respiration, regulatory functions, carbon assimilation and nitrogen metabolism as well as cellular transport. Photosynthesis and respiration related proteins were the most affected in $\Delta ndbA::ndbA$ strain as components of photosystem II (PSII), including PsbA (D1), PsbB (CP47), PsbD (D2), PsbC (CP43), PsbO, PsbV, PsbE, PsbQ and Psb32, were at elevated level as compared to WT (Table 1). In addition, the proteins involved in PSII assembly as sl0933, slr0147 and slr0151 were also upregulated in overexpression line. The Cyt b$_6$f complex proteins PetA, B and C were upregulated only in the $\Delta ndbA::ndbA$ strain compared to WT. In addition, the amounts of several soluble electron carriers, including Cyt c$_6$ and several Ferredoxins (Feds 2, 3, 5 and 7) were elevated in the $\Delta ndbA::ndbA$ strain compared to WT.

As to the regulatory proteins of thylakoid electron transfer, the amounts of the flavidiron proteins Fhv2 and Fhv4, involved in the protection of PSII (Zhang et al. 2009, Zhang et al. 2012, Bersanini et al. 2014), were distinctively elevated in $\Delta ndbA$ under LAHG conditions (Table 1). Interestingly, these same proteins were downregulated in $\Delta ndbA::ndbA$ compared to WT (Table 1) together with downregulation of the orange carotenoid protein (OCP) (Table 1), which performs non-photochemical quenching (NPQ) in several cyanobacterial species (Wilson et al. 2006, Wilson et al. 2007, Boulay et al. 2010) (for a review, see Kirilovsky and Kerfeld 2013). Conversely, the fluorescence recovery protein (FRP) required for deactivation of OCP during NPQ was downregulated in $\Delta ndbA$ but upregulated in $\Delta ndbA::ndbA$ (Table 1). The expression of several phycobilisome (PBS)-related proteins (CpcD, CpcA, ApcF, CpcG, CpcC1 and Cpc2) was elevated in the $\Delta ndbA::ndbA$ strain compared to WT (Table 1). The three enzymes involved in Chl and bilin biosynthesis as HemiF, HemiH, Ho1 and Chla, were upregulated. Similarly, the amounts of proteins involved in later steps of Chl biosynthesis, including Chlb, Chln and Chll were higher in $\Delta ndbA::ndbA$ compared to WT, whereas in Chlg-amount was lower in both $\Delta ndbA::ndbA$ and $\Delta ndbA$ strains compared to WT. Interestingly, also geranylgeranyl reductase (ChlP), responsible for reduction of geranylgeranyl chain in Chl biosynthesis and free diphosphate in tocopherol and phyloquinone derivatives biosynthesis, was upregulated (Table 1). In addition, carotenoids biosynthesis pathway enzymes Pyr (slr1255) and CrtU showed upregulation, while astaxanthin biosynthesis branch represented by CrtO was downregulated.

In addition to photosynthetic components, the absence or overproduction of the NdbA protein in the TM under LAHG conditions exerted an effect on the expression of several proteins involved in C$_3$ assimilation. Both inducible HCO$_3^-$ uptake systems, the ABC-type bicarbonate transporter BCT1 (CmpB, C) (Omata et al. 1999) and the Na$^+$ dependent bicarbonate transporter SbtA (Shibata et al. 2002), were upregulated in the $\Delta ndbA$ mutant compared to WT (Table 1). Also the amount of low-CO$_2$-inducible, high-affinity CO$_2$ uptake complex NDH-1$_3$ (for a review, see Battchikova et al. 2011, Peltier et al. 2016) was higher in the $\Delta ndbA$ mutant compared to WT, indicated by upregulation of the NDH-1$_3$–specific subunits CupA and CupS (Table 1). The $\Delta ndbA::ndbA$ strain behaved differently. Proteins related to the carbon concentration mechanisms (CCM) including BCT1 (indicated by CmpA, CmpB and CmpC) and SbtA, SbtB as well as the NDH-1$_3$ complex (represented by NdH3 and CupA) were downregulated in $\Delta ndbA::ndbA$ as compared to WT (Table 1).

Under LAHG, the amount of several proteins involved in N assimilation were elevated in the $\Delta ndbA::ndbA$ strain compared to WT. These included NrtA and NrtD, which are subunits of the NRT transporter. In addition, nitrogen reductase proteins, like ferredoxin-nitrate reductase NarB and NarM, were upregulated (Table 1). Further, the amounts of transporters that function in P, uptake (PstS1, PstS2 and SphX) as well as in K uptake (KdpA, KdpB and KdpC) were higher in both mutant strains compared to WT. The CopMRS and CopBAC systems responsible for copper homeostasis in the cell were downregulated in the $\Delta ndbA::ndbA$ strain while SynAtx1, the
| Function                        | Protein         | FC ΔndbA/WT LAHG | ANOVA (P-value) | FC ΔndbA:ΔndbA/WT LAHG | ANOVA (P-value) |
|--------------------------------|-----------------|------------------|----------------|------------------------|----------------|
| PSII                           | slr1311 PbxA2 (D1) | 1.3              | 1.98E-02       | 1.5                    | 2.54E-03       |
| sll0849 PbxB (D2)              | 1.5              | 7.72E-03         | 2.0           | 1.35E-03               |                |
| sll0851 PbxC (CP43)            | 1.1              | 1.16E-01         | 1.5           | 3.84E-05               |                |
| srr3451 PbxE                   | 1.3              | 1.60E-03         | 2.1           | 2.94E-06               |                |
| sll0258 PbxV                   | 1.3              | 3.57E-03         | 1.8           | 2.15E-04               |                |
| sll0427 PbxO                   | 1.2              | 9.41E-02         | 1.6           | 2.71E-04               |                |
| sll1638 PbxQ                   | 1.2              | 4.94E-02         | 1.4           | 3.35E-03               |                |
| sll1390 Pbx2                   | 1.1              | 4.44E-01         | 1.4           | 1.27E-04               |                |
| sll0933 PSII assembly factor   | −1.1             | 4.35E-01         | 1.4           | 3.04E-02               |                |
| sfr0147 PSII assembly factor   | 1.1              | 4.57E-01         | 1.4           | 2.39E-03               |                |
| sfr0151 PSII assembly factor   | −1.0             | 7.03E-01         | 1.4           | 8.84E-04               |                |
| Cyt b6f                         | sll1317 PetA     | 1.1              | 1.25E-01       | 1.5                    | 8.65E-04       |
| sll0342 PetB                   | 1.1              | 1.11E-01         | 1.5           | 1.96E-03               |                |
| sll1316 PetC                   | 1.0              | 6.33E-01         | 1.5           | 2.54E-03               |                |
| Soluble electron carriers      | sll1796 Cytcc, Petj | −1.8              | 1.24E-01     | 2.8                    | 1.86E-02       |
| sll1382 Fed2                   | −1.1             | 1.36E-01         | 2.2           | 6.90E-04               |                |
| sfr1828 Fed3                   | −1.1             | 3.99E-01         | 1.7           | 3.22E-02               |                |
| ssl2559 Fed5                   | 1.2              | 1.53E-01         | 1.4           | 2.07E-02               |                |
| sll0662 Fed7                   | 1.1              | 5.35E-01         | 1.6           | 1.85E-02               |                |
| Photoprotection of PSII        | sll0217 Flv4     | 2.0              | 2.70E-02       | −1.8                   | 4.92E-02       |
| sll0219 Flv2                   | 2.1              | 1.51E-02         | −1.5          | 2.56E-02               |                |
| sfr1963 Ocp                    | −1.0             | 9.52E-01         | −1.5          | 2.28E-06               |                |
| sfr1964 Frp                    | −1.3             | 1.57E-02         | 1.5           | 1.29E-02               |                |
| Phycobilosomes                 | sfr1459 ApcF     | 1.0              | 7.20E-01       | 1.4                    | 2.69E-04       |
| ssl1580 CpcC1                  | 1.1              | 1.21E-01         | 1.4           | 2.26E-03               |                |
| ssl1471 CpcG                   | 1.9              | 2.63E-04         | 2.1           | 6.15E-06               |                |
| ssl3093 CpcD                   | 1.1              | 2.52E-01         | 1.4           | 3.98E-04               |                |
| ssl1578 CpcA                   | 1.1              | 4.12E-01         | 1.4           | 2.42E-02               |                |
| ssl1579 CpcC2                  | 1.2              | 4.25E-03         | 1.6           | 9.35E-05               |                |
| Chlorophyll and bilin biosynthesis | sll1185 HemF   | −1.0             | 7.31E-01       | 1.7                    | 1.47E-05       |
| sll0839 HemH                   | 1.1              | 2.54E-01         | 1.6           | 7.56E-04               |                |
| sll1184 Ho1                    | −1.1             | 5.38E-01         | 2.1           | 1.23E-04               |                |
| sll1214 ChlA1                  | −1.1             | 1.06E-02         | 1.7           | 8.28E-05               |                |
| sfr0056 ChlG                   | −1.6             | 2.30E-02         | −1.9          | 6.43E-03               |                |
| sfr0749 ChlL                   | −1.2             | 3.51E-01         | 1.8           | 2.21E-02               |                |
| sfr0750 ChlN                   | −1.2             | 2.58E-02         | 1.8           | 1.94E-05               |                |
| sfr0772 ChlB                   | −1.1             | 1.19E-01         | 2.7           | 8.09E-06               |                |
| sll1091 ChlP                   | −1.2             | 1.14E-02         | 2.0           | 1.06E-04               |                |
| Carotenoids biosynthesis       | sfr1255 Pys, CrtB | −1.2             | 7.01E-02       | 1.6                    | 2.86E-03       |
| sfr0088 CrtO                   | 1.0              | 9.24E-01         | −1.6          | 2.00E-03               |                |
| sll0254 CruE                   | −1.4             | 3.48E-02         | 1.9           | 1.40E-04               |                |
| Carbon concentration mechanism | sfr1512 SbtA     | 1.3              | 1.88E-02       | −2.0                   | 7.44E-04       |
| sfr1513 SbtB                   | 1.1              | 1.70E-01         | −1.6          | 1.50E-03               |                |
| sll1734 CupA                   | 1.3              | 2.91E-02         | −1.7          | 1.19E-03               |                |
| sll1732 NdhF3                  | 1.9              | 7.16E-02         | −3.4          | 1.22E-02               |                |
| sfr0040 CmpA                   | 3.1              | 1.58E-01         | −6.1          | 4.58E-02               |                |
| sfr0041 CmpB                   | 3.4              | 2.67E-02         | −5.9          | 5.85E-03               |                |
| sfr0043 CmpC                   | 3.0              | 1.75E-02         | −2.9          | 7.62E-03               |                |
| Nitrogen assimilation          | ssl1451 NrtB     | 1.0              | 9.62E-01       | 1.5                    | 9.57E-04       |
| sll1452 NrtC                   | −1.2             | 1.54E-02         | 1.4           | 3.86E-05               |                |
| sll1453 NrtD                   | −1.2             | 5.70E-02         | 1.6           | 5.48E-04               |                |
| sll1454 NarB                   | 1.5              | 5.08E-01         | 1.8           | 7.34E-04               |                |
| sll1455 NarM                   | 1.1              | 6.45E-01         | 1.4           | 8.17E-03               |                |
| P uptake                       | ssl0679 SphX     | 2.1              | 2.52E-05       | 2.0                    | 1.84E-01       |
| ssl0680 PstS1                  | 1.7              | 2.69E-03         | 1.4           | 4.67E-01               |                |
| sfr1247 PstS2                  | 4.5              | 1.08E-04         | 1.7           | 4.80E-01               |                |
| K uptake                       | sfr1728 KdpA     | 2.5              | 1.24E-04       | 1.6                    | 2.68E-03       |
| sfr1729 KdpB                   | 2.2              | 4.24E-06         | 1.8           | 7.77E-05               |                |

(continued)
protein interacting with ATPases and ensuring copper transport to thylakoid lumen, was upregulated. TM located CurT protein also showed upregulation in the ΔndbA mutant compared to WT (Table 1). These included FutC, involved in ferric iron uptake (Katoh et al. 2001) and the iron–sulfur cluster biogenesis regulator SufR (Shen et al. 2007). Furthermore, the amounts of Cyt c₆ containing a heme group as well as a CruE enclosing Rieske 2Fe-2S center involved in the biosynthesis of carotenoids (Mohamed and Vermaas 2006) were present at lower level in the ΔndbA mutant compared to WT (Table 1).

Several histidine kinases (Hik) demonstrated different expression in the ΔndbA::ndbA strain compared to WT under LAHG conditions. The amount of Hik35 was elevated whereas the amounts of Hik36 and Hik34 were diminished (Table 1). Hik36 and Hik34 were likewise downregulated in ΔndbA compared to WT. In the ΔndbA::ndbA strain, the response regulators Rre29 and Rre27 were elevated at protein level but the amounts of Rre32 and Rre37 were lower compared to WT (Table 1), and Rre32 was downregulated also in ΔndbA. The two regulatory proteins TrxA and SigA were upregulated in ΔndbA::ndbA compared to WT (Table 1).

### Photosynthetic characterization of the NdbA mutants under PA and LAHG conditions

For proper comparisons, the Chl fluorescence and P700 measurements were first performed with PA-grown WT, ΔndbA and ΔndbA::ndbA cells using a Dual-PAM fluorometer. As demonstrated in Fig. 7, no distinct difference was observed between the strains either in the effective PSII yield, Y(II), (Fig. 7A) or in the effective PSI yield, Y(I), (Fig. 7B) measured during illumination with actinic light (50 μmol photons m⁻² s⁻¹). Next, the same measurements were performed with the cells grown under LAHG conditions. In the WT and in ΔndbA, the effective PSI yield, Y(II), was not detectable whereas the ΔndbA::ndbA cells showed a low but considerable Y(II) level (Fig. 7C). The effective PSI yield, Y(I), measurements demonstrated no clear differences between WT, ΔndbA and ΔndbA::ndbA cells under LAHG conditions (Fig. 7D).

Functionality of PSI was further measured from WT, ΔndbA and ΔndbA::ndbA cells by measuring the maximal amount of oxidizable P700 (Pₘ) when cells were grown either under PA or LAHG conditions (Table 2). The Pₘ values of the WT, ΔndbA and ΔndbA::ndbA cells did not differ significantly under PA conditions. On the contrary, the Pₘ value of ΔndbA was significantly lower compared to WT under LAHG conditions whereas in the ΔndbA::ndbA strain the Pₘ value was drastically higher compared to the two other strains studied under the same conditions.

### Discussion

The three NDH-2 enzymes in *Synechocystis* (NdbA, NdbB and NdbC) have been postulated to have regulatory roles rather than being directly involved in bioenergetic electron transfer...
chains that produce ATP (Howitt et al. 1999). However, their exact roles and even the cellular localization have remained largely elusive. Quite recently, the function for NdbB in vitamin K1 biosynthesis (Fatihi et al. 2015) as well as the role of NdbC in carbon allocation, its location in the PM and necessity under LAHG conditions (Huokko et al. 2017) have been demonstrated. This indicates that even though NDH-2s in Synechocystis catalyze the oxidation of NADH, they seem to be implicated in different metabolic pathways. In the present study, we addressed the physiological role of the third NDH-2, NdbA, which we first localized to the TM of Synechocystis (Fig. 3A), thus indicating diverse cellular compartments and reinforcing distinct functional roles for the three different NDH-2s.

NdbA is dispensable in Synechocystis under PA conditions

The deletion or overexpression of NdbA did not influence the growth of Synechocystis under PA conditions (Fig. 2A) which is in agreement with earlier studies by Howitt et al. (1999). In line with this, the proteome analysis of cells grown under PA conditions demonstrated only few changes caused by either the deletion or overexpression of NdbA (Supplementary Tables S3, S4). The insignificance of NdbA under photoautotrophy in Synechocystis is supported by the fact that only a small amount of NdbA is present in WT thylakoids since it was found only with immunodetection technique and remained below detection limit of mass spectrometer (Figs. 3B, 6).

**Table 2** Pm values from WT, ΔndbA and ΔndbA::ndbA grown under photoautotrophic (PA) conditions and under light—activated heterotrophic growth (LAHG) conditions

|          | Pm        |
|----------|-----------|
| WT PA    | 0.08 ± 0.004 |
| ΔndbA PA | 0.07 ± 0.005 |
| ΔndbA::ndbA PA | 0.08 ± 0.001 |
| WT LAHG  | 0.04 ± 0.003 |
| ΔndbA LAHG | 0.03 ± 0.002*  |
| ΔndbA::ndbA LAHG | 0.07 ± 0.003*  |

Values are means ± SD; n = 3. *Statistically significant differences between WT and the ΔndbA mutants (P < 0.05).

Fig. 7 Photosynthetic characterization of WT (black squares), ΔndbA (red circles) and ΔndbA::ndbA (blue triangles) grown under photoautotrophic (PA) and under light-activated heterotrophic growth (LAHG) conditions. (A) the effective PS II yield, Y(II), under PA; (B) the effective PSI yield, Y(I), under PA; (C) the effective PS II yield, Y(II), under LAHG, and (D) the effective PSI yield, Y(I), under LAHG. Cells were dark-acclimated for 10 min before measurements and the Chl concentration was adjusted to 15 μg ml⁻¹, and measurements were done under illumination of 50 μmol photons m⁻² s⁻¹ as actinic light. Mean ± SD. n = 3.
**NdbA optimizes the growth of Synechocystis under LAHG conditions without affecting the regulation of sugar catabolism**

The deletion of NdbA resulted in retarded growth of *Synechocystis* under LAHG conditions (Fig. 2C). Further, the growth defect of ΔndbA was not caused by the inability to uptake glucose since no difference in the growth between WT and the NdbA mutant strains was observed under MIXO conditions (Fig. 2B). The mass spectrometry analysis (Fig. 6) as well immunodetection (Fig. 3B) data indicate that the amount of NdbA significantly increases in WT thylakoids under LAHG conditions compared to its expression under PA conditions (Figs. 3B, 6), being the first indication about the importance of NdbA under LAHG conditions.

The proteome analysis demonstrated no differences in the amounts of sugar catabolic proteins in the ΔndbA mutant compared to WT, which may be explained by insignificant change in the expression of SigE, the general inducer of the genes functioning in sugar catabolism (Osanai et al. 2005) (Table 1). Thus, the growth retardation of ΔndbA under LAHG conditions, as compared to WT, hardly results from problems in glucose catabolism. Indeed, upon LAHG *Synechocystis* uses glucose as a sole energy source (Plohnke et al. 2015), and the activities of sugar catabolic enzymes, glucokinase and pyruvate kinase, have been reported to be at elevated level (Knowles and Plaxton 2003). Also Gap1, an essential enzyme for glycolysis, is upregulated at the protein level under LAHG conditions (Kurian et al. 2006). Under heterotrophic conditions the oxidative pentose phosphate pathway is the most important glycolytic route (Wan et al. 2017) as it is the only source of intermediates for nucleotide biosynthesis in darkness (Kruger and Von Schaewen 2003), consuming 90% of assimilated external glucose (Yang et al. 2002). All these essential enzymes for LAHG were present at WT level in both NdbA mutants, providing evidence that NdbA is not involved in regulation of sugar catabolism in *Synechocystis*.

The apparent intactness of sugar catabolism pathways in the ΔndbA mutant, opposite to that in the NdbC deletion mutant (Huokko et al. 2017), further supports the different functions of NDH-2s in *Synechocystis*. The PM-located NdbC is crucial for regulation of sugar catabolism and maintenance of growth under LAHG conditions (Huokko et al. 2017) while different mechanisms must be involved in the enhancement of LAHG growth of *Synechocystis* by the TM-localized NdbA. According to proteomics results, very few proteins were downregulated in the NdbA deletion mutant under LAHG as compared to WT (Table 1). These included the iron transport protein FutC, iron–sulfur cluster biogenesis regulator SufR (Shen et al. 2007) together with iron containing Cyt c₆ and CrtE (Table 1). Such downregulation may exert a crucial effect on cell growth rate as iron is one of the most essential micronutrients for maintenance of optimal cellular metabolism and growth, for example as a building block of the iron–sulfur clusters of many regulatory and redox active proteins. This can also partially explain the reduced photosynthetic capacity of PSI in the NdbA deletion mutant under LAHG (Table 2).

**Overexpression of NdbA maintains higher Chl and PBS content under LAHG conditions**

It has been shown previously that the growth of *Synechocystis* under LAHG conditions results in significantly lower amount of Chl (Barthel et al. 2013) and pigment-binding proteins (Plohnke et al. 2015) than growth under PA conditions. After 4 d of growth under LAHG conditions, ΔndbA demonstrated even paler green phenotype than WT whereas ΔndbA::ndbA remained considerably greener than WT (Fig. 5A). Closer inspection of the absorption spectra revealed that the color difference between WT and ΔndbA cultures (Fig. 5A, B) was caused by lower OD₇₅₀ in ΔndbA. On the contrary, overexpression of NdbA in ΔndbA::ndbA resulted in higher Chl, phycobilin and carotenoid content (Fig. 5B) as well as a higher amount of proteins involved in PBS formation and in Chl and carotenoid biosynthesis compared to WT (Table 1). Of the enzymes related to Chl biosynthesis, ChlA₆, ChlB, ChlL and ChlN were the most upregulated ones at protein level in the ΔndbA::ndbA strain compared to WT under LAHG conditions (Table 1). These proteins (ChlB, ChlL and ChlN) form the light-independent protochlorophyllide reductase (DPOR) which catalyzes the conversion of photochlorophyllide to chlorophyllide, and this reaction is the major regulatory step of the light-independent Chl synthesis (LICS) pathway (Fujita 1996, Kada et al. 2003). Upregulation of the iron transporter FutC in the NdbA overexpression strain ΔndbA::ndbA (Table 1) is possibly related to their enhanced biosynthesis of Chl.

It was recently demonstrated that LICS is necessary for the adequate Chl accumulation when *Synechocystis* cells are grown under light/-dark-rhythm and its importance was shown to increase when glucose was added to the growth medium (Fang et al. 2017). It is thus clear that the excess of NdbA maintains pigment biosynthesis in *Synechocystis* cells, especially by stimulating the LICS pathway. The downregulation of Chl synthase ChlG may be considered as the protective mechanism against too high accumulation of Chl, which becomes harmful for the cell if not bound to the protein complexes. The simultaneous upregulation of geranylgeranyl reductase ChlP suggests increased synthesis of tocopherol and phytoquinone derivatives that protect the cell against harmful activity of ROS (Latifi et al. 2009, Shpilyov et al. 2013). At the same time, two other pathways leading to bilin (HemF, HemH and Ho1) and carotenoid (Pys and CrtE) biosynthesis showed upregulation which further supports elevated need for protection against ROS in the excess of NdbA under LAHG conditions.

**NdbA plays a role in expression and functionality of the photosynthetic machinery under LAHG conditions**

*Synechocystis* cells grown under LAHG conditions develop only rudimentary and poorly organized thylakoids (Barthel et al. 2013, Plohnke et al. 2015) (Fig. 4B). However, the excess of NdbA in the ΔndbA::ndbA strain, as compared to WT, nearly doubled the TM content under LAHG condition, inspected from the TEM images (Fig. 4C). The WT *Synechocystis* harbored 4–6 visible thylakoid sacks per cell, whereas the NdbA
overexpression strain, ΔndbA::ndbA, demonstrated 8–10 thylakoid sacks in the cell image.

No effective PSII yield, Y(II), was detected from the LAHG grown WT and ΔndbA (Fig. 7C), being in accordance with earlier observation that although both PSII and PSI accumulate in thylakoids under LAHG conditions, the PSII complexes are fully inactive (Barthel et al. 2013). Interestingly, the ΔndbA::ndbA strain retained small but distinct effective PSII yield, Y(II), under LAHG conditions (Fig. 7C) together with elevated amounts of PSII subunits and several PSII assembly factors compared to WT (Table 1). In addition, elevated expression of CurT protein was detected in the ΔndbA::ndbA overexpression strain under LAHG (Table 1), which corresponds to increased number of thylakoids in the cell. In *Synechocystis*, CurT is responsible for shaping the thylakoids and is required for generation of biogenesis centers where PSII complex components are translated and assembled (Heinz et al. 2016). This is in agreement with increased amount of PSII subunits and assembly factors (Table 1) as well as its small but detectable function (Fig. 7C) in the ΔndbA::ndbA strain under LAHG conditions in comparison to WT.

Moreover, proteins whose expression under LAHG conditions most strikingly responded to the quantity of the NdbA protein in the TM included specific PSII regulatory proteins (Table 1). First, they comprised proteins that mediate NPQ in cyanobacteria (Wilson et al. 2006, Wilson et al. 2007, Boulay et al. 2010, Kirilovsky and Kerfeld 2013). OCP, which dissipates excess excitation energy captured by PBS as heat, was downregulated in ΔndbA::ndbA (Table 1) but the amount of FRP, required for inactivation of OCP, showed distinct upregulation in ΔndbA::ndbA and downregulation in ΔndbA as compared to WT (Table 1). It was earlier demonstrated that the alterations in the amounts of both OCP and FRP (high OCP to FRP ratio) could be applied as photoprotective mechanism in cyanobacterial cells (Kirilovsky 2015). Second, the expression of the Flv2 and Flv4 proteins, which function in photoprotection of PSI (Zhang et al. 2009, Zhang et al. 2012, Bersanini et al. 2014), was attenuated in ΔndbA::ndbA but was elevated in ΔndbA as compared to WT (Table 1). Thus, the distinct regulation of the expression of the PSI photoprotective proteins in the NdbA mutant strains (Table 1) correlates not only with the amount of the NdbA protein in the TM (Figs. 1B, 6) but also with the effective PSII yield Y(II) under LAHG conditions (Fig. 7C). These findings indicate that in the excess of NdbA and under LAHG conditions, *Synechocystis* cells strive for enhancing electron flow through PSI further to the photosynthetic electron transport chain even though the PSI activity remains extremely low.

Unlike PSII, PSI was found partly functional under LAHG in *Synechocystis* (Fig. 7D), as also observed earlier (Barthel et al. 2013). There was, however, no significant difference in the effective PSI yield, Y(I), between WT and the two NdbA mutants (Fig. 7D), due to the fact that there was only minor electron input originating from PSII in all the studied strains under LAHG conditions (Fig. 7C). Despite this, the intracellular NdbA amount in TM had a distinct effect on the maximum amount of photo-oxidizable P700, Pm, (Table 2), demonstrating that NdbA is required to maintain functional PSI under LAHG conditions. Increased Pm, value in the NdbA overexpression strain ΔndbA::ndbA under LAHG conditions is also in line with elevated expression of Chl biosynthesis proteins under the same conditions. It has been demonstrated that de novo synthesis of Chl is a rate limiting factor for the translation of the PSI core subunits, and thus, the maintenance of PSI functionality is likely controlled via the regulation of Chl biosynthesis (Eichacker et al. 1996). In addition, observed upregulation of the main iron transporter FutABC (Katoh et al. 2001, Kranzler et al. 2014) in ΔndbA::ndbA (Table 1) is in accordance with the maintenance of PSI because the biogenesis of functional PSI requires most iron among all photosynthetic protein complexes. All these results support the increased functional capacity of PSI under LAHG when NdbA is overexpressed.

In addition to PSII and PSI activities, the NdbA amount in TM under LAHG conditions correlates with the protein amount of major components of the linear electron transfer chain: the Cyt b6f complex together with soluble electron carriers cytochrome c6 (Cyt c6) and several ferredoxins were at elevated level in the ΔndbA::ndbA compared to WT (Table 1). Increased abundance of Cyt c6, the alternative electron transfer component to plastocyanin (PC), implies copper deficiency. In parallel, two systems regulating copper homeostasis in the cell, CopBAC and CopMRS, were downregulated in ΔndbA::ndbA (Table 1), whereas another protein SynAtx1, involved in protection of *Synechocystis* against free cupric ions in cytoplasm, was upregulated in ΔndbA::ndbA. SynAtx1 is the copper metallochaperone and interacts with CtaA or PacS to ensure transport of redundant copper ions to the cytosol or lumen, respectively (Giner-Lamia et al. 2012, Giner-Lamia et al. 2014). It is thus highly conceivable that copper availability is a limiting factor for PC synthesis in the excess of NdbA under LAHG conditions, and is thus complemented with Cyt c6, which is a typical response in cyanobacteria and green algae under copper deficiency (Zhang et al. 1992).

Taken together, it is relevant to conclude that NdbA is required for the adjustment of redundant expression and functionality of the photosynthetic machinery under LAHG conditions. The requirement of some photosynthetic activity upon a short daily exposure of cells to light is apparently important to coordinate the metabolic functions of the cells under LAHG conditions.

**NdbA is involved in regulation of C\textsubscript{i} uptake under LAHG conditions**

Another group of proteins whose expression under LAHG conditions follows the amount of the NdbA protein in TM (Figs. 3B, 6) comprises the proteins involved in CCM of *Synechocystis* (Table 1). Clearly, the overexpression of NdbA causes downregulation of the C\textsubscript{i} uptake protein complexes, like BCT1, SbtA, SbtB and NDH-1, under LAHG conditions whereas the deletion of NdbA in the TM of ΔndbA causes slight increase in C-acquisition components as compared to WT (Table 1).
CO₂ fixation is not expected to be required to support the growth of *Synechocystis* under LAHG conditions due to extensive utilization of glucose (Plohnke et al. 2015). Indeed, the depletion of the cell from energy-consuming C₅ acquisition mechanisms, as occurs in ΔndbA strains, is likely to provide possibilities for investigating the available energy in more crucial metabolism than CCM under LAHG conditions.

## Hypothetical regulatory function of NdbA under LAHG

NDH-2s in *Synechocystis* have been demonstrated to oxidize NADH and transfer electrons to quinone acceptors (Howitt et al. 1999). However, only cells grown under PA and mixotrophic conditions were previously studied and no intracellular location or specific function was addressed for NdbA. Our results provide compelling evidence that NdbA, shown here to be an intrinsic protein in the TM (Fig. 1), is an important component for the maintenance of functional PSI and PSI under LAHG conditions (Fig. 7C, Table 2). This was particularly apparent in the NdbA overexpression strain ΔndbA::ndbA concerning PSI, which is likely to result mainly from improved regulation (e.g. the Fe-S centers; Tiwari et al. 2016), as such drastic differences were not observed in the amount of PSI subunits (Table 1). Contribution of NdbA to PSI functionality is particularly demonstrated by the high $P_m$ value (Table 2), describing the maximal content of oxidizable P700, when NdbA is produced in excess under LAHG conditions. However, improved function of photosystems as such does not give any growth benefit (Fig. 2C) and thus the importance of the short daily illumination period for successful LAHG growth most likely relies on supporting the regulatory functions.

As the growth under LAHG conditions is maintained by sugar catabolism (Plohnke et al. 2015) that produces NADH in high amounts as a substrate for NdbA, the depletion of NdbA is expected to reduce and NdbA overexpression to enhance the electron supply to TM located electron transfer chain. The importance of the functionality of photosynthetic electron transfer upon the short daily illumination periods might become important for production of proper signaling components required for optimal gene regulation at transcriptional level. Changes in the redox state of various components of the electron transport chain, production of distinct reactive oxygen species by the photosystems as well as various metabolites activated on reducing side of PSI are likely to relay information for transcriptional regulation of genes in photosynthetic cyanobacteria (Hihara et al. 2001, Wilde and Hihara 2016). NdbA, an intrinsic thylakoid protein with redox activity, is likely to be involved in such signaling processes to modulate the gene expression of *Synechocystis* cells at protein level (Table 1). However, such a function does not provide an unambiguous explanation for the function of NdbA in promoting growth under LAHG conditions, due to the fact that the overexpression of NdbA showed no further acceleration of growth (Fig. 2).

Considering the physiological background for the retardation of growth under LAHG conditions, it is most relevant to focus on proteins whose expression was hampered in the absence of NdbA. The most striking and specific consequence of the deletion of NdbA under LAHG conditions, in comparison to WT *Synechocystis* cells, was the reduced expression of several proteins involved in iron utilization (Table 1) and in the functionality of the iron–sulfur cluster containing PSI (Table 2). On the contrary, the shift of WT cells from PA to LAHG conditions induced the expression of the NdbA protein, which could hardly be detected by MS approaches under PA conditions. In addition to NdbA, only a few proteins showed significantly higher expression in WT than in ΔndbA under LAHG conditions, with nearly all of them being related to Fe homeostasis. This suggests that under LAHG conditions the inabilities in iron metabolism are likely responsible for the major physiological hurdles resulting in retarded growth in the absence of NdbA.

## Materials and Methods

### Strains and culture conditions

A glucose-tolerant *Synechocystis* sp. PCC 6803 (WT) was used as the reference strain. The ΔndbA mutant was obtained by disruption of ndbA gene by erythromycin resistance cassette and the ΔndbA::ndbA strain by inserting functional ndbA gene to the ΔndbA mutant (for more details see Mutagenesis section). Pre-experimental cultures were grown in BG11 medium buffered with 20 mM HEPES-NaOH (pH 7.5) under continuous white light of 50 $\mu$mol photons m<sup>−2</sup> s<sup>−1</sup> at 30°C, under air enriched with 3% CO₂ with agitation of 150 rpm. Experimental cultures were grown in BG11 medium buffered with 20 mM HEPES-NaOH (pH 7.5) under ambient CO₂ concentration and continuous illumination of 50 $\mu$mol photons m<sup>−2</sup> s<sup>−1</sup> (photoautotrophic growth = PA), under mixotrophic growth conditions (MIXO), where in addition to continuous illumination of 50 $\mu$mol photons m<sup>−2</sup> s<sup>−1</sup> also 10 mM glucose was added to BG11 medium, while upon LAHG conditions the illumination was restricted to 10 min per 24 h and 10 mM glucose was provided in the growth medium. All experimental cultures were grown in growth chambers with cool-white LEDs (AlgeaTron AG130 by PSI Instruments, Czech) at 30°C with agitation of 150 rpm without antibiotics. OD<sub>750</sub> was measured using Lambda 25 UV/Vis spectrometer (PerkinElmer). For physiological, structural and activity measurements, as well as for protein extraction, cells were harvested from pre-experimental phase, inoculated to OD<sub>750</sub> = 0.1 and shifted to experimental conditions for 4 d. For activity measurements, the cells were harvested and resuspended in fresh BG11 medium at the desired Chl concentration. To monitor the growth, cells were inoculated to fresh BG11 medium at OD<sub>750</sub> = 0.1 in the beginning of the experiment.

### Mutagenesis

The ndbA gene (siro857) interrupted with erythromycin resistance cassette and the gene flanking regions were amplified by PCR from the ΔndbA mutant constructed by Howitt et al. (1999) using the following primers: forward primer 5'-TATTGCGGCCAGCTTATGAGC-3' and reverse primer 5'-GCCAA GCTTCTACCTTGTTC-3'. The resulting PCR product was transformed into WT cells using a protocol described by Eaton-Rye (2011). Transformants were segregated by gradually raising the concentration of erythromycin (from 5 $\mu$g ml<sup>−1</sup> to 25 $\mu$g ml<sup>−1</sup>). Complete segregation of the mutation was confirmed by PCR (Supplementary Fig. S1).

The ΔndbA::ndbA strain was constructed by using the same protocol as described above for transforming the ΔndbA mutant with a construct containing the ndbA gene under psbAII promoter with adjacent kanamycin resistance cassette as a selective marker. The construct was amplified with primers: forward primer 5'-CATCCGGGCCCAGGCGGACGACAAGCATTTAGG-3'
and reverse primer 5’-CATCGCGGACCTCACAATCCACTGATTTTGTCCACCTA-3’ and inserted into the chromosomal psbAI site by homologous recombination. Transformants were segregated by gradually raising the concentration of kanamycin (from 10 μg ml⁻¹ to 50 μg ml⁻¹). The correct insertion and the segregation were verified by PCR analysis using primers described above (Supplementary Fig. S1B).

**Room temperature whole cell absorption spectra**
Room temperature whole cell absorption spectra were measured using Oli's 17 UV/VIS/NIR Spectrophotometer (On-Line Instrument Systems, Inc.). Fry's correction was applied to correct the raw spectra. The OD₃₅₀ of cells was adjusted to 0.3 before measurement.

**Fluorescence measurements and P700 absorbance**
The Chl fluorescence from intact cells was recorded with a pulse amplitude modulated fluorimeter Dual-PAM-100 (Walz, Germany). Before measurements, cell suspensions at a Chl concentration of 15 μg ml⁻¹ were dark-acclimated for 10 min. Saturation pulses of 5,000 μmol photons m⁻² s⁻¹ (300 ms) and a strong far-red light (720 nm, 75 W m⁻²) were applied to samples when required. 50 μmol photons m⁻² s⁻¹ was applied as actinic light. The effective yield of PS II Y(II), was calculated as (Fₚ - Fᵣ)/Fₚ. The P700 signal was recorded simultaneously with fluorescence. The maximal change in the P700 signal (Pₚₑ) upon transformation of P700 from fully reduced to fully oxidized state was achieved by applying a saturation pulse on the FR background illumination. The effective yield of PSI, Y(I), was calculated as (Pₚₑ - P)/Pₚₑ.

**Western blotting; protein isolation, electrophoresis and immunodetection**
Total protein extracts of *Synechocystis* cells were isolated as described by Zhang et al. (2009). Proteins were separated by 12% (w/v) SDS-PAGE containing 6 M urea, transferred to a PVDF membrane (Immobilon-P, Millipore) and analyzed with the protein-specific antibodies. The membrane fractions to confirm the localization of NdbA were prepared as described by Zhang et al. (2004). A specific antibody against NdbA was raised by Agrisera.

**Transmission electron microscopy**
TEM-analysis was performed in the Laboratory of Electron Microscopy (University of Turku). Samples were prefixed with 5% glutaraldehyde in a 0.16 M s-collidine buffer (pH 7.4) and postfixed with 2% OsO₄ in 0.16 M s-collidine buffer (pH 7.4) and postfixed with 2% OsO₄. After this samples were dehydrated, embedded and cut to thin sections (70 nm). These were stained with 1% uranyl acetate and 0.3% lead citrate. A JEM-1400 Plus Transmission Electron Microscope (JEOL) was used in the analysis.

**Mass spectrometry—data dependent acquisition (DDA)**
For liquid chromatography-tandem mass spectrometry (LC-ESI-MS/MS) analysis the total proteins were isolated and digested as described by Vuorijoki et al. (2016) with one modification: ammonium bicarbonate buffer NH₄HCO₃ was replaced by Tris-HCl buffer (pH 8.0) of equal molarity for extraction and digestion steps.

The LC-ESI-MS/MS analyses were performed on a nanoflow HPLC system (Easy-nLC1200, Thermo Fisher Scientific) coupled to the Q Exactive HF mass spectrometer (Thermo Fisher Scientific) equipped with a nano-electrospray ionization source. The four biological replicates from each condition were injected on an analytical C18 column (75 μm × 40 cm, ReproSil-Pur 1.9 μm 120 Å, C18-AQ, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany). The mobile phase consisted of water with 0.1% formic acid (solvent A) or acetonitrile/water [80:20 (v/v)] with 0.1% formic acid (solvent B). Peptides were separated by a two-step 110 min gradient from 5% to 26% B in 70 min followed by 26% to 49% B increase in 30 min. MS data were acquired automatically by using Thermo Xcalibur 3.1 software (Thermo Fisher Scientific). A data dependent acquisition method consisted of an Orbitrap MS survey scan of mass range 300–1,800 m/z followed by HCD fragmentation for 12 most intense peptide ions. The spectra were registered with a resolution of 120,000 and 15,000 (at m/z 200) for full scan and for fragment ions, respectively, and normalized collision energy of 27%. The automatic gain control was set to a maximum fill time of 100 ms and 250 ms to obtain maximum number of 3e6 and 1e5 ions for MS and MS/MS scans, respectively.

Data files were searched for protein identification against *Synechocystis* database retrieved from Cyanobase (Kaneko et al. 1996) (3672 entries, October 23, 2012) using Proteome Discoverer 2.2 software (Thermo Fisher Scientific) connected to an in-house server running the Mascot (Perkins et al. 1999) algorithm (Matrix Science). The precursor value was restricted to monoisotopic with a mass tolerance of ±4 ppm and fragment ion mass tolerance of ±0.02 Da. Two missed cleavages were allowed and decoy searches were performed. For the validation of the spectrum identifications, we used a Percolator algorithm (Käll et al. 2007) with relaxed false discovery rate of 0.05. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Deutsch et al. 2017) via the PRIDE partner repository with dataset identifier PXD011671 and 10.6019/PXD011671 (Vizcaino et al. 2016). Quantitative analysis was done in Progenesis software with global normalization and using relative quantification of proteins with at least two peptides with no conflicts per protein.

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**Author contributions**
The concept of the study - T.H.&E.M.A, acquisition, analysis and data interpretation - T.H., D.M.P. & E.M.A, writing of the manuscript T.H., D.M.P. & E.M.A.

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