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An intermolecular disulfide-based light switch for chloroplast *psbD* gene expression in *Chlamydomonas reinhardtii*

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Abstract

Expression of the chloroplast *psbD* gene encoding the D2 protein of the photosystem II (PSII) reaction center is regulated by light. In the green alga *Chlamydomonas reinhardtii*, D2 synthesis requires a high-molecular-weight complex containing the RNA stabilization factor Nac2 and the translational activator RBP40. Based on size exclusion chromatography (SEC) analyses, we provide evidence that light control of D2 synthesis depends on the dynamic formation of the Nac2/RBP40 complex. Furthermore, 2D redox SDS-PAGE assays revealed an intermolecular disulfide bridge between Nac2 and Cys_{11} of RBP40 as the molecular basis for attachment of RBP40 to the complex in light-grown cells. This covalent link is reduced in the dark, most likely via the NADPH-dependent thioredoxin reductase C (NTRC), suggesting crosstalk between chloroplast gene expression and chloroplast carbon metabolism during dark adaption of algal cells.

Introduction

Owing to the endosymbiotic origins of the chloroplast, its gene expression machinery is basically of prokaryotic origin. During the evolutionary development of chloroplasts, however, this machinery was extensively modified by the recruitment of nucleus-encoded regulatory factors which nowadays constitute an intracellular network dedicated to the coordination of gene expression in the nucleus and the organelle (for a recent review see Barkan 2011). While recent years have seen the identification and characterization of a number of these *trans*-acting factors, much less is known about their precise molecular modes of action with regard to light-dependent regulation.

In this context, the idea of redox control of chloroplast gene expression has attracted much attention, since it provides an appealing basis for a direct link between photosynthetic activity and the expression of photosynthesis-related chloroplast genes (Dietz and Pfannschmidt 2011). Indeed, many elements of chloroplast gene expression, including RNA transcription, stabilization, processing and splicing, and translation have been shown to be affected directly or indirectly by the redox state of the organelle (for an overview see Barnes and Mayfield 2003). Translation, however, appears to represent the rate-limiting step for the synthesis of chloroplast-encoded proteins (Eberhard et al. 2002; Zerges and Hauser 2009).

In the green alga *Chlamydomonas reinhardtii*, synthesis of the large subunit of ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco) encoded by the *rbcL* gene has been shown to be regulated via the redox state of the chloroplast glutathione pool, which in turn is modulated by light-induced oxidative stress (Irihimovitch and Shapira 2000). Interestingly, the RbcL protein possesses an intrinsic non-specific RNA binding activity located within its N-terminal region (Yosef et al. 2004). It has therefore been postulated that the binding of RbcL to its own mRNA blocks its translation if either its redox-controlled interaction with the chloroplast chaperone system or Rubisco subunit assembly is disturbed (Cohen et al. 2005).

The most elaborate – but also most controversial – model for redox-controlled translational regulation in chloroplasts has been described in *C. reinhardtii* for the *psbA* gene that encodes the D1 protein of the photosystem II (PSII) reaction center (Barnes and Mayfield 2003; Zerges and Hauser 2009). This model postulates that redox-controlled binding of a protein complex to the 5´ UTR of the *psbA* mRNA leads

to recruitment of ribosomes. The heart of this complex is the RNA-binding protein RB47, whose activity is modulated by RB60, a disulfide isomerase homolog (Kim and Mayfield 2002). RB60 was shown to form intermolecular disulfide bonds with RB47 *in vitro*, suggesting tight cooperation of these factors also *in vivo* (Alergand et al. 2006). It was proposed that light-dependent reduction of the involved thiol groups in RB60 provides the molecular basis for light-dependent increases in D1 synthesis (Trebitsh et al. 2000).

We have previously shown that the expression of the chloroplast *psbD* gene in *C. reinhardtii* is under the control of a high-molecular-weight (HMW) complex containing the RNA stabilization factor Nac2 and the translational activator RBP40 (Schwarz et al. 2007). Furthermore, translation of *psbD* mRNA depends on an U-rich element within its 5´ UTR which serves as a binding site for the translational activator RBP40 (Nickelsen et al. 1999; Ossenbühl and Nickelsen 2000). Deletion of this element (ΔU) results in the complete loss of D2 synthesis (Nickelsen et al. 1999; see also Fig. 1a, b) but is partially restored in genetically selected second-site suppressor lines, namely *suΔU+9*, *suΔU-3* which harbor point mutations in a downstream RNA stem-loop structure encompassing the AUG start codon (Klinkert et al. 2006). In these lines, the *psbD* mRNA can be translated in the absence of RBP40 binding, leading to a model in which Nac2-assisted binding of RBP40 to the U-rich element affects the RNA conformation at the initiation codon, and thereby makes the initiation site accessible to the translational machinery (Schwarz et al. 2007). Thus, both RBP40 and the RNA stem-loop have the capacity to form a molecular switch that regulates *psbD* gene expression and, as a direct consequence of the so-called CES (control by epistasis of synthesis) process, the accumulation of the entire PSII in *C. reinhardtii* (Minai et al. 2006).

Here, we report on the molecular mechanisms that underlie light-controlled regulation of *psbD* gene expression via the RBP40/RNA stem-loop switch. We provide evidence showing that RBP40 is required for this control and that the light-dependent formation of the active Nac2/RBP40 complex is mediated by the establishment of an intermolecular disulfide bridge between the two factors. The redox state of this connection appears to represent a key determinant for D2, and therefore PSII, synthesis.

Results

Light regulation of D2 synthesis depends on the RBP40/RNA stem-loop switch

To test whether the RBP40/*psbD* RNA stem-loop switch is involved in the well-known light-dependent regulation of D2 synthesis in *C. reinhardtii,* we analyzed light-dependent D2 synthesis rates in the deletion strain *ΔU* (which lacks the U-rich element) and the suppressor lines *suΔU-3* and *suΔU+9* which have lost the RBP40 binding site but contain a less stable RNA stem-loop (Malnoe et al. 1988; Klinkert et al. 2006). In pulse-labeling experiments, wild-type cells grown in the light exhibited an 2.8-fold increase in rates of D2 synthesis relative to cells which had been adapted to dark conditions for 38 h (Fig. 1a, b). As described previously, in the dark the ΔU strain showed no D2 protein synthesis, while both suppressor lines exhibited reduced D2 synthesis compared to the wild-type (Nickelsen et al. 1999). Moreover, in the light, D2 expression increased only 1.9-fold in *suΔu-3* and *suΔU+9* strains (Fig. 1a, b). These results suggest that in the suppressor lines both the overall rate of *psbD* mRNA translation and the degree of induction of D2 synthesis by light are affected. Northern analyses verified that the observed differences are due to translational effects, since no significant alterations in *psbD* mRNA levels occurred under the conditions tested (Fig. 1c). In conclusion, these findings suggest that RBP40 is required for efficient regulation of D2 synthesis by light since bypass of RBP40 function in the suppressor lines results in reduced levels of light control. This supports the hypothesis of a light switch which is constituted by the negatively acting *psbD* mRNA stem-loop at the AUG start codon and RBP40 which activates translation by changing the conformation of this RNA structure.

RBP40 contains a single Cys residue

How then does light affect this molecular switch? Redox reactions have been postulated to play critical roles during light activation of chloroplast gene expression (Barnes and Mayfield 2003; Dietz and Pfannschmidt 2011) and, interestingly, RBP40 had been identified as a target for glutathionylation under conditions of oxidative stress in a proteomic analysis in *C. reinhardtii* (Michelet et al. 2008). Inspection of the amino acid sequence of RBP40 revealed the presence of only a single cysteine residue at amino acid position 11 (Cys_{11}) which could serve as a target for

glutathione binding (Fig. 2a). However, Cys_{11} is located within the predicted N-terminal transit sequence of RBP40 which should be cleaved off upon import by the chloroplast and, thus, should not be present in the mature protein (Barnes et al. 2004). To test for the presence of a Cys residue in mature RBP40, stromal proteins from *C. reinhardtii* were treated with the thiol-alkylating reagent PEG5000-maleimide for various times, and subsequently analyzed by immunoblotting. As shown in Figure 2b, an alkylation-dependent size shift of RBP40 was observed, indicating that Cys_{11} is indeed still present in the mature RBP40. This implies either that RBP40 is imported into the chloroplast by an alternative pathway which does not involve N-terminal processing of proteins or it contains an unusually short transit sequence of less than 11 amino acid residues (Schwenkert et al. 2011). Both possibilities are compatible with previous *in vitro* import experiments, which have detected no size change in RBP40 after transport into chloroplasts (Barnes et al. 2004).

To test whether the redox state of Cys_{11} directly affects the RNA-binding activity of RBP40, UV crosslinking experiments with full-length recombinant RBP40 (rRBP40) and a *psbD* 5´ UTR RNA probe were performed under different redox conditions (Fig. 2c, supple. Fig 1). The addition of neither oxidized (GSSG) nor reduced (GSH) glutathione to the reaction mixture had any influence on RNA recognition (Fig. 2c). Moreover, alkylation with NEM had no significant effect on RNA binding, indicating that binding of RBP40 to RNA is not dependent upon the redox state of Cys_{11}.

Light- and redox-dependent formation of the Nac2/RBP40 complex

We have previously shown that RBP40 forms a complex with the RNA stabilization factor Nac2 and that this interaction specifies recognition of the *psbD* 5´ UTR by RBP40; on its own RBP40 binds to any RNA, at least *in vitro* (Ossenbühl and Nickelsen 2000; Barnes et al. 2004; Schwarz et al. 2007). We therefore wished to know whether the interaction with Nac2 is affected by the redox state of Cys_{11}. To this end, we analyzed the distribution of stromal RNA/protein (RNP) complexes by size-exclusion chromatography (SEC, Johnson et al. 2010; Schwarz and Nickelsen 2010). When wild-type cells were grown in the light, the previously described Nac2/RBP40 complex was identified by both Nac2 and RBP40 antibodies in the range of 550 kDa (Schwarz et al. 2007, Fig. 3a, fractions 6-9). In addition, even larger complexes in the range of 1000 kDa were detected only with the RBP40 antibody (Fig. 3a, fractions 4 and 5). These latter complexes have not been observed in

previous experiments using time-consuming glycerol gradient centrifugation for RNP complex separation, probably because they are relatively labile. Since RNA co-immunoprecipitation experiments have previously shown that ribosomal RNA can be precipitated by an RBP40 antibody these RBP40-specific HMW complexes might represent associations with ribosomes/ribosomal subunits during the initiation phase of translation when Nac2 has already left the *psbD* mRNA (Schwarz et al. 2007). This idea is further supported by data revealing that the ribosomal protein S1 and, thus, at least the small ribosomal subunit partially co-elutes with these larger RBP40-containing complexes (Fig. 3a).

Intriguingly, when dark-adapted cells were analyzed, RBP40 accumulated only in the low molecular weight (LMW) range, peaking at ~160 kDa (Fig. 3a, fractions 9-15). Concomitantly, the Nac2 signal shifted towards the later fractions 8-10, corresponding to a complex of smaller size in the range of 440 kDa (Fig. 3a). This suggests that, in the dark, most of RBP40 is detached from the Nac2 complex and, as a consequence, *psbD* mRNA translation would be turned down. Hence, the dynamic formation of the Nac2/RBP40 complex could provide the molecular basis for the observed light-dependent regulation of D2 synthesis (Fig. 1). To determine whether formation of this complex is redox-dependent, we performed SEC analysis on RNP complexes from light-grown cells in the presence of reduced glutathione. As shown in Figure 3a, these reducing conditions resulted in the detachment of RBP40 from the Nac2 complex, although the effect was less pronounced than that observed in dark-grown cells (Fig. 3a, fractions 8-15). Nevertheless, the data strongly suggest that the redox state does have a critical role in Nac2/RBP40 complex formation.

Moreover, SEC analysis of RNP complexes from the suppressor line *suΔU+9* revealed the presence of HMW Nac2/RBP40 complexes similar to the situation in the wild-type (Fig. 3b). This indicates that interaction of RBP40 with its cognate binding site on the *psbD* 5´ UTR is not a prerequisite for Nac2/RBP40 complex formation, which is consistent with the earlier finding that this complex can form even in a mutant strain lacking the *psbD* mRNA (Schwarz et al. 2007).

The observed redox control of RBP40 association with the Nac2 complex raises the question whether photosynthetic electron flow is directly involved in controlling the synthesis of D2. To check this, two photosynthetic mutants with defects in either PSII or PSI were examined with regard to formation of Nac2/RBP40 complexes in the light. In *mbb1*, a nuclear factor is mutated that is required for the stabilization of the

chloroplast *psbB* mRNA encoding the CP47 subunit of PSII (Vaistij et al. 2000), as Nac2 is for *psbD* stability. As shown in Figure 3b, the distribution of both Nac2 and RBP40 following SEC analysis resembled that found for light-grown wild-type cells, indicating that the absence of PSII does not affect Nac2/RBP40 complex formation per se. In the *psaA trans*-splicing mutant *raa1*, PSI is absent, causing severe oxidative stress when cells are grown in the light (Merendino et al. 2006). Under these conditions, partial disassembly of the Nac2/RBP40 complex was observed as indicated by the shift of both the Nac2 and the RBP40 signal towards lower molecular weights during SEC (Fig. 3b). This suggests that oxidative stress might lead to down-regulation of D2 and, consequently, of PSII synthesis.

Light- and redox-dependent disulfide bridge formation between Nac2 and RBP40

The data obtained so far support the idea of a light-dependent control of Nac2/RBP40 complex formation which might involve Cys_{11} of RBP40. To test this more directly, two-dimensional SDS-PAGE analyses were carried out in which stromal proteins were first fractionated by SDS-PAGE in the absence of reducing agents, i.e. preserving preformed disulfide bridges (Ströher and Dietz 2008), and then orthogonally electrophoresed under reducing conditions. Consequently, polypeptides that contain no S-S groups in their native state come to lie on a diagonal across the second-dimension gel, while intermolecular or intramolecular disulfide bridges cause deviations from the diagonal to the left or right, respectively (Ströher and Dietz 2008).

When stromal proteins from light-grown wild-type cells were analyzed by following this procedure, some RBP40 was detected on the diagonal, but substantial amounts were also found in the HMW range up to ca. 170 kDa (Fig. 4a). When stromal proteins were pretreated with reduced glutathione, no such HMW signals were detectable, indicating that RBP40 forms an intermolecular disulfide bridge via its single cysteine Cys_{11} (Fig. 4b). On the other hand, most Nac2 was found on the diagonal at 140 kDa, but lesser amounts migrated in the range of the RBP40 signal at 170 kDa (Fig. 4a). Reduction prior to electrophoresis in the first dimension eliminated this 170 kDa HMW form, confirming that its formation is redox-dependent (Fig. 4b). These findings are consistent with the existence of a direct disulfide bridge between Cys_{11} in RBP40 and one of the several Cys residues present in Nac2 (Fig.

S2). The analysis of the *nac2-26* mutant, which fails to accumulate any Nac2 protein, further substantiated this idea: no HMW RBP40 signals were obtained in this strain (Fig. 4c).

Strikingly, HMW RBP40 signals were also lacking when stromal proteins from dark-adapted wild-type cells were assayed, suggesting that the disulfide bridge linking Nac2 and RBP40 is reduced in the dark (Fig. 4b). Furthermore, the Nac2 signal appeared to be shifted towards lower molecular weight on the right side of the diagonal, suggesting enhanced formation of intramolecular disulfide bridges in Nac2 in the dark (Fig. 4b).

We also analyzed Nac2/RBP40 disulfide bond formation in the genetic backgrounds used to study complex formation by SEC. In the suppressor *su⊿U+9*, Nac2 and RBP40 signals at 170 kDa were actually enhanced, indicating efficient binding of RBP40 to Nac2 despite the absence of its cognate binding site on the *psbD* 5'UTR (Fig. 4c). Furthermore, several additional intermediate RBP40 signals appeared whose nature remains elusive. In *mbb1*, enhanced Nac2/RBP40 binding was also detected, verifying that the effect seen in the *nac2-26* mutant is not due to a deficiency in PSII but is Nac2-specific (Fig. 4c). Finally, reduction of the Nac2-RBP40 disulfide bridge was found to occur in light-grown *raa1* cells, suggesting that oxidative stress leads to down-regulation of D2 synthesis via the Nac2/RBP40 redox switch (Fig. 4c). In conclusion, these data reveal a clear correlation between Nac2/RBP40 complex formation as visualized by SEC analysis and the formation of a disulfide bridge between Nac2 and RBP40.

Blue or red light do not affect Nac2/RBP40 complex formation

In higher plants, both chloroplast transcription and translation have been shown to be regulated by exposure to low levels of blue light (Gamble and Mullet 1989; Barneche et al. 2006). In particular *psbD* gene transcription depends on a blue-light-responsive promoter element which is recognized by a specific sigma factor, namely sig5 (for an overview see Lerbs-Mache 2011). However, in the chloroplast of *C. reinhardtii* only a single sigma factor has been shown to operate, and accordingly no obvious changes in *psbD* mRNA levels were observed under the light conditions applied in this work (Carter et al. 2004; Bohne et al. 2006, Fig. 1c). Nevertheless, we tested whether exposure of dark-adapted cells to low-level blue or red light induces Nac2/RBP40-disulfide bridge formation. As shown in Figure 5, irradiation with neither red nor blue

for 3 h does induce formation of the Nac2/RBP40 complex. This suggests that disulfide bridge formation is not dependent on signal relays activated by blue or red light.

Chloroplast NTRC might be involved in reduction of the Nac2/RBP40 disulfide bridge in the dark

The emerging picture of light-dependent regulation of D2 synthesis postulates a central role for the redox state of the Nac2/RBP40 complex. In the light, assembly of this complex requires formation of a disulfide bond between the two proteins. In the dark or under oxidative stress, this bond is reduced and, consequently, RBP40 is detached from Nac2. While oxidative stress is likely to lead to the previously observed glutathionylation of RBP40 (Michelet et al. 2008), it has remained obscure how reduction in the dark can be achieved.

One candidate for this role is the recently identified NTRC (chloroplast NADPH-dependent thioredoxin reductase C) enzyme (Serrato et al. 2004). NTRC reduces disulfides in the dark using electrons from NADPH, which is generated by the oxidative pentose phosphate pathway (Kirchsteiger et al. 2009). To test the possibility that NTRC might be involved in redox regulation of the Nac2/RBP40 complex in the dark, 2D redox PAGE was performed with stromal proteins from light-grown cells that had been preincubated with recombinant rNTRC from the cyanobacterium *Anabena* sp. PCC 7120 in the presence of 250 µM NADPH (Fig. 6a). Whereas NADPH alone had no effect on the covalent link between Nac2 and RBP40, the disulfide bridge was reduced when the cyanobacterial enzyme was added. In contrast, rNTRC from rice had no effect on the Nac2/RBP40 complex (data not shown), suggesting that specific recognition of the disulfide target has diverged during evolution. Nevertheless, in *C. reinhardtii* it does appear that the Nac2/RBP40 complex represents a target for chloroplast NTRC, which may therefore be the enzyme that mediates down-regulation of D2 synthesis in the dark in this species.

To substantiate the idea that dark-grown cells contain an activity that reduces the Nac2/RBP40 disulfide bridge, we mixed stromal protein extracts from light- and dark-grown wild-type cells in a 1:1 ratio. When this mixture was assayed, a drastic decrease in the level of the Nac2/RBP40 complex was observed which cannot be explained by a dilution effect (Fig. 6b). In the presence of 250 µM NADPH, this effect was even more pronounced, strongly suggesting that dark-adapted chloroplasts from

C. reinhardtii contain an activity, probably the algal NTRC, which severs the link between Cys_{11} of RBP40 and Nac2, and thereby turns down *psbD* gene expression.

Discussion

We have previously postulated that the translational activator RBP40, together with an RNA stem loop structure encompassing the AUG start codon, form a molecular switch with the capacity to regulate chloroplast *psbD* gene expression (Klinkert et al. 2006). Here, we demonstrate that this molecular switch is indeed involved in controlling D2 synthesis in a light-dependent manner (Fig. 7). Suppressor lines in which the requirement for RBP40 is bypassed, exhibited reduced levels of light-induced D2 synthesis (Fig. 1b). This argues for a light-dependent resolution of the RNA structure by RBP40. We have previously shown that recruitment of RBP40 by the Nac2 complex specifies its interaction with the *psbD* 5´ UTR (Schwarz et al. 2007). Intriguingly, this recruitment process and the subsequent formation of a Nac2/RBP40 complex is light-dependent and, thus, most likely forms the critical step during dark/light transitions in patterns of *psbD* gene expression (Fig. 3b).

Moreover, the data reveal a direct interaction of RBP40 with Nac2 via a light-dependent disulfide bridge involving the single Cys residue in RBP40 at position 11, suggesting that the redox state of Cys_{11} is the main target for the light control mechanism. To date, no RBP40 knock-out mutant lines are available, which hampers site-directed genetic approaches to confirm the role of Cys_{11} *in vivo*. However, the redox state of Cys_{11} apparently has no direct influence on the RNA-binding activity of RBP40, which is consistent with the localization of Cys_{11} in the N-terminal segment of RBP40, relatively remote from its predicted RNA-binding domain which starts at position 39 (Barnes et al. 2004, Fig. 2a). This RNA-binding domain is made up of four conserved repeats, each spanning 70 amino acids, and is structurally related to other RNA-binding domains of the RBD or KH type (Barnes et al. 2004, Fig. 2a). In agreement with this, a truncated version of RBP40 lacking the first 18 N-terminal amino acids – including Cys_{11} – has been shown to retain general RNA-binding activity (Barnes et al. 2004).

The question arises as to which Cys residue in the Nac2 protein interacts with Cys_{11} of RBP40. Nac2 encodes a total of eleven Cys residues at various positions, some of which might form intramolecular disulfide bonds, as suggested by the 2D redox PAGE analyses (Fig. 4; suppl. Fig. 2). Interestingly, the most probable disulfide

bridge is predicted to be formed between Cys_{981} and Cys_{1008}, which are both located within the tetratricopeptide repeat (TPR) domain of Nac2. This domain has previously been shown to play a critical role for Nac2 function, probably by mediating the interaction with other subunits of the Nac2 complex (Boudreau et al. 2000). In addition, a putative dinucleotide-binding domain is predicted at position 402 – 413 of Nac2 which might be involved in the modulation of the redox state of one or more of its Cys residues. However, only a systematic evaluation of these sites can uncover the residue that forms the link with RBP40.

The most interesting question concerns the light-mediated redox switch at Cys_{11}. The current models for redox control of chloroplast gene expression usually involve light-catalyzed reduction processes which are linked to the photosynthetic electron transport (PET) via either PSI and thioredoxin or the redox state of the plastoquinone pool (Barnes and Mayfield 2003). In case of Nac2/RBP40 complex formation, PET is apparently not directly involved in the regulatory process since Cys_{11} oxidation is also observed in the PSII mutant *mbb1*. This is consistent with previously measured wild-type levels of D2 synthesis rates in this mutant (Vaistij et al. 2000). PSI deficiency leads to oxidative stress in the light, which obviously affects NAC2/RBP40 complex formation and results in a shutdown of *de novo* PSII synthesis, thereby avoiding harmful photosynthetic electron overflow. The reductive detachment of RBP40 from Nac2 under oxidative stress conditions is likely to be mediated via gluthationylation of RBP40 as has been reported previously (Michelet et al. 2008, Fig. 4c).

In the dark, however, a different regulatory redox system appears to operate on the Nac2/RBP40 disulfide bridge, namely the NTRC system. NTRC uses NADPH as source of reducing power, which can be produced during darkness by the oxidative pentose phosphate pathway (Neuhaus and Emes 2000). Thus it was proposed that NTRC allows redox regulation in the chloroplast during the night, a notion supported by the hypersensitivity of the Arabidopsis NTRC knock out mutant to prolonged darkness (Pérez-Ruiz et al. 2006). Recently the regulation of the ADP-Glc pyrophosphorylase (AGPase) involved in starch synthesis in *A. thaliana* was shown to be mediated by NTRC (Michalska et al. 2009). Our data suggest that this enzyme, which is active in the dark, is also involved the regulation of chloroplast *psbD* gene expression – at least in *C. reinhardtii*. Thus, NTRC would directly link the regulation of chloroplast gene expression to carbon metabolism in the chloroplast, i.e. the oxidative pentose phosphate pathway.

In conclusion, the following scenario is likely to describe the molecular events which underlie light-dependent regulation of D2 synthesis (Fig. 7). In the light, *psbD* mRNA translation is activated by RBP40 which is tightly bound via its Cys_{11} residue to Nac2, and is thereby targeted to its cognate binding site within the *psbD* 5´ UTR. The electron acceptor during formation of the disulfide bridge is not yet known. As discussed by Wittenberg and Danon (2008), reactive oxygen species, GSSG and O_2 might serve the purpose. RBP40 binding alters the RNA conformation at the initiation codon, making it accessible to the translation machinery. In the dark, reduction of the disulfide bond between Nac2 and RBP40 via NTRC leads to detachment of RBP40 from the Nac2 complex, resulting in down-regulation of D2 synthesis. Thus, the redox state of the Nac2/RBP40 disulfide bridge appears to represent the key control point for regulation of D2 synthesis, which – in light of the CES principle of PSII assembly – represents the key player in determining PSII levels in the green alga *C. reinhardtii* (Minai et al. 2006).

Methods

Strains and culture conditions

C. reinhardtii strains were grown in continuous light (30 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 23°C in Tris-acetate-phosphate medium containing 1% sorbitol (TAPS, Gorman and Levine 1965). For dark adaption, cells were transferred to complete darkness for 38 h prior to analysis.

Analysis of nucleic acids

Whole-cell RNA was prepared with TriReagent (Sigma-Aldrich) according to the manufacturer's instructions, and 2-µg aliquots were electrophoretically fractionated on gels, blotted onto positively charged nylon membranes and hybridized to *atpB* and *psbD* probes. Probes were generated by PCR using DIG-11-dUTP (Roche Diagnostics) and primers specific for the respective target genes (psbD: 5'-GTAATACGACTCACTATAGGGCCACAATGATTAAAATTAAA-3', 5'-GTTGGTGTCAACTTGGTGG-3' / atpB: 5'-ATGTTGTCCAGCGTGCGC-3', 5'-TTACTTCTTGGGCAGGAG-3'). Visualization of hybridization signals was performed by ECL using AP-conjugated anti-DIG-antibody and CDP* substrate (Roche Diagnostics).

Pulse labelling of proteins

Chlamydomonas liquid cultures were grown in TAPS medium to a density of ~ $2 \cdot 10^6$ cells/mL, pelleted, resuspended in TAPS medium in which all sulfur-containing ingredients were replaced by the respective chloride salts (TAPS-S), and incubated for 16 hours at 23°C in the light. Cells were pelleted, washed, and resuspended in TAPS -S/-T (lacking both sulfur salts and trace elements) and grown in the dark for 2 hours. Cells were then washed again and resuspended in TAPS -S/-T to a concentration of 80 µg chlorophyll per mL. Aliquots (225 µl) of the cell suspension were incubated with cycloheximide (10 µg/mL) for 10 minutes. Subsequently, 100 µCi $H_2{}^{35}SO_4$ (Hartmann Analytic, Braunschweig) was added to each, followed by incubation for 15 minutes in the light as before. After centrifugation, sedimented cells were frozen in liquid nitrogen. Cells were resuspended in 10 mM HEPES-KOH pH 7.5, 10 mM EDTA in the presence of CompleteMini protease inhibitors (Roche) and

disrupted by sonication (30 sec, RT). The homogenate was then centrifuged at 20000 g for 30 min. The pellet was resuspended in 10 mM HEPES-KOH pH 7.5, 10 mM EDTA. Samples were fractionated by electrophoresis on a 16% sodium dodecyl sulfate-polyacrylamide gel containing 8 M urea.

UV cross-linking of RNA to recombinant RBP40

For expression of recombinant RBP40 protein, the DNA sequence encoding amino acids 1-382 was PCR-amplified from a cDNA clone using the primer pair BamHI-RBP40 (5'- aaggatccATGCTGACCTTGAGACGTGC-3') and RB38-DN44revSalI (5'-ttgtcgacCTAGTAGCGGGCGCCC-3'), and inserted into the plasmid pQE30 (Qiagen) via *Bam*HI/*Sal*I restriction sites. Protein expression in *E. coli* M15 blue cells (Stratagene) was induced by addition of IPTG to a final concentration of 1 mM, followed by growth at 37°C for 3 h. The recombinant protein was purified according to the GE Healthcare protocol for purification of histidine-tagged recombinant proteins under native conditions using Ni-Sepharose 6 Fast Flow (GE Healthcare). In preparation for the binding reactions described below, the purified protein was incubated for 1 h at RT with 25 mM GSSG, 50 mM GSH, or a 50-fold molar excess of NEM, respectively, in a buffer containing 100 mM HEPES/KOH pH 7.8, 25 mM $MgCl_2$ and 300 mM KCl, followed by desalting using Amicon Ultra centrifugal filtration devices (Millipore) with a 10-kD molecular mass cutoff, in accordance with the manufacturer's instructions.

The DNA template for *in vitro* synthesis of the *psbD* RNA probe was generated by PCR using T7psbD5 (5'-gtaatacgactcactatagggCCACAATGATTAAAATTAAA-3'; T7 RNA polymerase promoter in lower case letters) and psbDUTR3 (5'-ACCGATCGCAATTGTCAT-3') as primers. RNA synthesis was catalyzed by T7 RNA polymerase (Fermentas) in the presence of [α-32P]UTP (3000 Ci/mmol; Hartmann Analytic), according to the manufacturer's protocol. After removal of the template by treatment with DNase I (Promega), the RNA was extracted with phenol-chloroform and precipitated with ethanol in the presence of ammonium. Binding reactions (20 µl) were performed at RT for 5 min and contained 500-1000 kcpm of ^{32}P-labeled RNA probe, 20 mM HEPES/KOH pH 7.8, 5 mM $MgCl_2$, 60 mM KCl, and 3 µg of pretreated protein. After irradiation, the free RNA probes were digested by treatment with 10 U RNase One (Promega) for 30 min at 37°C, and the samples were fractionated by SDS-PAGE, and analyzed by phosphorimaging.

Gel filtration analysis of native proteins

For analysis of native protein complexes, chloroplasts were isolated from *cw15* strains according to Zerges and Rochaix (1998), and lysed in non-reducing breaking buffer (10 mM EDTA, 10 mM Tricine-KOH pH 7.5, and Roche CompleteMini protease inhibitors). Membrane material was removed by centrifugation on a 1 M sucrose cushion (100000 g, 30 min). Reducing conditions, if indicated, were achieved by adding 5 mM GSH to the stroma-containing supernatant prior to concentration using Amicon Ultra filtration devices (Millipore). Samples (~2 mg protein) were loaded through an SW guard column onto a 2.15 × 30-cm G4000SW column (Tosoh), and elution was performed with gel filtration buffer (50 mM KCl, 5 mM $MgCl_2$, 5 mM □-aminocaproic acid, 20 mM Tricine-KOH pH 7.5), at a flow rate of 2 mL/min (Johnson et al. 2010). All steps were performed at 4 °C

Diagonal 2D redox SDS–PAGE

Stromal proteins (100□µg) from *cw15* strains were isolated according to Zerges and Rochaix (1998) in the absence of reducing agents. To prevent thiol reoxidation, proteins were alkylated with 0.1 M iodoacetamide in the dark (15 min, 4 °C). An appropriate volume of non-reducing Laemmli buffer was added, and the samples were separated by SDS-PAGE in the first dimension (10 % polyacrylamide) resolving gel. After electrophoresis, gel lanes were excised and incubated in SDS running buffer containing 0.1 M DTT (10□min, RT), before incubation with 0.1 M iodoacetamide in the same buffer (10 min, RT). The gel strips were then horizontally applied to another 10% SDS-PA gel, and electrophoresis was performed in the second dimension (Ströher and Dietz 2008; Stengel et al. 2009). Immunoblotting was carried out with antibodies raised against Nac2 and RBP40 (Schwarz et al. 2007).

PEGylation

Stromal proteins (20 µg) were treated with 10 mM methoxypolyethylenglycol-maleimide (5 kDa, PEG-MAL; Laysan) in alkylation buffer (0.1 M Tris-HCl pH 7.0, 1 mM EDTA) for 0, 10, 20 and 30 minutes, at 4 °C in the dark. The reaction was stopped by addition of Laemmli buffer in the presence of 0.1 M DTT (Balsera et al.

2009). Sauer:bis-Tris SDS-PAGE (10%) and MOPS running buffer were used to separate the proteins. RBP40 was detected by immunoblot analysis.

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Figure legends

Figure 1: Rates of D2 synthesis depends on light conditions.

(**a**) Thylakoid membrane proteins of indicated strains were pulse labeled with ^{35}S-sulfur, fractionated by SDS-PAGE in 16 % gels containing 8 M urea, and visualized by autoradiography. D: cells were grown in the dark for 38 h prior to analysis; L: cells were grown in continuous light at 30 µmol $m^{-2}s^{-1}$. (**b**) Densitometric quantification of D2 protein synthesis rates shown in (**a**). The results are representative of three independent experiments. D2 signals were normalized relative to the AtpA signal marked by the *star* in (**a**). The relative increase of D2 synthesis upon light induction is indicated.

(**c**) Northern blot analysis of *psbD* and *atpB* transcripts from the indicated strains grown under the same conditions as in (**a**).

Figure 2: Thiol labellingand RNA binding activity of RBP40.

(**a**) Schematic representation of the RBP40 polypeptide showing the disposition of the four repeats involved in RNA binding relative to the single Cys residue (Cys_{11}). The C-terminal end of the putative transit peptide predicted by the TargetP 1.1 algorithm (30 amino acids, http://www.cbs.dtu.dk/services/TargetP) is indicated by the scissors. (**b**) Time course of Cys_{11} alkylation. Stromal proteins (20 µg) were treated with 10 mM mPEG5000-maleimide for the periods indicated, and then subjected to immunoblot analysis with an anti-RBP40 antibody. (**c**) RNA-binding activity of full-length recombinant RBP40 protein (rRBP40). Aliquots (3 µg) of rRBP40 were pretreated with 25 mM GSSG, 50 mM GSH, or a 50-fold molar excess of NEM, UV-crosslinked to a radiolabelled *psbD* 5´UTR probe and fractionated by SDS-PAGE.

Figure 3: Light-dependent formation of Nac2/RBP40 complexes.

Wild-type (**a**) and mutant (**b**) cells were grown under the conditions indicated on the left margin (see Fig. 1 for details) and subjected to SEC. Fractioned proteins were subjected to Western blotting and labeled with the antibodies indicated on the right. The samples marked LR was treated with 5 mM gluthatione prior to SEC. Fraction numbers and molecular weights are indicated at the top.

Figure 4: Light-dependent formation of a disulfide bridge between Nac2 and RBP40. (**a**) Aliquots (100 µg) of stromal proteins from light-grown wild-type cells were fractionated by 2D redox SDS-PAGE, and Nac2 and RBP40 were localized by immunoblot analysis. The diagonal along which polypeptides that form no S-S bonds are expected to lie is indicated. For further explanation, see text. (**b**) Sections of 2D redox gels showing immunodetected Nac2 and RBP40 signals after 2D electrophoresis of samples from strains grown under the indicated conditions. The samples marked LR were reduced with 5 mM glutathione prior to electrophoresis in the first dimension. For details see Figs. 1 and 3.

Figure 5: Blue and red light do not affect Nac2/RBP40 disulfide bridge formation.
Stromal proteins were isolated from dark-adapted wild-type cells that had been exposed to low levels (5 µmol m^{-2} s^{-1}) of either blue (BL) or red light (RL) for 3 h, and subjected to 2D redox PAGE. Nac2 and RBP40 proteins were localized by immunoblot analysis.

Figure 6: The Nac2/RBP40 disulfide bridge can be reduced by rNTRC.
(**a**) Stromal proteins isolated from light-grown wild-type cells were incubated with 250 µM NADPH in the presence or absence of 2 µM rNTRC enzyme from *Anabena* sp. PCC 7120, and analyzed by 2D redox PAGE. The rNTRC enzyme was prepared as reported in Pascual et a. (2011).
(**b**) A 1:1 mixture of 50 µg each of stromal proteins from dark-adapted and light-grown cells was incubated in the presence or absence of 250 µM NADPH prior to 2D redox PAGE. Nac2 and RBP40 proteins were detected by immunoblot analysis.

Figure 7: Working model for redox regulation of *psbD* gene expression.
In the light, RBP40 binds to Nac2 via an intermolecular disulfide bridge and, as a consequence, the RNA conformation at the AUG start codon is altered. This allows ribosomes access to the initiation site and enables efficient translation of *psbD* mRNA. In the dark, the disulfide bridge between Nac2 and RBP40 is reduced via NTRC leading to detachment of RBP40 from Nac2 and down-regulation of D2 synthesis. For further explanation see text.

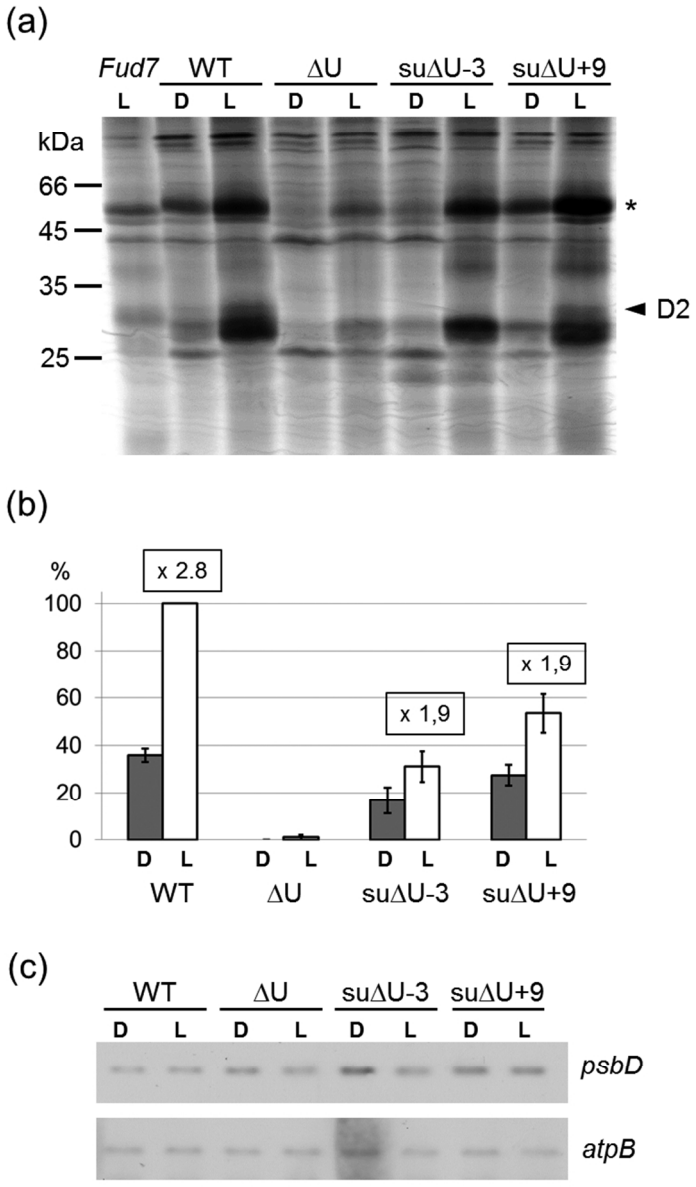

Figure 1
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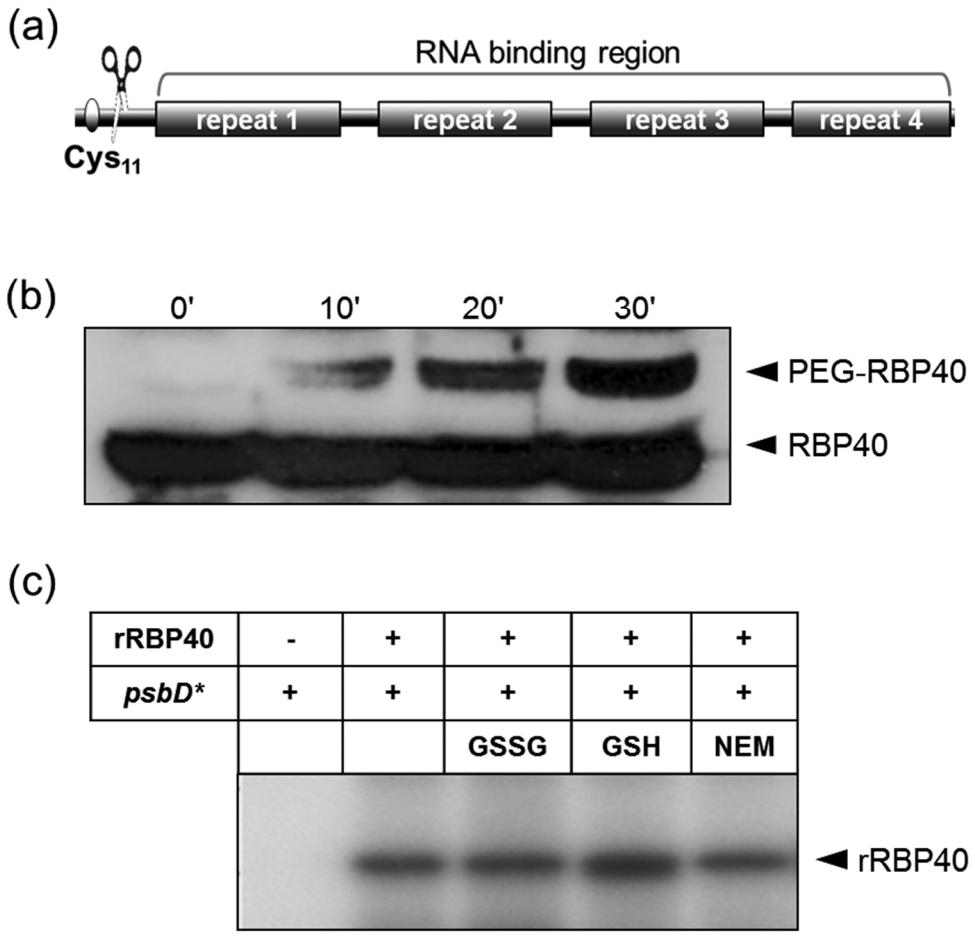

Figure 2
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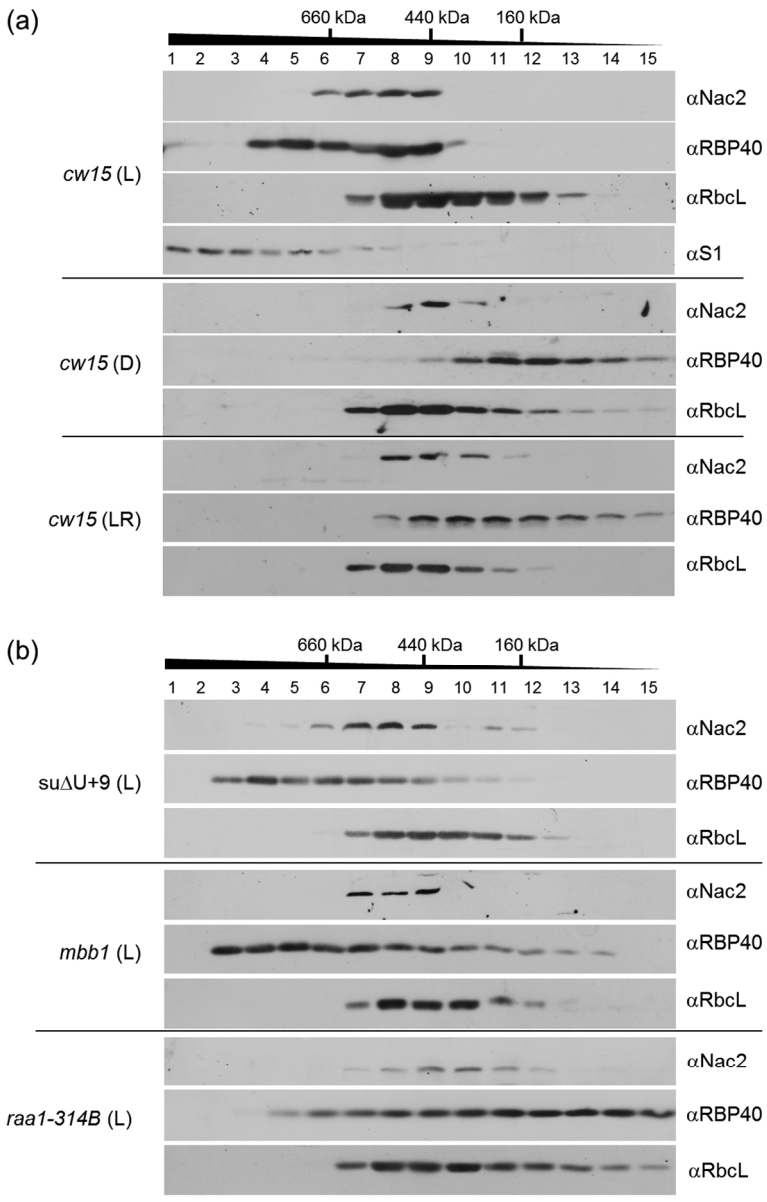

Figure 3
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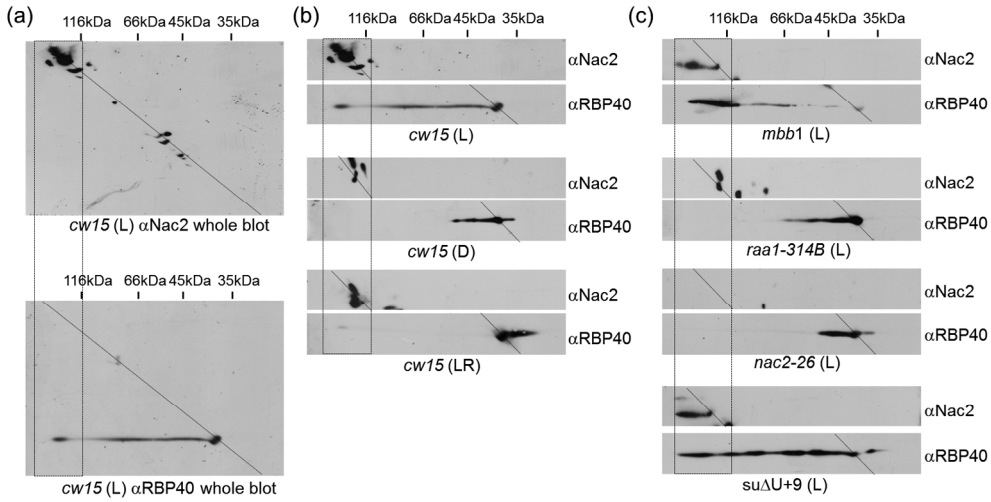

Figure 4
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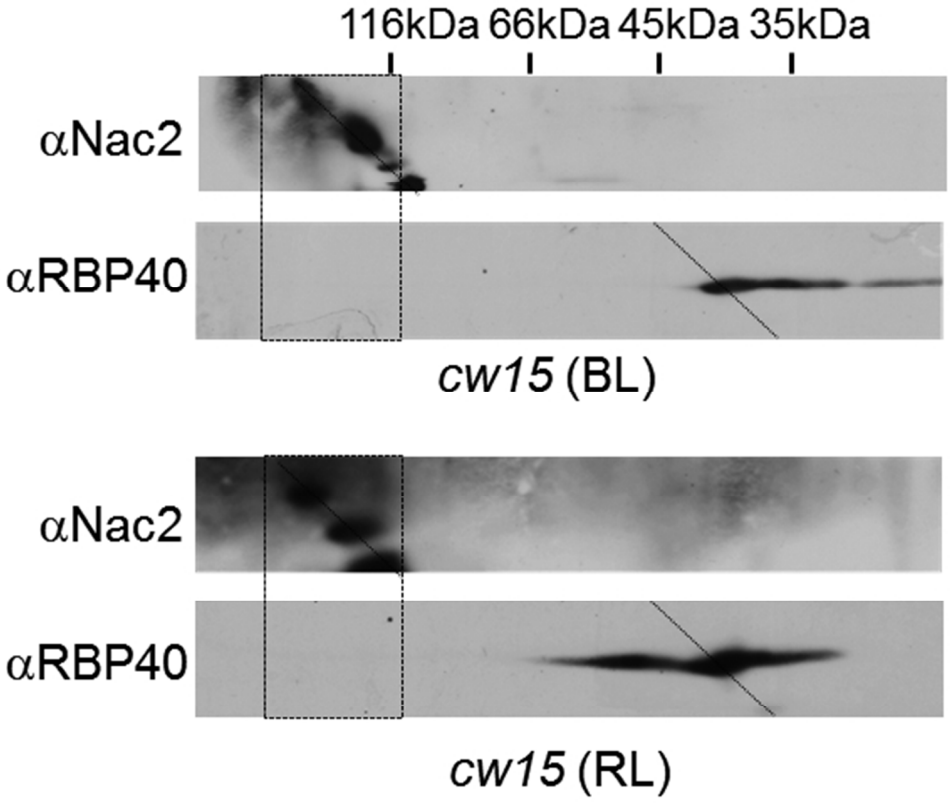

Figure 5
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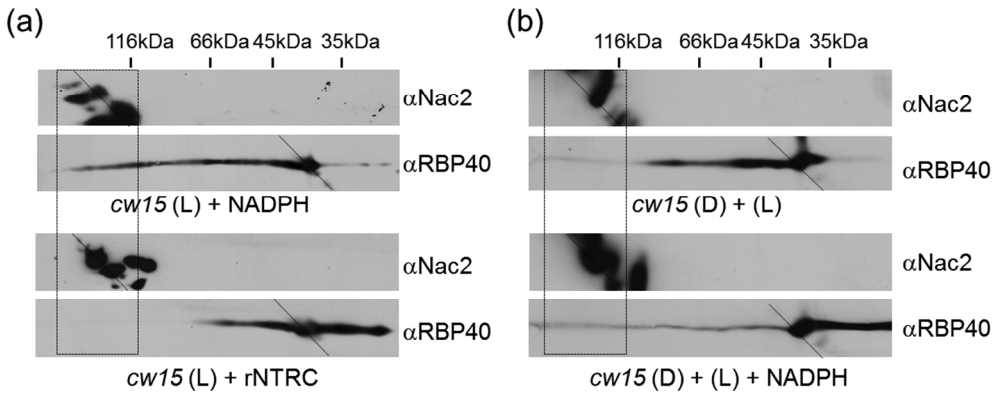

Figure 6
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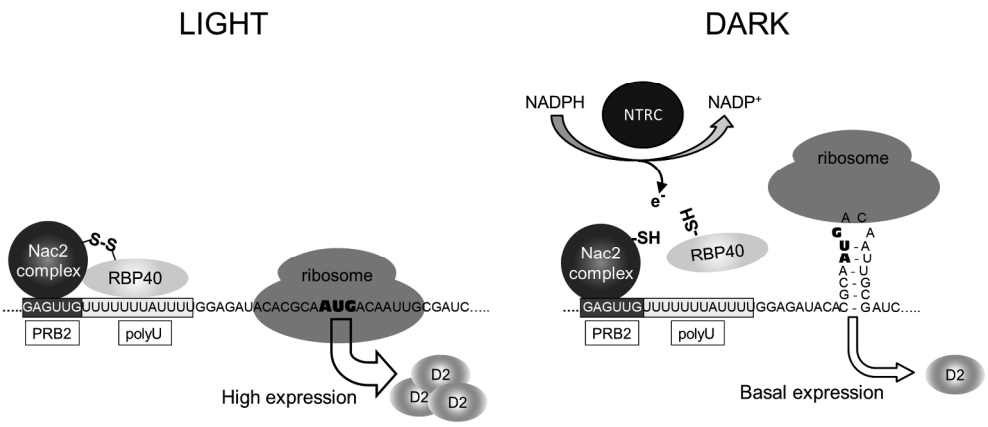

Figure 7
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