Title

Identification of a gene controlling levels of the copper response regulator 1 transcription factor in Chlamydomonas reinhardtii

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Identification of a negative regulator of the transcription factor CRR1 pathway in *Chlamydomonas reinhardtii*

Short title:
The FBXO3 protein CEHC1 negatively regulates CRR1

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Abstract

Oxygen prevents hydrogen production in Chlamydomonas, in part by inhibiting transcription of hydrogenase genes. We developed a screen for mutants showing constitutive accumulation of *HYDA1* transcripts in normoxia. A reporter gene required for ciliary motility, placed under the control of the *HYDA1* promoter, conferred motility only in hypoxia. By selecting for mutants able to swim even in normoxia, we obtained strains that express the reporter gene constitutively. One mutant identified a gene encoding an F-box protein 3 (FBXO3) known to participate in ubiquitylation and proteasomal degradation pathways in other eukaryotes. Transcriptome profiles revealed that the mutation, termed *cehc1-1* (constitutive expression of hydrogenases and copper-responsive genes) leads to increased expression of genes known to be targets of CRR1, a transcription factor in the nutritional copper signaling pathway and in the pathway for response to hypoxia. *CRR1* was required for upregulating expression of the *HYDA1* reporter gene in response to hypoxia and for the constitutive expression of the reporter gene in *cehc1-1* mutant cells. The CRR1 protein, normally degraded in Cu-supplemented cells, was stabilized in *cehc1-1* cells, supporting the conclusion that CEHC1 facilitates the degradation of CRR1. Our results describe a previously unknown pathway for the negative regulation for CRR1 and possibly other pathways leading to complex metabolic changes.

Introduction

Using diverse physiological strategies, the unicellular photosynthetic alga *Chlamydomonas reinhardtii* acclimates to fluctuations in environmental factors including oxygen and carbon dioxide content; light quality and quantity; and concentrations of salt and organic molecules in the soil/freshwater habitats where it grows (reviewed by Johnson and Alric, 2013; Saroussi et al. 2017; Burlacot and Peltier, 2018; Burlacot et al. 2019). One acclimation strategy is to use hydrogenases to maintain redox balance in photosynthetic and fermentative metabolism, processes that have been investigated in detail using the powerful experimental tools of this system (reviewed by Ghirardi et al. 2007; Grossman et al. 2010; Atteia et al. 2013; Catalanotti et al. 2013; Yang et al., 2015; Grechanik and Tsygankov, 2022). Hydrogenases evolved as components of anaerobic energy metabolism in the evolution of early eukaryotes during the period of low atmospheric O_2 and have been retained during evolution of most unicellular eukaryotic lineages including certain algae (Gould et al. 2019). Chlamydomonas cells

accumulate O_2-sensitive [Fe-Fe] hydrogenases in the chloroplast stroma where the enzymes transfer electrons from ferredoxin1 (PETF1, Fd) to protons, generating H_2 (Winkler et al., 2010; Sawyer et al. 2017; Sawyer and Winkler, 2017).

Given the multiple roles for hydrogenases in photosynthetic and fermentative metabolism, it is likely that complex mechanisms have evolved for regulating expression of genes involved in hydrogen production. Dark hypoxia treatment induces increased expression of many genes as shown by analyses of transcript abundance (Mus et al. 2007; Hemschemeier et al. 2013; Subramanian et al. 2014; Blaby-Haas et al. 2016) and by quantitative proteomic analyses (Terashima et al. 2010; Nikolova et al. 2018). Among the genes whose expression is upregulated are nuclear genes *HYDA1* and *HYDA2*, encoding [Fe-Fe]-hydrogenases (Happe and Kaminski, 2002; Forestier et al. 2003) as well as *HYDG1* and *HYDEF1* genes, encoding assembly factors (Posewitz et al. 2004) needed for hydrogenase assembly and activity in the chloroplast stroma (Sawyer et al. 2017). *HYDA1* and *HYDA2* transcript levels decrease within minutes in the presence of oxygen. Reporter gene constructs have been used to demonstrate that the increase in *HYDA1* transcripts in response to hypoxia is regulated at the level of transcription (Stirnberg and Happe, 2004; Sun et al. 2013). Although *HYDA1/2* gene expression is inhibited by oxygen, data from RNA-seq experiments with cells grown under aerobic conditions shows that genes encoding hydrogenases and hydrogenase assembly factors are expressed in a diurnal manner, peaking within a few minutes of dark onset when fermentation may begin and again just prior to onset of light when photosynthesis begins (Zones et al. 2015; Gould et al. 2019; Strenkert et al. 2019).

The transcription factor CRR1, identified by its role in regulation of the response to copper depletion (Quinn et al. 2000), is also a positive regulator in the pathway for response to hypoxia (Quinn et al. 2002). *crr1* mutants fail to grow in anoxia, even in copper-replete medium (Eriksson et al. 2004). Its squamosa-promoter binding protein (SBP) domain binds to GTAC-containing copper response elements (CuRE) in promoters of target genes (Kropat et al. 2005; Sommer et al. 2010). For response to anoxia, a second overlapping element termed hypoxia response element (HyRE) is required (Quinn et al., 2002), as is a metallothionein-like Cys-rich domain at the C-terminus of CRR1 (Sommer et al. 2010; Hemschemeier et al. 2013), suggesting a mechanism for CRR1 activity in the integration of copper response and anoxia response

pathways. The *HYDA1* promoter contains CuRE elements needed for induction of the gene in hypoxia; a screen for positive regulators of the promoter yielded a mutation in *CRR1* (Pape et al. 2012). Several studies have confirmed that CRR1 activates *HYDA1* gene expression in response to hypoxia (Quinn, 2002; Pape et al. 2012; Hemschemeier et al. 2013).

To identify components of the hypoxia signaling pathway(s) controlling hydrogenase expression, we designed a screen utilizing ciliary motility (Zhang and Lefebvre, 1997). Wild-type cells swim with two cilia that beat to propel the cells through liquid medium. Normal motility depends on the function of radial spokes, protein complexes that regulate dynein activity in the ciliary axoneme (Zhu et al. 2017). The *pf14* mutation results in ciliary paralysis due to a mutation in the *RSP3* gene encoding radial spoke protein 3 (Williams et al. 1989). Our screen used a reporter gene with a one kb fragment (including the 5'UTR) from upstream of the *HYDA1* gene to drive the expression of the *RSP3* gene (p*HYDA1-RSP3*; Sun et al. 2013). When expressed in *pf14* cells, the reporter gene conferred motility only in the absence of oxygen (Figure 1A). Transformant strains remained immotile under aerobic conditions but became motile after transfer to hypoxic conditions for 12 h, indicating that expression of the reporter gene was induced by hypoxia.

Because the transformant strains could not swim in aerobic conditions, a simple and sensitive genetic screen was used to identify mutants defective in the suppression of *HYDA1* expression in oxygen. After spontaneous or chemical mutagenesis, the immotile cells were grown aerobically in liquid culture and screened for motile cells that could swim up to the meniscus (Figure 1B; Sun et al. 2013). Many of these mutant cells had sequence alterations in the *HYDA1* promoter that allowed expression of RSP3 in the presence of oxygen. This screen also produced a constitutively motile mutant defective in a factor that acts in trans to regulate the activity of the *HYDA1* promoter in the transgene. The mutation, termed *cehc1* for constitutive expression of hydrogenases and copper-responsive genes, resides in a previously uncharacterized gene encoding an F-box protein in the *FBXO3* family (Kipreos and Pagano, 2000). We determined that this protein is a negative regulator of the CRR1 transcription factor that controls expression of many genes in the copper assimilation pathway and the hypoxia response (Merchant et al. 2006; Sommer et al. 2010; Castruita et al. 2011; Pape et al. 2012; Hemschemeier et al. 2013). The *CEHC1* gene also appears to be involved in additional pathways controlling other metabolic responses.

Results

Constitutively swimming mutant B6-F up-regulates expression of the *RSP3* reporter gene

Aerobically grown cells of the parental strain B6 (CC-4508; Supplemental Table 1), carrying one copy of the *pHYDA1-RSP3* reporter gene together with a selectable marker gene conferring resistance to paromomycin (Sizova et al. 2001), showed the immotile phenotype expected of *pf14* mutants (Figure 1C, left). After chemical mutagenesis, one mutant, B6-F, was motile and swam up to the meniscus from the bottom of the tube (Figure 1C, right). Increased expression of the ectopic wild-type *RSP3* gene accompanied this motility, as shown by an immunoblot assay to detect the presence of the HA-tagged RSP3 protein (Figure 1D). The motility phenotype of strain B6-F correlates with and is likely dependent on the upregulated expression of the reporter gene.

A single mutation confers the motility phenotype on B6-F cells

A cross of motile B6-F (CC-4509) cells with immotile CC-613 cells, both of which contain an endogenous *pf14* mutation, showed that the new mutation in B6-F cells is linked within 25 cM of the reporter gene (Sun et al. 2013; see Methods). The *aphVIII* selectable marker gene conferring paromomycin resistance segregated 2:2 in all tetrads and segregated exclusively with motile cells in the PD (parental ditype) tetrads, indicating tight linkage between the plasmid carrying the p*HYDA1-RSP3* reporter gene and the pSI103 plasmid (Sizova et al. 2001) used for co-transformation to generate strain B6. An F1 progeny of the B6-F strain was back-crossed to the parent strain B6. The 2:2 segregation of motile to immotile progeny indicates that the causative mutation resided in a single genetic locus. For reasons described below, we designated the mutation as *cehc1* for constitutive expression of hydrogenases and copper responsive genes.

The *cehc1* mutation maps to chromosome 1

We mapped the *cehc1-1* mutation using progeny from crosses to a polymorphic reference strain, CC-1952 and SNP markers (Figure 2). The mutation was mapped to a ~1.1 Mb region of chromosome 1 (Figure 2A, double-headed blue arrow; Supplementary Tables 2 and 3; Kathir et

al. 2003). To identify the lesion within the mapped region, genomic DNA samples from strains B6 and B6-F were sequenced and the sequence was compared with the JGI v5.5 reference genome sequence (strain CC-503) (Merchant et al. 2007; Blaby et al. 2014). One SNP unique to B6-F within the 1.1 Mb region was detected, a T to C transition mutation localized to nucleotide 1,029,527 within gene Cre01.g005900 (Figure 2B). Consistent with the genetic linkage detected between the p*HYDA1-RSP3* reporter gene and the mutation leading to its constitutive expression, the site of insertion of the reporter gene construct was found on chromosome 1 at coordinate 5,034,215, or ~4 Mb away (Figure 2A, black arrow).

Gene Cre01.g005900 rescues the motility phenotype of *cehc1-1*

To determine whether the *cehc1-1* mutation is recessive, we generated stable diploid cells heterozygous for both *cehc1-1* and for the p*HYDA1/RSP3* reporter gene. These diploid strains showed the immotile phenotype, indicating that the reporter gene was not expressed and that the mutation is recessive (Supplementary Tables 4 and 5). We then used transformation to determine whether the SNP mutation in Cre01.g005900 is responsible for the motile phenotype of *cehc1-1* cells. A library of wild-type DNA cloned in a lambda phage vector was screened with a PCR-generated DNA fragment from the 3' UTR of gene Cre01.g005900. Clone 17-1, which contained a 15.5 kb wild-type genomic fragment that covered the Cre01.g005900 gene model, the adjacent gene, and two partial gene models (Figure 2B) was transformed into B6-F cells along with the p*Hyg3* plasmid as a selectable marker (Berthold et al. 2002). Phenotypically rescued strains containing both mutant and wild-type copies of the *CEHC1* gene were expected to display the immotile *pf14* phenotype along with decreased expression of the HA-tagged *RSP3* reporter gene. Some transformant colonies had immotile cells (Supplementary Table 6) and some of these strains were tested for expression of the HA-tagged *RSP3* reporter gene using immunoblotting (Figure 2C). The HA-tagged RSP3 protein was expressed in strain B6-F and in a control strain transformed with an unrelated phage DNA (TFNT7). Expression of RSP3 was greatly reduced in three rescue strains (RESQ58, 60, 66) transformed with phage 17-1 DNA containing the intact wild-type Cre01.g005900 gene. Plasmid subclones containing the intact gene or a gene with an HA-tag coding sequence inserted directly following the start codon also resulted in transformants with a paralyzed phenotype (Supplementary Table 6). An immunoblot of proteins obtained from independent rescue strains (B6-F_resc 1, 2, 3) expressing the HA-tagged copy of Cre01.g005900

showed that RSP3 protein expression was reduced compared to strain B6-F, whereas an HA-tagged protein of 55 kDa, the expected size for the protein encoded by Cre01.g005900, was expressed (Figure 2D). Thus, expression of the wild-type Cre01.g005900 gene prevents constitutive expression of the p*HYDA1-RSP3* reporter gene, leading to loss of motility and confirming that Cre01.g005900 is *CEHC1*. We did not detect a difference in the rescue phenotype conferred by the unaltered gene and the HA-tagged gene, suggesting that the HA-tag inserted at the N-terminus does not affect the activity of the protein in this pathway.

The *cehc1-1* lesion results in a splicing defect

Gene model Cre01.g005900 contains 13 introns, the first of which is affected by the T to C transition mutation in B6-F (Figure 2E). The 5' consensus donor splice site of the first intron is changed from GT to GC. To determine whether the mutation affects splicing of the transcript, we used reverse transcription PCR to amplify a fragment spanning the intron (Supplementary Table 7). The resulting cDNA fragment from RNA of strain B6-F (*cehc1-1*) was larger than the fragment from strain B6 (Figure 2F). Sequencing of the cDNA verified that the intron 1 sequence of 86 bp was retained in transcripts from strain B6-F but was missing in transcripts from strain B6. The unspliced transcripts likely do not produce a functional product because an in-frame premature stop codon within the intron follows the SNP mutation. Re-initiation of translation at the first start codon downstream of the mutation (22 nt downstream, within intron 1) would produce a protein in the incorrect reading frame. Data from RNA-seq (see below) revealed that the mutant sequence can function at a low level as a splice donor, perhaps due to inherent flexibility of the splicing machinery (Papasaikas and Valcárcel, 2016). Excision of intron 1 was found in all transcripts in the wild-type strain (Figure 2G). In contrast, correct intron excision occurred for approximately 9% of transcripts in the mutant strain, showing that the *cehc1-1* mutation is a hypomorphic as opposed to a null allele. Compared to the wild type levels, the mutant appears to retain about 20% of correctly spliced transcript. Overall transcript levels (spliced and unspliced) were two-fold higher in the mutant strain for reasons that are unclear, although it could imply the action of a feedback mechanism in which loss of the CEHC1 protein leads to accumulation of its transcript.

The *cehc1-1* mutation affects an FBXO3-like protein

Gene model Cre01.g005900 encodes a predicted protein of 474 amino acids with a molecular mass of 52,195 D and a pI of 6.34. Subcellular localization predictions are ambiguous. The TargetP (Emanuelsson et al. 2000) algorithm did not detect localization signals and predicted "other" localization with high likelihood (0.9977). Predalgo (Tardif et al. 2012) predicts a chloroplast localization, but the score (0.5304) is barely higher than the score for secretory pathway localization (0.4703). Basic Local Alignment Search Tool (BLAST) searches showed that the amino acid sequence is conserved across a wide range of eukaryotes including metazoans, with the highest sequence similarity (35% – 45% identity) in proteins from the clade Viridiplantae including green algae and nonvascular and vascular land plants (Supplementary Figure 1). Analysis of the Chlamydomonas protein along with putative homologs from Arabidopsis and humans using InterPro 85.0 software (Blum et al. 2020) and Alphafold software (Varadi et al. 2022) identified three conserved domains in the same order (Figure 3A, B). From N-terminus to C-terminus, these include an F-box or F-box-like domain, a KNR4/Smi1-like domain, and an ApaG domain. The *Homo sapiens* FBXO3 protein has been shown to be a component of an SCF type (Skp1, Cullin, F-box protein) ubiquitin E3 ligase (Ilyin et al. 2000; Chen et al. 2013). The Arabidopsis SKIP16 (SKP/ASK-interacting protein 16) was identified in a yeast-two hybrid screen to interact with ASK2, a Skp1 homolog (Risseeuw et al. 2003). These comparisons suggest that the Chlamydomonas CEHC1 protein functions in an SCF complex. Identification of conserved domains in the *C. reinhardtii* proteome by InterProScan revealed that only Cre01.g005900 encodes a protein with all three domains.

The *cehc1-1* mutation alters gene expression

In *cehc1-1* mutant cells, the *RSP3* reporter gene is expressed constitutively from its activated *HYDA1* promoter. To determine whether the *cehc1-1* mutation results in changes in the expression of other genes, strains B6 (*CEHC1*), B6-F (*cehc1-1*), and B6-F_resc (*cehc1-1*; *CEHC1*-TG) (trans gene) were grown in triplicate under aerobic conditions and RNA was isolated using conditions to minimize exposure to hypoxia prior to cell lysis (Figure 4). Transcript levels for specific genes were assayed using qPCR (Figure 4A). All three strains showed similar levels of transcripts from the *AMYB1* gene encoding beta amylase, used as a control for detecting the intracellular O_2 availability in sampled cells (Mus et al. 2007). Levels of

HYDA1 transcripts were elevated by ~15-fold in *cehc1-1* cells relative to *CEHC1*. The *cehc1-1* mutation appears to be responsible for upregulated expression of *HYDA1* because rescue with the wild-type gene in strain B6-F_resc restored *HYDA1* transcripts to the low levels observed in *CEHC1* cells.

Because the *HYDA1* promoter is known to be a target for upregulation by the CRR1 transcription factor (Quinn et al. 2002; Pape et al. 2012), we examined expression of known CRR1 target genes *CPX1* and *FDX5* (Kropat et al. 2005; Lambertz et al. 2010; Castruita et al. 2011; Pape et al. 2012; Kropat et al. 2015; Blaby-Haas et al. 2016). An increase in transcripts was observed in *cehc1-1* mutant cells; transcripts returned to low levels in the B6-F_resc strain. We also tested transcript levels for genes (or proteins) previously shown to be upregulated in hypoxia including *HYDA2* and *HYDG1* (Mus et al. 2007; Terashima et al. 2010; Hemschemeier et al. 2013; Blaby-Haas et al. 2016). Although not previously noted as targets of the CRR1 transcription factor, both genes showed increased transcript levels in *cehc1-1* cells as compared to *CEHC1* and these levels were rescued by the wild-type gene.

The endogenous *RSP3* gene in all three strains is the *pf14* allele, an ochre nonsense mutation. It produces a transcript that is similar in size and abundance to that produced by the wild-type gene (Williams et al. 1989). A second *RSP3* gene in all the strains is the wild-type allele driven by the HYDA1 promoter. The qPCR results showed that the *RSP3* transcript level in motile *B6-F (cehc1-1)* cells is elevated ~4-fold compared to that of immotile *CEHC1* and rescue cells (Figure 4A). This relatively small increase in transcript level associated with motility underscores the sensitivity of the *RSP3* reporter gene and the power of the motility assay for genetic discovery of negative regulators. Because the *cehc1-1* mutation leads to constitutively elevated expression of the *HYDA1* gene and other genes known to be targets of the transcription factor CRR1, a key regulator of the copper response pathway in Chlamydomonas, we designated the mutation as *cehc1* for constitutive expression of hydrogenases and copper responsive genes. Because the screen we employed is most useful for identifying negative regulatory genes, the mutants would be expected to show elevated expression of genes downstream of the negative element.

To gain a comprehensive view of the effect of the *cehc1-1* mutation on the transcriptome, the RNA samples were analyzed using RNA-seq and mapped to the JGI v6.1 genome (see Methods;

Supplementary Dataset 1). The transcriptome data were subjected to three filters: genes whose expression differed by at least two-fold between the B6 (*CEHC1*) and B6-F (*cehc1-1*); genes for which the FPKMs were greater than 5.0 in at least one strain; genes whose altered expression in the *cehc1-1* mutant strain were rescued by at least 50% in the B6-F_resc strain. Transcripts from 279 genes upregulated in the *cehc1-1* mutant and 500 genes down-regulated in the mutant (Supplementary Dataset 2) met these criteria. The greatest differences in transcript levels observed between the *CEHC1* and *cehc1-1* strains were in the set of genes upregulated in the mutant; 89 genes were upregulated between 4- and $>1x10^4$-fold in the mutant strain, whereas 37 were down-regulated, between 4- and 42-fold. Controls for the experiment included transcript levels for genes previously shown to have stable expression levels (Shi et al. 2017; Zones et al. 2015) (Supplementary Table 10). For six such genes, the *cehc1-1*/*CEHC1* ratio ranged from 1.5 to 0.7-fold. As noted above for qPCR results, the *AMYB1* transcript levels were consistent with exposure of all the cultures to similar levels of aeration. Also confirming the qPCR results is the relatively small increase in *RSP3* transcripts (~2-fold) required to generate motility in the B6-F strain. Transcript levels for the *CEHC1* gene differed by 2-fold between the mutant and wild-type strains but were higher in the rescue strain as might be expected for the strain carrying an extra copy of the gene.

Overlap with genes responding to dark hypoxia

The *cehc1-1* mutation alters expression of the hypoxia-induced *HYDA1* gene. We took advantage of a previous RNA-seq study by Hemscheimeier et al. (2013a) to identify other genes regulated by hypoxia that show altered expression in the *cehc1-1* mutant. Of the 779 genes regulated by at least 2-fold in aerobically grown *cehc1-1* cells, 260 genes overlapped with a set of genes showing at least 2-fold altered expression in the previous study of wild-type cells (strains CC-5070 and CC-5071, averaged) exposed to dark hypoxia for 6 h (Figure 4B; Supplementary Dataset 3, columns F and V). The overlapping genes included 94 genes upregulated in the *cehc1-1* mutant and 166 down-regulated genes. The results confirm that CEHC1 plays a role in regulating expression of many but not all genes regulated by oxygen levels. The list of transcripts upregulated both by hypoxia and by the *cehc1-1* mutation includes a second hydrogenase gene, *HYDA2,* as well as *HYDG1* and *HYDEF1*, encoding hydrogenase assembly factors (Posewitz et al. 2004). Transcripts from three members of the HCP (hybrid cluster protein) gene family

(HCP1, HCP3 and, HCP4), were upregulated in the *cehc1-1* mutant and returned to wild-type levels in the rescue strain. The HCP enzymatic activity includes conversion of nitric oxide into nitrous oxide and the enzyme may play a role in NO based signaling (Hagen, 2019). Increased expression of *HCP3* and *HCP4* in response to dark hypoxia was noted previously (Mus et al. 2007; Hemschemeier et al. 2013; Olson and Carter, 2016).

The majority (> 90%) of the 166 genes that were downregulated by two-fold or more in the *cehc1-1* mutant were also repressed in dark hypoxia while only 13 genes showed upregulation in these conditions (Supplementary Dataset 3). These results imply that CEHC1 may function as a positive and a negative regulator at the same time, depending on the regulatory pathway.

Overlap with CRR1 target genes

The *RSP3* reporter gene contains within its *HYDA1* promoter a CRR1 binding site or Cu responsive element (CuRE) (Kropat et al. 2005). To assess the possible role of CRR1 in regulating genes in the CEHC1 pathway, transcript levels from the remapped Hemschemeier study (2013) in aerobic conditions vs. 6 h dark anaerobic conditions were compared between two *crr1-2* mutant strains and two strains rescued with a wild-type *CRR1* copy. A Venn diagram in Figure 5 (Supplementary Dataset 4) shows 126 genes upregulated or down-regulated by more than four-fold in the *cehc1-1* mutant strain (CEHC1-regulated genes). A subset of these genes with assigned symbols in the JGI v6.1 genome annotations are indicated in colored boxes. We observed overlap with 28 genes identified as CRR1 targets. Many of these genes have putative CuREs upstream of the genes. The RNA samples in our study were from cells grown in aerated, copper-replete medium, conditions that should suppress CRR1 activity. The finding that genes regulated by CRR1 are similarly regulated by loss of CEHC1 activity is consistent with the hypothesis that CEHC1 acts in the same pathway as CRR1 and that loss of CEHC1 function leads to increased CRR1 activity. The increased activity is likely due to a post-transcriptional mechanism because the levels of *CRR1* transcripts were relatively constant in the *CEHC1*, *cehc1-1*, and rescue strains (Supplementary Table 10). Relevant to this conclusion is the observation of little change in *CRR1* transcript levels in plus or minus copper, indicating that the copper response is regulated at the level of the CRR1 polypeptide (Kropat et al. 2005).

Many genes defined as CRR1 targets are also upregulated in response to dark hypoxia conditions (Quinn et al. 2002; Hemschemeier et al. 2013). We observed a similar requirement for the CRR1 protein for upregulation of the *pHYDA1-RSP3* reporter gene in response to dark hypoxia (Supplementary Figure 4A). To assess the possible role of CRR1 in regulating genes in the CEHC1 pathway, transcript levels from the remapped Hemschemeier study (2013) in aerobic conditions vs. 6 h dark anaerobic conditions were compared between two *crr1-2* mutant strains (CC-5068 and CC-5069) and a two strains rescued with a wild-type *CRR1* copy (CC-5070 and CC-5071). An O_2 responsive CRR1 target was defined as a gene whose expression change in the *crr1-2* mutant was less than 25% of the expression change in the wild-type cells in the presence vs. absence of O_2 (Fig 5; Supplementary Dataset 4, column AA). Eighteen O_2-responsive CRR1 target genes overlapped with Cu-responsive CRR1 targets whose expression is altered by the *cehc1-1* mutation, reaffirming a role for CEHC1 in the CRR1 pathway.

The gene expression results summarized in Venn diagrams in Figures 4 and 5 suggest that CEHC1 plays a role in the CRR1 and hypoxia regulatory pathways. However, expression of many additional genes (in the blue sections of the diagrams) is affected by the *cehc1* mutation. These genes may represent targets of other regulatory pathways in which CEHC1 participates.

Interaction of the *CEHC1* and *CRR1* genes

To examine whether the *CEHC1* and *CRR1* genes function in the same signaling pathway, we determined the role of these genes in expression of the reporter and other genes. Strains were constructed with all four possible combinations of the wild-type and mutant alleles (Supplementary Tables 1, 5). Immunoblots of total cell proteins from aerobically grown cells showed elevated expression of the reporter gene in strains with the *cehc1-1*; *CRR1* combination, the same combination that exists in the B6-F strain (Figure 6A). Expression of *HA-RSP3* is lost in the presence of the *crr1-1* mutation (*cehc1-1*; *crr1-1*), indicating that *crr1-1* is epistatic to *cehc1-1*. A similar epistasis was observed for the expression of endogenous genes upregulated in the *cehc1-1* mutant (Figure 6B). That is, the phenotype of the *cehc1-1* mutant requires the action of the *CRR1* gene product. Progeny from a single tetratype tetrad were grown aerobically in copper-replete medium and RNA was isolated for qPCR analysis of relative transcript levels. Each strain expressed similar levels of *AMYB1* transcripts, indicating similar levels of aeration.

As observed for expression of the RSP3 reporter protein, only the *cehc1-1*; *CRR1* strain showed upregulated expression of the *CRR1* target genes *HYDA1*, *FDX5* and *CPX1*, as well as of the hypoxia regulated genes *HYDA2* and *HYDG1*. The up-regulation of transcript levels of these endogenous genes in *cehc1-1* strains is dependent on *CRR1*.

CRR1 transcripts are equally abundant in both copper-replete (+Cu) and -Cu cells (Kropat et al. 2005). Abundance of the CRR1 protein was monitored using anti-CRR1 antibodies (see Methods; Supplementary Figures 2 and 3; Supplementary Results for Supplementary Figure 3). Immunoblot analysis indicates that the protein is present in wild-type but not *crr1* cells (Figure 6C; Supplementary Figures 4B,C; Supplementary Figure 3E). It accumulates in -Cu cells and is lost within minutes when Cu is added to the culture (Figure 7A; Supplementary Figure 3A). In contrast, it is stabilized in hypoxia conditions (Figure 7D; Supplementary Figure 3F). Consistent with its proposed function, it is found in the nuclear fraction of cells grown in copper-deficient medium (Supplementary Figure 3C). We also conclude that levels of CRR1 protein are regulated at a post-transcriptional level.

Among tetrad progeny with different combinations of *CEHC1* and *CRR1* alleles, an elevated level of CRR1 in copper-replete cells was observed in cells with the *cehc1-1*; *CRR1* allele combination (Figure 6C; Supplementary Figure 4B, C; Supplementary Figure 3E), the same combination found in cells with elevated expression of the reporter gene and CRR1 target genes (Figure 6A, B). Cells with the *CEHC1* allele, including B6 and phenotypically rescued cells, have much lower levels of CRR1. Copper addition to -Cu wild-type cells resulted in CRR1 loss within minutes, whereas the same treatment of *cehc1-1; CRR1* cells resulted in partial CRR1 loss (Figure 7B; Supplementary Figure 3D). The intermediate level of CRR1 protein present in the *cehc1-1* mutant strain after copper addition may reflect a low level of CEHC1 protein remaining in *cehc1-1* cells caused by splicing flexibility (Figure 2G). The result is consistent with a function for CEHC1, a candidate component of an SCF ubiquitin ligase, in the pathway leading to CRR1 destabilization.

The role of proteasomes in Cu-dependent destabilization of CRR1 was tested by pre-treatment of -Cu cells with bortezomib, an inhibitor of the catalytic activity of the proteasome (Fig. 7C; Supplementary Figure 3B). Pre-treatment allowed CRR1 to accumulate after Cu addition,

indicating that regulation by Cu of CRR1 function involves its rapid degradation by the proteasome.

The *cehc1-1* mutation does not affect cell growth

To examine the possible role of CEHC1 in the regulation of cell growth, tetrad progeny with all combinations of the wild-type and mutant alleles at the *CEHC1* and *CRR1* loci were grown in minimal medium in the light. In growth curves obtained for two different sets of tetrads, we observed no consistent differences among the tetrad progeny (Supplementary Figure 5). With the caveat that residual splicing may allow accumulation of a small amount of the CEHC1 protein in *cehc1-1* mutant cells, the results suggest that full expression of the CEHC1 protein is not required for normal cell growth, despite the large number of genes whose expression is up- or down-regulated in *cehc1-1* mutants.

Discussion

Our genetic screen identified a negative regulator of the *HYDA1* expression pathway. Conditional motility dependent on hypoxia was provided by the *HYDA1-RSP3* reporter gene expressed in *pf14* cells. The selection of motile cells from among 10^9 chemically-mutagenized immotile cells revealed mutants with constitutive activity of the *HYDA1* promoter even in aerobic conditions, allowing the cells to produce wild-type RSP3 protein. The *CEHC1* gene is not closely linked to the reporter gene construct (Figure 3), consistent with a defect in a trans-acting factor that negatively regulates the pathway, thereby leading to increased transcription of *HYDA1*. Because the *cehc1-1* mutant allele retains 20% of the wild-type transcript levels (Figure 2G), we cannot determine whether the *CEHC1* gene is essential.

The ability of the *RSP3* gene to induce motility when transformed into cells with cilia paralyzed by the *pf14* mutation provides a versatile tool to study Chlamydomonas gene promoters. A screen for motile cells simply requires that they swim to the top of a liquid culture, meaning that very large numbers of mutagenized cells can be screened for a desired phenotype by inoculating sufficient numbers of culture tubes. The *RSP3* reporter was used previously as a promoter trap (Haring and Beck, 1997) and to report the induction of both strong and weak promoters

including those from the *PSAD1*, *CYC6*, and *CAH1* genes (Ferrante et al. 2011). In a fusion construct with the *NIT1* promoter, it was used to find mutations in regulatory genes controlling ammonia repression of *NIT1* gene expression (Zhang and Lefebvre, 1997).

It is not surprising that a screen for negative regulators would identify a protein that functions in ubiquitylation leading to proteosomal degradation. The possible role of the CEHC1 protein in ubiquitylation is suggested by its homology with mammalian and plant proteins with three conserved domains that place it within the FBXO3 (Fbxo3) class of F-box-like proteins (Fig. 3, Lu et al. 2020; Jin et al. 2004). The Smi1/Knr4 domain (PF09346) takes its name from a *Saccharomyces cerevisiae* protein termed a "hub" protein due to its physical and/or genetic interaction with ~100 other proteins (Martin-Yken et al. 2016). It is part of the SUKH domain superfamily of proteins proposed to serve as scaffolds for binding proteins for post-translational modifications such as ubiquitylation (Zhang et al. 2011). The ApaG or DUF525 (PF04379) domain, first described in the ApaG proteins from gram-negative bacteria, is also present in some eukaryotic proteins where it is thought to mediate protein-protein interactions via a Fibronectin type III fold (Farr et al. 1989; Krzysiak et al. 2016) (Cicero et al. 2007).

The identities and arrangement of predicted structural domains in the CEHC1 protein are similar to those of human FBXO3 (NP-036307.2) (Ilyin et al. 2000; Jin et al. 2004), a protein that functions in the ubiquitin-proteasome system. When bound to a substrate, F-box proteins form a complex with SKP1, an adaptor for the CUL1-RBX1 class of Cullin-RING E3 ubiquitin ligases (Harper and Shulman, 2021). A Cullin protein scaffold together with a RING ("really interesting new gene") domain protein regulates transfer of the ubiquitin from a ubiquitin-carrying enzyme to the target protein (Cardozo and Pagano, 2004; Deshaies and Joazeiro, 2009; Buetow and Huang, 2016). Through a variety of C-terminal domains, different F-box proteins provide specificity for recognition of post-translationally modified target proteins.

Activities of mammalian FBXO3 provide insights into the possible role of the CEHC1 protein. The human protein FBXO3 co-immunoprecipitated with CUL1, SKP1, and RBX1, indicating that it is a component of a Cullin-based E3 ubiquitin ligase (Shima et al. 2008). Murine and human FBXO3 play a role in transcriptional activation of proinflammatory cytokines in the innate immunity system when signals from microbial infection stimulate cell surface receptors

(Mallampalli et al. 2013). Associated factors (TRAFS) transduce signals leading to increased transcription of cytokines. In resting cells, TRAFS are maintained at low levels as a result of their binding to FBXL2, a component of an SCF-type E3 ligase, leading to their polyubiquitylation and proteasomal degradation (Chen et al. 2013). In turn, levels of FBXL2 are regulated by phosphorylation, binding of FBXO3, polyubiquitylation and proteasomal degradation (Chen et al. 2013). In addition to its role in promoting cytokine production, FBXO3 has multiple binding partners and acts as a positive regulator in pathways affecting other transcription factors (reviewed by Zhang et al. 2022).

The CEHC1 protein shows greatest sequence conservation with proteins encoded in numerous genomes in the Viridiplantae lineage such as the *Arabidopsis thaliana* SKIP16 protein (SKP1/ASK-interacting protein 16, At1g06110) (Risseeuw et al. 2003; Supplementary Figure 1). SKIP16 interacts with SKP1-like proteins ASK2 (At5g42190) and ASK 4 (At120140) (Risseeuw et al. 2003) (Arabidopsis Interactive Mapping Consortium, 2011; BioGRID: https://thebiogrid.org) in yeast two-hybrid experiments, suggesting that SKIP16 is likely a component of a RING domain E3 ligase and involved in a protein ubiquitylation pathway. Although SKIP16 transcripts are widely expressed in *A. thaliana* tissues (Klepikova et al. 2016), no function has yet been described for this gene.

If the CEHC1 protein functions similarly to FBXO3 homologs in other systems, it will bind a substrate protein that becomes polyubiquitinated and then degraded by proteasomes, causing its cellular levels to fall. For purposes of discussion, we will assume that CEHC1 acts on CRR1 itself, although it could act on some other proteins required for CRR1 function.
When CEHC1 protein function is lost through mutation, CRR1 would not be subject to degradation and its steady state levels would increase, resulting in increased expression of the CRR1 regulon, including increased transcription of *HYDA1*. In aerobic conditions and in copper-supplemented cells, the CEHC1 protein likely functions to keep the levels of CRR1 low and thus acts as a negative regulator of the pathway. Mutation of *CEHC1* leads to constitutive presence of CRR1, increasing transcription of the p*HYDA1-RSP3* reporter gene, the endogenous *HYDA1* gene, and indicator genes including *CYC6* encoding cytochrome c_6, CPX1 encoding coproporphyrinogen oxidase and *CHL27A* (formerly *CRD1*) encoding a chlorophyll biosynthesis enzyme (Quinn et al., 2000, 2002). Mutations in *CRR1* impaired but did not eliminate *HYDA1*

gene expression, indicating the presence of other pathways for controlling expression of this gene (Pape et al., 2012; Hemschemeier et al., 2013).

Support for a model in which CEHC1 and CRR1 act in the same pathway includes our results from genetic interaction studies, gene expression studies, and analysis of protein levels using immunoblotting. Presence of *CRR1* is required for upregulating expression of the p*HYDA1-RSP3* reporter gene in response to anoxia (Supplementary Figure 4A). It is also required in normoxia for expression of the reporter gene (Figure 6A) and the endogenous genes known to be targets of CRR1 (Figure 6B) but this occurs only in the *cehc1-1* mutant indicating that CEHC1 acts upstream of CRR1. The RNA-seq data showed that known target genes of CRR1 are upregulated (or down-regulated) in the *cehc1-1* mutant and that this expression change is rescued in mutant cells transformed with the wild-type *CEHC1* gene (Figure 5; Supplemental Data Sets 1-4). The levels of *CRR1* transcripts do not change in the *cehc1-1* mutant (Supplementary dataset 1; Supplementary Table 10), a result consistent with the observation that CEHC1 regulates half-life of the CRR1 protein (Figure 7B; Supplementary Figure 3D, E) rather than transcription or half-life of the mRNA. Loss of CEHC1 activity in the *cehc1-1* mutant would be expected to stabilize CRR1, leading to higher levels of *HYDA1* transcripts, as we observed both for the reporter gene driven by the *HYDA1* promoter and for the *HYDA1* endogenous gene (Figures 2C, D; 4A; Supplementary Dataset 1). Using an inhibitor of proteasomes, bortezomib, we showed that CRR1 is subject to proteasomal degradation upon Cu supplementation (Figure 7C; Supplementary Figure 3B), a result consistent with a model in which CEHC1 acts in ubiquitylation leading to proteosomal degradation of CRR1.

Although the genetic interaction between the *CEHC1* and *CRR1* genes is clear, our results do not demonstrate that CEHC1 interacts physically with CRR1. Indeed, we do not know how CRR1 is modified in +Cu or in +O_2 cells in order to be recognized for degradation. Studies in other systems have revealed numerous post-translational modifications leading to recognition of target proteins by E3 ligases (reviewed by Skaar et al. 2013; Lee et al. 2023). The modified protein is presumably the form of CRR1 that would interact with CEHC1. It is possible, and perhaps likely, that the Cu and O2 sensors are different molecules, which feed different signaling inputs. For instance, CRR1 may directly bind Cu, resulting in a conformational change, or a Cu-dependent enzyme (i.e. a Cu sensor) might activate a kinase that phosphorylates CRR1. O_2 may directly

oxidize the protein, acting on cysteinyl thiols, or activate a modification enzyme, either as a substrate or as a regulator. The O2 sensor might result in hydroxylation of CRR1, by analogy to mammalian hypoxic signaling (Ivan et al. 2001; Jaakkola et al. 2001). Either of these two modifications could be recognized by CEHC1 (Skaar et al. 2013). Hydroxylation is one potential modification we have considered for the following reasons: 1) Prolyl hydroxylases are targets of CRR1 (Castruita et al., 2011), 2) Ni or Co, which turn on the CRR1 regulon even in +Cu, $+O_2$ cells (Quinn et al., 2003) and stabilize CRR1 (data not shown) are prolyl hydroxylase inhibitors, and 3) O_2 is a substrate of these enzymes. Combinatorial recognition could drive increased specificity for the ancestral co-occurrence of hypoxia and Cu-deficiency in nature (Crichton and Pierre 2001; Ridge et al. 2008; Decaria et al. 2011). The slightly different mobility of CRR1 in -Cu vs. -O2 (Figure 7D, Supplementary Figure 3F) might be an indication of these different signaling inputs.

We do not know the set of post-translational modifications on CRR1. and whether the priming modification is the same in +Cu and $+O_2$. Targeted proteomics of CRR1 is a priority to address these questions before we can test for a direct interaction between CRR1 and CEHC1. This is now possible in the *cehc1* background because we can accumulate the presumably modified form(s) of CRR1 in either condition. The stabilization of CRR1 in the *cehc1-1* mutant gives us the opportunity to capture modified forms of CRR1 and distinguish signal-specific post-translational modifications.

The human homolog of CEHC1, FBXO3, acts to degrade another F-box protein, FBXL2, which contains leucine-rich repeats (Chen et al 2013). Although the Chlamydomonas genome contains an F-box protein with leucine-rich repeats (Cre01.g047650), it is not known whether it interacts with CEHC1. It is likely, as is the case for most E3 ligases, that CEHC1 has several substrates and participates in multiple signaling pathways, which may be unrelated to Cu or O_2 signaling. Further experiments will be needed to identify such pathways and other substrates of CEHC1. In this context, the RNA-seq results showed that in *cehc1-1* mutants, 96 differentially expressed genes (DEGs) in the *cehc1-1* mutant are not known to be regulated by CRR1 (Figure 5) and could be regulated in other pathways controlled by CEHC1. We did not identify functional enrichment among this set of genes. However, in the v6.1 genome update, only 6445/17,712 (36%) of genes have been assigned a functional description. The 96 DEGs could be interesting

targets for future studies. As was found for the mammalian FBXO3 protein, it is possible that CEHC1 has other targets in addition to transcription factors. To understand the full function of the *CEHC1* gene and whether it is essential, it would be useful to compare the *cehc1-1* hypomorphic allele with a null mutant. Insertional knock-out mutations in this gene were not available for this study are not yet available and we have not been successful in creating a mutant using CRISPR.

Green algae like Chlamydomonas offer an attractive prospect for renewable biohydrogen production. Among the impediments for development of this potential energy source is a lack of knowledge of the molecular mechanisms controlling expression of genes required for hydrogenase production. Selection and characterization of the *cehc1-1* mutation revealed the role of a negative regulator in controlling a suite of genes known to be both important for hydrogenase activity and negatively regulated by oxygen. Selecting new mutants using the motility screen could result in identification of other genes acting in negative control of the hydrogenase pathway. Because of its upregulated expression of numerous genes that support hydrogenase assembly and function, the *cehc1-1* mutant strain also provides a starting point for efforts to engineer an oxygen-tolerant hydrogenase that could be expressed in the *cehc1-1* mutant background.

Methods

Growth Conditions

Cells were maintained on Tris-Acetate-Phosphate (TAP) with Hutner's trace elements or Sager-Granick (M) medium (Harris,1989) agar for long-term storage https://www.chlamycollection.org/methods/media-recipes/. Strains with the *ac17* mutation were maintained on TAP agar or ½ R agar (M medium with a three-fold increase in potassium phosphate and supplemented with 11 mM sodium acetate). Strains with the *arg2* mutation were kept on medium with 0.02% L-Arg in TAP. Cultures on agar medium were maintained at 24°C in light (4800 lux). Liquid cultures were grown in TAP or M media bubbled with filtered air, at 24 C on a 14-hr light/10-hr dark cycle, illuminated with white light (4800 lux) from fluorescent tubes. For the qPCR experiments in Figures 4 and 6 and for the RNAseq experiments analyzed in

Supplemental Data sets 1-4 and shown in Figures 4 and 5, strains were grown in triplicate cultures in TAP medium under a 14h:10h light/dark regimen at 24°C. Light at ~146 $\mu mol.m^{-2}.s^{-1}$ was provided by two 4-ft fluorescent 40W cool white bulbs placed over the cultures and two 4-ft LED 42W 3700 luminous flux lights placed to the side of the cultures. For experiments shown in Figure 7 and Supplementary Figure 4, cells were grown (24 C, 160 rpm) in TAP medium with revised trace elements (Kropat et al., 2011) and continuous illumination at a photon flux density of 60-70 $\mu mol.m^{-2}.s^{-1}$ from 2:1 mixture of cool white (4100K) and warm white (3000K) bulbs.

Genetic Analysis

Gametogenesis, mating, and tetrad analysis was performed at 24° C using standard protocols (Harris, 1989, Chapter 10). In crosses of motile CC-4509 cells with immotile CC-613 cells, the progeny were scored as PD (2 motile:2 immotile; NPD (4 immotile); TT (1 motile:3 immotile). Linkage was calculated using the formula 1/2T + 3NPD/(PD + NPD +T). A ratio of 43:1:33 indicated that *CEHC1* is linked within 25 cM of the site of genomic insertion of the reporter gene construct (Sun et al. 2013).

Molecular mapping PCR

Genomic DNA from tetrad progeny of the B6-F x CC-2290 cross was extracted using Puregene Core Kit (Qiagen 1042601). PCR reactions were carried out as described (Kathir et al. 2003) using primers from the molecular mapping kit (Chlamydomonas Resource Center, University of Minnesota; Kathir et al. 2003; Rymarquis et al. 2005) or primers designed for this project by BLAST comparison of DNA sequences from the reference genome (Phytozome v5.6; Merchant et al. 2007) with those from the polymorphic Chlamydomonas strain CC-2290 (Supplementary Tables 2 and 3).

Generation and Analysis of Diploid Strains

Stable diploids were selected using the *nit1* mutation in the CC-4509 strain and the *ac17* mutation in strain CC-4322. The mating culture was plated on M medium lacking acetate and ammonium (substituting KNO_3 for NH_4NO_3) to select for stable diploid *AC17 NIT1* colonies.

After five days growth under constant light, colonies (potential stable diploid cells) were picked into liquid selective medium for analysis of motility using a stereomicroscope. To determine whether both mating type loci were present in a potential diploid line, DNA was isolated and used as a template for the PCR with primers for mt^+ and mt^-. Loss of heterozygosity for chromosome 1 was tested by using the PCR with primers designed (Supplementary Tables 4 and 5) to amplify DNA fragments containing SNP differences between the genome of the reference strain and the genome sequence of strain CC-4509, generated in this project. The amplified fragments were sequenced to determine the presence of one or both alleles at the SNP site.

Cloning and tagging the *CEHC1* gene

A WT copy of gene Cre01.g005900 was cloned by screening a lambda phage library of WT Chlamydomonas DNA (Schnell and Lefebvre, 1993) with a hybridization probe consisting of a 500 bp fragment from the 3' end amplified by using PCR. Restriction mapping of the resulting clones showed that phage 17-1 contained the complete gene. A HindIII/SpeI fragment extending from Cre01: 1029777 – 1024634 in Phytozome v5.6 genomic DNA sequence was cloned into pBlueScript KS (+) to create plasmid p*CEHC1*-5.1H/S. The plasmid was modified by inserting three copies of a sequence encoding the HA epitope into the SacI site located immediately downstream of the ATG start codon. In addition, a 322 bp HindIII fragment was cloned into the HindIII site to extend the promoter region. The final clone is designated p*CEHC1*-5.5H/S(1)3xHA5'. A selectable marker gene was added by digesting the plasmid with ClaI and KpnI and ligating it with a 1.7 kb fragment containing the aph7" gene from *Streptomyces hygroscopicus* (Berthold et al. 2002). This gene was amplified from plasmid pHyg3 by using a forward primer at the 5' end modified to include a ClaI site and a reverse primer at the 3' end that included the KpnI site. This plasmid was designated p*CEHC1*-5.5H/S(1)3xHA5'+Hyg3. Plasmids were digested with KpnI for use in transformation experiments.

Chlamydomonas Transformation

Glass bead co-transformation with plasmid pSI103 (Sizova et al. 2001) and selection on paromomycin was carried out as described (Sun et al. 2013). For phenotypic rescue of *cehc1-1*

mutants, CC-4509 cells were co-transformed with a lambda phage or plasmid construct containing the Cre01.g005900 gene (p*CEHC1*-5.5H/S(1)3xHA5') together with the pHyg3 plasmid (Berthold et al. 2002). In other experiments, a plasmid containing both the HA-tagged Cre01.g005900 gene and the *aph*7" gene (p*CEHC1*-5.5H/S(1)3xHA5'+Hyg3) was used for transformation. The transformation mixture was plated on TAP medium containing 18 μg/ml Hygromycin B (Roche 10 843 555 001; Mannheim, Germany) using 0.9% agar to screen for colony morphology. Phenotypic rescue of the *cehc1-1* mutation should result in downregulated expression of the p*HYDA1-RSP3* reporter gene and loss of motility. Because colonies of motile cells spread to a larger diameter on soft agar as compared to colonies of immotile cells (Bloodgood, 1981), we picked colonies with a tightly packed morphology into liquid culture and assayed cell motility using phase contrast microscopy.

qPCR Quantification Using Roche Universal Probe Library

Triplicate cultures were grown and RNA was isolated as described in the "RNA-seq analysis" section below. Assays were carried out at the Biomedical Genomic Center (BMGC), University of Minnesota. The assay design utilized the Roche Universal ProbeLibrary (UPL) technology with hydrolysis probes and was based on the JGI v4.0 *C. reinhardtii* genomic DNA sequence (http://genome.jgi.doe.gov/Chlre4/Chlre4.home.html). Each assay design generated a sequence for the forward primer, reverse primer, amplification product (amplicon) and provided the UPL probe number. In most cases, the primer sets spanned an intron (Supplementary Table 8). To generate cDNA for the assays, total RNA samples were treated with DNase using the TURBO DNA-free kit (Applied Biosystems). The RNA was used as a template for first-strand cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen) following the protocol of the manufacturer and using an Applied Biosystems GeneAmp PCR System 9700.

To validate the primer probe set designs, a one-to-five dilution series of the cDNA sample was created in five wells of a 96-well plate with the starting concentration at 5 ng/μl. Four μl of a cocktail containing 2 μl of 10 mM forward primer, 2 μl of 10 mM reverse primer, 1 μl of 10 mM probe (Roche Universal ProbeLibrary), and 36 μl of 2.2X Master mix was dispensed into wells containing 4 μl aliquots of the dilution series or a 4 μl aliquot of distilled water for a negative template control. 3 μl of the dilution series and cocktail mix were dispensed into

duplicate wells on a 384-well ABI optical plate and the plate was loaded on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The FAM-Non Fluorescent Quencher detector and Rox passive reference were selected. The qPCR protocol was: 2 min activation at 60° C, 5 min denaturation at 95° C, followed by 45 cycles of 10 sec at 95° C and 1 min at 60° C. Different iterations of the primer probe set designs were tested with the dilution series. The set design closest to 10% efficient was chosen for the expression run. Any design <90% efficient or >110% efficient was rejected.

For quantitative PCR reactions, 3 µl of cDNA (24 ng) was dispensed into duplicate wells on a 384-well ABI optical plate. A working cocktail contained 10 µM forward primer (IDT), 10 µM reverse primer, 10 µM probe (Roche Universal ProbeLibrary), and 2.2X Master mix. 3 µl of cocktail was dispensed into the wells containing the cDNA samples and the reactions were carried out as described above.

The results of qPCR are presented as Ct (threshold cycle) values. Data were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001). All data were normalized to the transcript levels of RCK1 (Cre06.g278222; Schloss,1990) and calibrated to the transcript levels of a control strain.

Reverse Transcription PCR

Total RNA (1 µg) was treated with DNase I (Amplification grade, Invitrogen) at room temperature for 45 min and the enzyme was inactivated as per instructions of the manufacturer. First-strand cDNA synthesis was carried out with SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's protocol in a total volume of 50 µl. The RT primer (5 pmol, Supplementary Table 7) was used to initiate reverse transcription. A 5 µl aliquot of the reverse transcription product was used as the template for PCR amplification in a total volume of 50 µl, using FailSafe enzyme mix and FailSafe PreMix K from Epicenter Biotechnologies (Madison, WI) along with gene-specific primers (Supplemental Table 7). Amplification was performed following standard cycles: 95° C 3 min, 35 cycles of (95° C 30 sec, 53° C 30 sec, 72° C 30 sec), 72° C 5 min.

Next-generation Whole Genome Sequencing

Genomic DNA samples prepared using the CsCl centrifugation method (Schnell and Lefebvre, 1993) from strains CC-4507 and CC-4509 were used for whole genome next-generation sequencing. The sequencing libraries were prepared by the BMGC at the University of Minnesota, using Nextera Library Prep Kits (Illumina), following the manufacturer's instructions. Illumina cBOT (Illumina) was used for cluster generation, following the manufacturer's instruction. Both DNA samples were sequenced for paired-end, 100-bp read length using the Illumina GAIIX sequencer. Short reads were trimmed for a minimum quality score of 28 at the 3' end. A total of ~20.9 million pass-filter reads (274 bp average) were generated for strain B6 and ~27.6 million (320 bp average) for strain B6-F. The data provided a roughly 24-fold coverage for the 120 Mb nuclear genome (Merchant et al. 2007) of strain B6 and 37-fold coverage for strain B6-F. Reads were aligned to the *C. reinhardtii* v5.6 genome at JGI using BWA (Burrows-Wheeler Aligner) (Li and Durbin, 2009) and Bowtie (Langmead et al. 2009) with default settings. SNPs were identified by comparing uniquely mapped reads generated from Bowtie alignment between the two DNA samples. SNP sites with a coverage of less than 6 reads for either strain were excluded from further analysis. For cases of multiple base calls, the required allele ratio at the position was >85%. After filtering, a total of 49 SNPs were identified between the genomes.

Preparation of CRR1 antibodies

CRR1-A: A portion of CRR1 (439-800 including the SBP domain and the extended SBP domain, Epitope 3, Supplementary Figure 2) was expressed in *Escherichia coli* with an amino-terminal his_6-tag and purified in 50 mM Tris-Cl pH 8, 100 mM NaCl, 10 mM 2-mercaptoethanol, 6 M urea at the UCLA Protein Expression facility. The purified protein was used to immunize rabbits at Covance (now Labcorp), Denver, PA. The purified antigen was also used by Covance to affinity purify antibodies from pooled serum generated from the immunization protocol.
CRR1-B: A mixture of peptides corresponding to residues 1-21 (Epitope 1) and 34-50 (Epitope 2), conjugated to keyhole limpet hemocyanin, were used to immunize rabbits at Labcorp, Denver, PA. Validation of both antibodies is presented in Supplementary Figure 3 and discussed in the Supplementary Results.

Immunoblot Analysis

Method 1: Protein extracts from intact cells were prepared by pelleting $1.8x10^7$ cells, removing the supernatant, adding 180 µl of 1.5x sample buffer (93.75 mM Tris-Cl, pH 6.8, 3% SDS, 15% glycerol, 7.5% 2-mercaptoethanol), and immediately boiling the mixture for 90s. For detection of CRR1, concentrated cell culture was resuspended in M medium containing 1X protease inhibitor cocktail before boiling (Sigma P8340). Before loading the gel, the sample tubes were centrifuged at 14,000g for 2 min to pellet cell debris and gel lanes were loaded with 20 ul of the solution (1.8 x 10^6 cells per lane). Proteins were fractionated using SE250 Mighty Small II (Hoefer Scientific Instruments) on SDS-polyacrylamide minigels (1.5 mM thick, 8% or 9% monomer) and transferred to Immobilon-P membranes (Millipore IPVH00010) using a Bio-Rad Mini Trans-Blot (#1703930) with 0.192 M Tris-Glycine buffer (pH 8.3) in 20% methanol at 102 V for 70 min. HA-tagged proteins were detected using the ECL Western Blotting Analysis System (Amersham #RPN2109) protocol. Blots were incubated for three hours in anti-HA High Affinity antibody from rat IgG (clone 3F10; Roche), used at 1:1200 dilution in TBS Tween 20 (0.1%, pH7.6) containing 2.5% (w/v) dried milk. The secondary antibody was rabbit anti-rat IgG conjugated to peroxidase (Sigma-Aldrich A5795), used at a 1:8000 dilution in TBS-T with 2.5% dried milk buffer for one hour. For detection of CRR1, the primary antibody was CRR1-B at 1:1000 dilution. Secondary antibody was GAR-POD (Jackson Immunoresearch Lab., Inc. 111-035-144) at 1:50,000. After washing, blots were incubated in detection solutions 1 and 2 (ECL kit) and exposed to Blue Ultra Autorad film (GeneMate # F-9029-8X10). As a loading control, we used a rabbit antibody against OEE1 (gift from the Merchant laboratory) at a 1:3000 dilution. The secondary antibody was goat anti-rabbit-peroxidase (Chemicon International AP183P) at a 1:22,000 dilution. Alternatively, we used a mouse monoclonal antibody against actin (clone C4, MP Biomedicals, LLC) at a1:500 dilution. The secondary antibody was goat anti-mouse-peroxidase(Sigma Aldrich) used at a 1:25,000 dilution. Prestained molecular weight standards were from Thermo Scientific (Spectra Multicolor High Range Protein Ladder #26625). Proteins in the gel were silver-stained (Wray et al. 1981). All immunoblot experiments were repeated at least three times; a typical result is shown in the figures.

Method 2: For analysis of total proteins, 15 ml of a culture (density between 4-8 x 10^6 cells/ml) was centrifuged at 1650*g*. Total protein was extracted in one of two ways. 1) The resulting cell pellet was resuspended in 300 µl of a buffer composed of 10mM Na-phosphate (pH 7), and

EDTA-free cOmplete Protease Inhibitor Cocktail (Roche). Samples were flash frozen in liquid nitrogen and stored at -80° C prior to processing. The sample was thawed on wet ice, re-frozen and re-thawed to break the cells open. 2) The resulting cell pellet was resuspended in a solution containing 50 mM TEAB pH8.5 and 5% SDS and heated at 55 C for 15 min to generate cell lysate. Protein concentrations were determined using a BCA Protein Assay Kit against BSA as a standard and diluted with 2x sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.0005% bromphenolblue). Proteins were separated on SDS-containing polyacrylamide gels (7.5 % or 10% monomer) using 10-20 μg of protein for each lane, as indicated in the legend for Supplementary Figure 3. The separated proteins were transferred by semi-dry electro-blotting to nitrocellulose membranes (Amersham Protran 0.1 or 0.45 micron NC as indicated in the legend, Supplemental Figure 3). The membrane was blocked for 30 min with 3-5% dried non-fat milk in phosphate buffered saline (PBS) solution containing 0.1% (w/v) Tween 20 (PBST) and incubated in primary antiserum. The PBS solution was used as the diluent for both primary and secondary antibodies. The membranes were washed in PBS containing 0.1% (w/v) Tween 20. Antibodies were directed against CRR1 (1:1000 dilution), histone H3 (Abcam ab1791; 1:1000 dilution), COXIIb (Agrisesra AS06 151 at 1:4000 dilution) and PsaF (gift from J.D. Rochaix; 1:2000 dilution). The secondary antibody was goat anti-rabbit conjugated to alkaline phosphatase (ThermoFisher, Cat # 31340, 1:5000) and processed according to the manufacturer's instructions.

RNA-seq Analysis

Triplicate cultures were grown in TAP medium. The cells were grown first in 10 ml cultures in 50 ml culture tubes to a density of 5 x10^6 cells/ml. The cultures were transferred to 300 ml contained in 500 ml flasks and aerated with air bubbled through Pasteur pipettes until the cell density reached ~5 x 10^5 cells/ml. Finally, cells were diluted to 2.5 x 10^5 cells/ml in 200 ml of fresh medium in 2800 ml Fernbach flasks (Pyrex 4420). The cultures were aerated by rotary shaking at 95 rpm for two days at which time the cell concentration was ~5 x 10^6 cells/ml. At 6 h into the light cycle, an aliquot of 3 x 10^7 cells was removed from each flask and placed in a 15 ml conical tube. Processing each cell sample took approximately 1.5 min during which time the cells were pelleted by centrifugation for 45 sec, the supernatant was decanted, and the cells were suspended in 3 ml lysis buffer. Total RNA was prepared using the LiCl precipitation method (Wilkerson et al. 1994). The RNA samples were subjected to quality control tests using a Ribo

Green Assay (Invitrogen) for quantification and an Agilent Nano chip (Agilent Technologies) to verify the RNA integrity. All samples surpassed an RNA Integrity Number of 8. A cDNA library was prepared from each RNA sample using the Illumina TruSeq RNA library preparation kit (Illumina) following the manufacturer's instructions. The libraries were gel size selected to have average inserts of ~200 bp.

The pooled libraries were sequenced in one lane on a HiSeq 2500 instrument using v4 chemistry and a 125 bp paired-end run. The lane generated ~220 million reads with all expected barcodes well represented. Average quality scores were above Q30 for all pass-filter reads. Illumina conversion software bcl2fasstq version 2.17.1.14 was used to demultiplex and generate FASTQ files. To trim the sequences, Trimmomatic version 0.33 was used with parameters LEADING:3, TRAILING:3, SLIDING WINDOW:4:16, MINLEN:63 (Bolger et al. 2014). Average size of reads was 126 bp after trimming.

RNA-seq reads from strains B6 (*CEHC1*), B6-F (*cehc1-1*) and B6-F_resc (*CEHC1* rescued) were mapped to the *C. reinhardtii* reference assembly (v6 assembly, v6.1 annotations, available from https://phytozome.jgi.doe.gov) with RNA STAR (v2.7.10b; Dobin et al. 2013) with the following options: --alignIntronMax 3000 –outMultimapperOrder Random –outSAMmultNmax 1. Normalized counts for all nucleus-encoded transcripts were calculated in terms of fragments per kb of transcript per million mapped reads (FPKMs) with cuffdiff (v2.0.2; Trapnell et al. 2013) with the following options: --multi-read-correct –max-bundle-frags 1000000000 –library-type fr-firststrand. Reproducibility within each set of triplicate samples was indicated by the correlation coefficient, with average R values greater than .999 for each set (Supplementary Table 9). Differential expression analysis was performed in R with the DESeq2 package (v1.38.3). Genes were identified as differentially expressed (DEGs) if they met the following four criteria: 1) ≥5 FPKMs in at least one strain, 2) ≥ five-fold change in FPKMs between strains B6 and B6-F, 3) a Benjamini-Hochberg adjusted p-value < 0.05 for the fold change between strains B6 and B6-F, and 4) at least 50% rescue of expression in the B6-F_resc strain (calculated as the difference in FPKMs between B6-F and B6-F_resc divided by the difference between B6-F and B6).

In order to facilitate comparison of the *CEHC1*-regulated transcriptome with that of *CRR1*-regulated transcriptome, RNA-seq data from two previous studies was reanalyzed in parallel with this study using the same reference assembly and annotations. The first study examined the relationship between *CRR1* and Cu-responsive genes (*crr1-2* +/–Cu) (Castruita et al. 2011), and the second study examined the relationship between *CRR1* and dark anoxia-responsive genes (*crr1-2* +/–O_2) (Hemschemeier et al. 2013). Raw sequencing data from the *crr1-2* +/-Cu study (PRJNA134525) and from the *crr1-2* +/-O2 study (PRJNA178958) were downloaded from NCBI SRA as follows: (GSM617187-96 = *CRR1* –Cu (strain CC-4532), GSM617197-206 = *CRR1* +Cu (strain CC-4532), GSM617207-10 = *crr1-2* –Cu (strain CC-5068), GSM617211-12 = *CRR1* rescued –Cu (strain CC-5070), GSM1030930 = *CRR1* rescued light oxic 0h (strains CC-5070 and CC-5071, averaged), GSM1030932 = *CRR1* rescued 6 h dark anoxic (strains CC-5070 and CC-5071, averaged), GSM1030933 = *crr1-2* light oxic (strains CC-5068 and CC-5069, averaged), GSM1030935 = *crr1-2* 6 h dark anoxic (strains CC-5068 and CC-5069, averaged)), and mapped to the *C. reinhardtii* v6 assembly as described above. DEGs were identified using the same criteria as was reported in the previous two studies, respectively. For the Cu study, the inclusion criteria were: 1) ≥10 FPKMs in at least one strain, 2) ≥ two-fold change in FPKMs from the *CRR1* strain between –Cu and +Cu, and 3) ≥ two-fold change in FPKMs between the *crr1-2* mutant strain the *CRR1* rescued strain in –Cu. For the O_2 study, inclusion criteria were: 1) ≥5 FPKMs in at least one strain, 2) ≥ two-fold change in FPKMs in the *CRR1* rescued strains between 6 h dark-anoxic and aerobic conditions, 3) <25% as much fold change in the *crr1-2* mutant strains between dark-anoxic and aerobic conditions relative to the *CRR1* rescued strains, and 4) a Benjamini-Hochberg adjusted *p*-value < 0.05 for the fold change in FPKMs in the *CRR1* rescued strains between 6 h dark-anoxic and aerobic conditions. A Venn diagram summarizing these results was produced with the VennDiagram package (v1.7.3) in R. A *p*-value for the significance of each overlap was calculated by means of the hypergeometric distribution using the stats package (v4.4.0) in R. All RNA-Seq data, including raw sequencing reads and normalized expression estimates, are available at the US National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository under accession number GSE252595

Supplementary Tables, Figures, Methods and Datasets

Supplementary Table 1. Chlamydomonas strains used in this study

Supplementary Table 2. SNP mapping of the *cehc1-1* mutation in strain B6-F

Supplementary Table 3. Primers for chromosome 1 mapping

Supplementary Table 4. Dominance/recessiveness testing of the *cehc1-1* mutation in strain B6-F

Supplementary Table 5. Primers for SNP markers developed in this study

Supplementary Table 6. Transformation rescue of the *cehc1-1* mutation in strain B6-F

Supplementary Table 7. Primers for Reverse Transcription (RT)-PCR

Supplementary Table 8. Primer/probe sets used in qPCR assays

Supplementary Table 9. Correlation Coefficients for replicate samples in RNAseq analysis

Supplementary Table 10. RNA-seq results for selected genes

Supplementary Method 1. Isolation of Chlamydomonas nuclei

Supplementary Figure 1. Conservation of Fbxo3-like proteins in plants

Supplementary Figure 2. CRR1 amino acid sequence, domain structure, and epitopes.

Supplementary Figure 3 and Supplementary Results. Validation of CRR1 antibodies and full size CRR1 immunoblots and controls from Figure 7

Supplementary Figure 4. Interaction of *CEHC1* and *CRR1* genes

Supplementary Figure 5. Effect of *CEHC1*, *CRR1* mutant and wild-type alleles on cell growth

Supplementary Dataset 1. Combined FPKMs for All Genes

Supplementary Dataset 2. All 2FC DEGs in *cehc1-1*

Supplementary Dataset 3. Overlap of *cehc1-1* and O_2 Regulated DEGs

Supplementary Dataset 4. Overlap of *cehc1-1* and *crr1-2* DEGs

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