RNA silencing of hydrogenase(-like) genes and investigation of their physiological roles in the green alga Chlamydomonas reinhardtii

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INTRODUCTION

Proteins containing Fe-S cofactors are ubiquitous in nature. They function predominantly in electron transfer and catalysis [1] and play crucial roles in photosynthesis, respiration and numerous metabolic pathways. In bacteria and selected eukaryotic algae, Fe-S-dependent [FeFe]-hydrogenases catalyse the reversible reduction of protons to molecular hydrogen. The catalytic site is formed by the so-called ‘H-cluster’, consisting of a [4Fe-4S] cluster coupled to a di-iron centre co-ordinated by cyanide and carbon monoxide ligands, and a dithiolate bridge [2]. The green alga Chlamydomonas reinhardtii expresses two hydrogenase genes, HydA1 and HydA2 [3], also annotated as HYD1 and HYD2, however their relative contribution to hydrogen production is not known.

Although Fe-S clusters can self-assemble on proteins in a reaction tube, in vivo this process requires dedicated assembly proteins which are conserved from bacteria to multi-cellular organisms [4]. Photosynthetic eukaryotes have inherited two genetically distinct assembly systems through endosymbiosis. Studies on the model plant Arabidopsis have identified a complete set of six SUF (sulfur mobilization) gene products in the plastids, as well as additional scaffold proteins, whereas a complete ISC (iron-sulfur cluster) pathway is located in the mitochondria (reviewed in [5]). The plant cytosol contains homologues of the CIA (cytosolic iron-sulfur cluster assembly) pathway, as identified in yeast, but with some differences. For example, the CFD1 scaffold protein is absent in the green lineage, and cluster assembly is thought to be mediated by a homodimer of the related NBP35 protein [6,7].

Another protein of the CIA machinery, called Nar1 in yeast, is highly conserved in all eukaryotes, and is likely to have a monophyletic origin [8]. Chlamydomonas HYD3 belongs to this group. The protein bears striking similarity to [FeFe]-hydrogenases, not only in amino acid sequence (49%), but also in domain structure, cysteine ligands and three-dimensional models of the active site [2,9]. The Nar1 proteins from yeast and Arabidopsis can bind two Fe-S clusters [10–12]. However, these hydrogenase-like proteins are assumed not to have hydrogenase activity because they lack certain conserved residues (e.g. C1 in H-cluster motif L1 [8]), and because most eukaryotes do not have the HydEFG genes for assembling the di-iron centre and its ligands, with the exception of algae that do have functional [FeFe]-hydrogenases [13]. Down-regulation of NAR1 expression in yeast leads to a defect in the assembly of clusters on cytosolic and nuclear Fe-S proteins [10], but the precise molecular function of Nar1 remains unknown. Two Nar1 homologues are found in humans, IOP (iron-only hydrogenase-like protein) 1 and IOP2. IOP1 was initially identified as a modulator of HIF (hypoxia-inducible factor)-1α activity [14], and was later shown to be required for cytosolic Fe-S protein biogenesis [15]. Mutants in plant and Caenorhabditis elegans homologues displayed an oxygen-sensitive phenotype [12,16], but whether the activity of cytosolic Fe-S enzymes is impaired in these mutants has not been investigated.

To investigate Fe-S protein biogenesis and its compartmentalization in the green lineage, we have previously catalogued putative Fe-S cluster assembly genes in the recently sequenced genome of the green algae Chlamydomonas reinhardtii [17]. Overall, Fe-S cluster assembly genes are well conserved from plants to algae, but
are usually single copy in *Chlamydomonas*, whereas *Arabidopsis* may have two or three copies. This is an advantage for reverse genetics studies, which can be cumbersome and time-consuming when no viable insertion mutant is available, the situation for many Fe-S cluster assembly genes in *Arabidopsis*.

The discovery of microRNAs and characterization of a microRNA-based silencing mechanism in *Chlamydomonas* has led to the development of tools for effective gene knockdown based on amiRNAs (artificial microRNAs) [18–21]. In the present study we have used this reverse genetics system to investigate the contribution of the three annotated HYD genes to hydrogen production, and to investigate whether HYD*3* has a role in Fe-S protein biogenesis and/or the hypoxia response.

**EXPERIMENTAL**

*Chlamydomonas* strains and culture conditions

The C. reinhardtii strain 325 arg− (cw15 asl− mt−) was obtained from Dr Michael Schroda (Plant Biochemistry, University of Freiburg, Germany). Cells complemented with the empty pChlamiRNA2 vector served as wild-type. Cells were cultured in TAP (Tris/acetate/phosphate) medium [22], supplemented with 100 μg/ml arginine if required, at 25°C, with shaking at 100 rev./min and ∼100 μmol photons m−2 s−1 light intensity. Anaerobic induction was performed by concentrating cultures to 100 μg/ml chlorophyll, and bubbling with nitrogen gas for 2 h. For analysis of hypoxanthine-dependent growth, cells were plated on TAP plates containing 2.35 mM hypoxanthine [23].

amiRNA silencing constructs

amiRNA sequences were designed using the Web MicroRNA Designer (http://wmd3.weigelworld.org/). The following 21-nt amiRNA sequences were used to target *HydA1*, *HydA2* and *HYD3* (CHLREDAFT Protein ID numbers 183963, 24189 and 117241 respectively; corresponding to NCBI Gene ID numbers 5718949, 5720168 and 5718937 respectively); the position in the coding sequence is given in brackets:

- *HydA1* amiRNA2 (606–626) 5′-CAGTGTCCTACCTTT-GGGAATAA-3′;
- *HydA2* amiRNA2 (298–318) 5′-CTGTCGCTATGCAGTATTA-3′;
- *HYD3* amiRNA1 (141–161) 5′-AAAAGGTGTGCTGGAGCTAGA-3′; and
- *HYD3* amiRNA2 (100–120) 5′-CAGGTTGAGCACTCTTGTGCA-3′.

Each amiRNA was synthesized, in forward and reverse orientation, as part of a 90-mer oligonucleotide cassette (Spel–amiRNA−spacers–amiRNA–Spel). Forward and complementary sequences of each 90-mer cassette were annealed and cloned into the SpeI site of the pChlamiRNA2 vector [19]. This places the amiRNA cassette between flanking sequences of the CRE-MIR1157 precursor and under the control of the HSP70A−RBCS2 promoter. Transformation of *Chlamydomonas* and auxotrophic selection were performed as described previously [19].

**qRT-PCR (quantitative real-time PCR)**

Total RNA was extracted using TRIzol (Invitrogen). RNA concentration and quality (A260/A280) was determined using a ND-1000 spectrophotometer (Nanodrop). cDNA was synthesized using RevertAid H Minus M-MuLV reverse transcriptase (Fermentas), according to manufacturer’s instructions, using an oligo(dT) primer, except for *HYD3*, for which a gene-specific primer was used (Supplementary Table S1 at http://www.BiochemJ.org/bj/431/bj4310345add.htm). qRT-PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma–Aldrich), with 5% DMSO added, using the Bio-Rad CFX96 Real-time PCR Detection system, both according to the manufacturers’ instructions. PCR mixes were prepared as master mixes, with template DNA being added to the final reaction volume. Reactions were carried out in triplicate (biological or technical replicates) and RACK1 (receptor of activated protein kinase C1; NCBI gene ID 5723548) amplification was used as a baseline. Changes in gene expression were calculated using the 2−ΔΔCT method [24]. Melting curve analysis was performed on all PCRs to ensure single DNA species were amplified, and the product sizes confirmed by agarose gel electrophoresis. Primer sets were designed to span an intron to distinguish PCR products derived from cDNA (Supplementary Table S1).

**Enzyme activity measurements**

In vitro hydrogenase activity was measured as described in [25]. Rates of respiratory oxygen consumption in the dark and photosynthetic oxygen evolution under saturating light conditions (250 μmol photons m−2 s−1) were measured in an 1.5 ml cell culture volume (25 μg/ml chlorophyll) using an oxygraph oxygen electrode (Hansatech).

AO (aldehyde oxidase) activity was analysed using an in-gel activity assay as described previously for *Arabidopsis* [26], adapted for *Chlamydomonas*. A total of 15 ml of cell culture, in mid-exponential growth, was centrifuged (4000 g for 5 min) and the cell pellet was resuspended in 150 μl of 100 mM KPO4, pH 7.5, 5 mM dithiothreitol and 20 mM EDTA on ice. All further procedures were carried out at 4°C. Samples were sonicated (Soniprep 150) for 3×20 s, with 1 min between sonication steps, and centrifuged at 16000 g for 30 min. The supernatant was recovered, and mixed with one volume of loading buffer (50 mM Tris/HCl, pH 6.8, 100 mM dithiothreitol, 10% (v/v) glycerol and 0.1% Bromophenol Blue), and 20 μl (50 μg of protein) was separated on a standard 12% (w/v) polyacrylamide gel (without SDS) at 150V for 2 h. Gels were equilibrated for 15 min in 100 mM KPO4, pH 7.5, and AO activity was visualized using 1 mM 1-naphthaldehyde and 1 mM indole-3-carboxylic acid as substrates and coupling of the reaction to the formation of purple formazan precipitate.

**Immunolabelling**

Cells from 0.5 ml of mid-exponential-phase culture were harvested by centrifugation (16000 g for 3 min) and resuspended in 90 μl of loading buffer [0.125 M Tris/HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol and 0.1% (w/v) Bromophenol Blue]. Mixtures were boiled for 3 min and debris pelleted at 16000 g for 10 min. Proteins were separated by SDS/PAGE (12% gels), blotted on to nitrocellulose membrane (Protran BA 83; Whatman), and labelled with anti-histone 3 (AbCam), anti-HydA1 (kindly provided by Professor Peter J. Nixon, Imperial College London, U.K.) or anti-PsaA (Agrisera) antibodies.

**Other methods**

Protein was quantified using the Bio-Rad Protein Assay (Bio-Rad); chlorophyll levels were determined in 100% (v/v) acetone extracts [27]; and densitometry was performed using ImageJ software [28].
Distinct in vivo functions of HYD genes in *Chlamydomonas*

Figure 1 Gene-specific silencing of HydA1, HydA2 and HYD3 expression by amiRNAs

(A) Positions of the amiRNA target sites (vertical grey bars) and qRT-PCR primers (arrow heads) in the coding sequences of HydA1, HydA2 and HYD3 (version 4 models of the draft genome). Relative induction of (B) HydA1 and (C) HydA2 transcript levels after anaerobic treatment in wild-type (WT) and amiRNA-transformed lines. Transcript levels before and after 2 h of anaerobic growth were determined by qRT-PCR, and normalized to RACK1 transcript levels, a constitutively expressed gene. Results represent the means ± S.E.M. for three experiments. (D) Relative HYD3 transcript levels in wild-type and amiRNA-transformed lines under standard conditions. Results were normalized to RACK1 expression and represent the means ± S.E.M. for three experiments. (E) Expression levels of the target genes in amiRNA-transformed lines compared with wild-type, after anaerobic induction for HydA1 and HydA2, in standard conditions for HYD3 (calculated from the results shown in (B–D)). miRNA, amiRNA construct.

RESULTS

Specific amiRNA-mediated silencing of hydrogenase(-like) genes in *Chlamydomonas*

amiRNAs were designed, following criteria described in Molnár et al. [19], against unique 21-nt target sequences identified in the 5′ regions of the HydA1, HydA2 and HYD3 coding sequences (Figure 1A). The HydA1 and HydA2 coding sequences are 69% identical, whereas HYD3 shares only 12% and 16% identity with HydA1 and HydA2 respectively. Two independent miRNAs were designed for each target gene in order to determine whether any effects seen were specific. The amiRNAs were cloned under the control of the constitutive promoter *HSP70A–RBCS2* into the *Chlamydomonas* expression vector pChlamiRNA2 [19]. Transformants were selected on TAP plates lacking arginine, and genomic insertion of the amiRNA cassette was confirmed by PCR. More than 100 positive transformants were obtained for each amiRNA construct. Because genomic insertion is random in *Chlamydomonas*, causing positional effects on the expression of transgenes, independent transformants were screened by reverse transcription PCR. Considerable variation in transcript abundance was observed and only four out of 20 transformed lines showed strongly decreased levels of the target transcript. No significant silencing was observed for lines bearing HydA2 amiRNA2, and this amiRNA construct was not used in further investigations.

To confirm the specificity of each amiRNA to its target transcript, the expression levels of HydA1, HydA2 and HYD3 were quantified in wild-type and selected amiRNA lines using qRT-PCR. HydA1 and HydA2 are known to be expressed at very low levels under aerobic conditions, but induced in the absence of oxygen or sulfur [3,29]. The expression of HYD3 has not been investigated, but RNA sequence data indicate that transcripts are of low abundance (S. Merchant, personal communication). We therefore analysed expression of all three genes in cultures grown in air and after 2 h of anaerobic treatment. Under these conditions, HydA1 and HydA2 transcript levels were induced approx. 4-fold in wild-type cells (Figures 1B and 1C), but HYD3 expression did not change (results not shown). In lines transformed with amiRNA1 or amiRNA2 against HydA1, the induction of HydA1 expression was only 1.4- and 2.5-fold respectively (Figure 1B), whereas HydA2 induction was abrogated in cells harbouring HydA2 amiRNA1, but not in cells with HydA1 amiRNAs (Figures 1B and 1C). HYD3 mRNA was specifically down-regulated to 7 ± 12% by HYD3 amiRNA1, and to 23 ± 10% by HYD3 amiRNA2 (Figures 1D and E). These qRT-PCR results show that amiRNA gene silencing in *Chlamydomonas* is effective to down-regulate the expression of inducible and low-abundance genes.

HydA1 represents the major hydrogenase activity under anaerobic conditions

Next, we analysed the effect of silencing each of the hydrogenase (-like) genes on hydrogenase protein levels and activity. Wild-type and amiRNA lines were sampled before and after 2 h of anaerobic induction for immunoblot analysis using antibodies raised against the 16 kDa C-terminal fragment of HydA1. This region of HydA1...
is 71% identical (82% similar) in amino acid sequence to HydA2, and cross-reactivity with HydA2 protein is likely, but remains to be confirmed. However, only HydA1-knockdown lines showed decreased accumulation of hydrogenase protein both in aerobic conditions and after 2 h of anaerobic treatment compared with wild-type samples (Figure 2A). Quantification of the signal by densitometry showed that HydA1-knockdown lines accumulated about one-quarter of the wild-type hydrogenase protein level. In HydA2- and HYD3-knockdown lines, HydA1 protein accumulation was similar to wild-type cells. Coomassie Blue staining confirmed equal protein loading.

Since the lower hydrogenase protein levels in the HydA1 amiRNA line (Figure 2A) could be due to an air leak and associated failure of induction, the expression of Fdx5, an anaerobically induced ferredoxin [30], was assessed by qRT-PCR, a n a n a e r o b i c a l l y i n d u c e d f e r r e d o x i n [30], was assessed by qRT-PCR using RNA extracted from the same samples. Fdx5 expression was induced 12-15-fold in all anaerobic cultures, including the HydA1 amiRNA line (results not shown, and see Figure 6).

To investigate the relative contribution of HydA1 and HydA2 to hydrogenase production in *Chlamydomonas*, the amiRNA lines were treated anaerobically for 2 h, followed by an *in vitro* assay to determine hydrogenase activity. In brief, cell extracts were incubated in a closed vial with Methyl Viologen as an electron donor, and gas samples were removed for analysis by gas chromatography. The HydA1 amiRNA line showed a 76 ± 15% decrease in hydrogenase activity compared with wild-type (Figure 2B), which correlates well with HydA1 protein levels (Figure 2A). Lines containing the amiRNA targeting HydA2 did not show any significant decrease in hydrogenase activity, even though HydA2 transcript levels were decreased by 97 ± 4% under these conditions (Figure 1E). Silencing of *HYD3* expression also did not affect total hydrogenase activity. Together, these results show that HydA1 is the major hydrogenase induced by anaerobic conditions in *Chlamydomonas*.

**HYD3 is required for normal growth**

To further investigate the function of HYD3, we analysed growth of the amiRNA lines. Cultures of wild-type and two independent HYD3-knockdown lines were grown concurrently in triplicate under aerobic conditions, and the chlorophyll content of standard culture volumes was determined as a measure for cell density and therefore growth (Figure 3). To control for chlorophyll differences, per-cell chlorophyll concentrations were calculated for wild-type and HYD3 amiRNA lines and were not observed to differ significantly (Figure 3, inset), nor was there an obvious difference in cell size (results not shown). The HYD3 amiRNA1 line displayed clear growth defects by 4 days post-inoculation compared with wild-type, and reaching saturation after 5 days at approx. 80% of the cell density of wild-type cultures. HYD3 amiRNA2 cell cultures initially grew more slowly, but reached similar levels of cell density to wild-type cells by day 5–7. The difference in growth between the amiRNA1 and amiRNA2 lines corresponded to the different degree of silencing of *HYD3* in these two lines (Figures 1D and 1E).

The growth of HydA1- and HydA2-knockdown lines were also assayed, but the growth curves were identical to wild-type under aerobic conditions (results not shown), as reported for mutants in the hydrogenase assembly genes *HydeFG* [13]. In contrast, the observed growth defect of the HYD3 amiRNA lines fits with the expectation that *HYD3* is an essential gene, like its yeast homologue *NAR1* [10].

**HYD3 is required for the activity of cytosolic Fe-S enzymes, but not organellar Fe-S enzymes**

Next, we investigated whether HYD3, like the yeast homologue *Nar1* and the human IOP1 protein, is required for the activity of cytosolic Fe-S proteins, but not for mitochondrial Fe-S proteins. The mitochondrial respiratory chain contains Fe-S proteins in Complex I, II and III, therefore the dark respiration rate was measured using an oxygen electrode. As expected, HYD3-knockdown lines had similar oxygen consumption rates as wild-type (Figure 4A). In photosynthetic eukaryotes like plants and algae, Fe-S proteins are also abundant in the plastids, for instance in Photosystem I, which has three [4Fe-4S] clusters. Oxygen evolution in the light was assayed to detect any lesions in photosynthetic electron transfer, but no decrease was observed in HYD3 amiRNA lines (Figure 4B). In agreement, the levels of PsaA, a...
core subunit of Photosystem I binding the Fx [4Fe-4S] cluster, were as wild-type (Figure 4C). In contrast, PsA is degraded in Fe-S protein assembly mutants in Arabidopsis chloroplasts [31,32]. Together with our result that knockdown of HYD3 has no effect on the chloroplast-localized hydrogenase activity, these results indicate that HYD3 is not required for the assembly of organellar Fe-S proteins.

Although Fe-S proteins are abundant and well studied in the endosymbiotic organelles, they are generally of low abundance and poorly characterized in the cytosolic/nuclear compartment. Moreover, the cytosolic enzymes Leu1 (an isopropylmalate isomerase) and sulfite reductase, which are commonly used as marker Fe-S enzymes in Saccharomyces cerevisiae, are located in the plastid. Searching the genome of Chlamydomonas, we identified a gene encoding AO (CHLREDRAFT Protein ID 518045), which has been used as a marker for cytosolic/nuclear compartment. A native gel assay using synthetic aldehydes as substrates revealed a single AO activity in Chlamydomonas cell extracts. The intensity of the activity staining was linear with the amount of protein (Supplementary Figure S1 at http://www.BiochemJ.org/bj/431/bj4310345add.htm), indicating that this assay is at least semi-quantitative. In HYD3 amiRNA lines, the AO activity is significantly decreased compared with wild-type lines, and the electrophoretic mobility is poor, indicating that the protein is partly folded (Figure 5A). Densitometric analysis of the staining intensity indicated that AO activity is more than 70% lower in the HYD3-knockdown lines. In contrast, transcript levels of AO were equal to wild-type (Figure 5B) supporting a post-transcriptional defect in AO assembly. The activity of AO is also dependent on FAD and Moco (molybdenum cofactor). However, HYD3/NAR1/IOP have not previously been implicated in the well-studied FAD or Moco biosynthesis pathways. Moreover, hydrogenase-like proteins are conserved in organisms that lack Moco enzymes altogether, suggesting that the lower AO activity in HYD3-knockdown lines is due to a lack of Fe-S clusters.

XDH (xanthine dehydrogenase) is similar in structure and cofactors to AO. The activity of XDH can use hypoxanthine as a nitrogen source [37], which depends on a functional WDHD. Wild-type and HYD3 amiRNA lines were plated on standard TAP, containing ammonium chloride, or on TAP with 2.35 mM hypoxanthine as the only nitrogen source (bottom panel). Cells were plated as drop spots from 5× serial dilutions of algal culture and grown for 13 days. Three independent HYD3 amiRNA2 transformant lines were spotted to assess the variation in growth with respect to the nitrogen source. miRNA, amiRNA construct.
activity of the cytosolic Fe-S-containing enzymes AO and XDH, but not for Fe-S proteins in the chloroplast or mitochondria.

**HYD3 is not involved in the hypoxia response**

In mammalian cells, plants and nematodes, IOPs have been implicated in the hypoxia response and/or oxygen sensing. Knockdown of IOP1 in mammalian cells increased the mRNA and protein levels of HIF-1α, the α-subunit of the hypoxia response regulator, under both normoxic and hypoxic conditions. The expression of both endogenous HIF-1α target genes and a hypoxia-response-element reporter gene were induced [14]. The hypoxia response in *Chlamydomonas* is well characterized, and the levels of more than 500 transcripts are known to increase significantly, including HydA1, HydA2 and the ferredoxin Fdx5 [29]. We therefore investigated the transcript levels of these three genes in *HYD3*-knockdown lines before and after 2 h of anaerobic induction using qRT-PCR. HYD3 amiRNA1 cells were able to induce HydA1 and HydA2 4–5-fold under these conditions; Fdx5 expression was induced 12–15-fold, similar to wild-type cells (Figure 6). The induction of HydA1 protein levels and hydrogenase activity are also not affected in *HYD3*-knockdown lines (see Figure 2). These results suggest that HYD3 in *Chlamydomonas* is not involved in regulating the transcriptional or post-transcriptional response to low oxygen.

**DISCUSSION**

The recently sequenced genome [17] and amiRNA-mediated gene-silencing tools [19,21] have significantly added to the already existing advantages of *Chlamydomonas* as a model system, being a haploid uni-cellular organism. In the present study, we show that amiRNA-mediated gene silencing can be highly specific in the case of HydA1 and HydA2, which are 69% identical in their coding sequence. In theory, degradation of a transcript could lead to multiple 21-nt fragments that, when converted into double-stranded RNA, could amplify the silencing effect and knockdown the expression of highly similar genes. However, no RNA-dependent RNA polymerases have been identified in the *Chlamydomonas* genome therefore it is unlikely that the targeted mRNAs would be involved in amplification and spread of the silencing signal.

Tracing the silencing effect from RNA to protein, we show that the levels of HydA1 transcript (≈36%), protein (≈25%) and enzyme activity (≈25%) correlated well. In our system, the amiRNAs are constitutively expressed under the control of the *HSP70A–RBCS2* promoter. The expression levels of the amiRNA are sufficient to down-regulate both uninduced, as well as induced, transcript levels (Figures 1, 2 and results not shown). In the case of HydA1 mRNA, the induction under anaerobiosis was not completely abrogated by its cognate amiRNA (Figure 1B), which could explain the residual hydrogenase activity (Figure 2B). In contrast, HydA2 transcript levels were not induced at all in the presence of HydA2 amiRNA1. It may be that HydA2 amiRNA1 is more effective (Figure 1E), or that HydA2 expression levels are generally much lower than those of HydA1. The latter possibility is supported by RNA sequence data (S. Merchant, personal communication) and by a quantitative proteomics study in which HydA1 and HydG, but not HydA2, were identified in isolated chloroplasts from cells grown under anaerobic conditions [38].

The *HYD3* gene product is not predicted to be targeted to an organelle (by the Predotar 1.03, TargetP 1.1 and WoLF PSORT programs). It is therefore reasonable to assume that HYD3 is limited to the cytosol, as shown for yeast Nar1 [10]. In addition, similar to Nar1 and its human homologue IOP1, *Chlamydomonas* HYD3 is required for the activity of cytosolic, but not organelar, Fe-S proteins. Using 32P-labelled studies in yeast, Nar1 was shown to function downstream of the cytosolic scaffold proteins Nbp35/Cfd1 [39]. Interestingly, Nar1 was not needed for cluster transfer *in vitro*. Therefore, it is thought that Fe-S clusters are directly transferred from Nbp35/Cfd1 to recipient proteins (including Nar1 which needs clusters for its own function), and that Nar1 plays an essential role in the cluster transfer process.

It is perhaps remarkable that several Nar1 homologues have been identified that are linked to oxygen sensing [12,14,16]. In addition, the growth phenotype of Nar1-depleted yeast cells is dependent on oxygen levels [16]. One possible explanation is that the hydrogenase-like proteins function as oxygen-sensitive regulators of Fe-S cluster transfer in the CIA pathway. An example of a sensing ‘enzyme’ derived from a strictly catalytic isoform is seen in [Ni-Fe]-hydrogenases: the H2-sensing hydrogenase in *Ralstonia eutropha* orchestrates a transcriptional response when hydrogen becomes available as an energy source [40]. However, we found that the transcriptional response of HydA1, HydA2 and Fdx5 in the *HYD3*-knockdown line was normal under (an)aerobic conditions. Moreover, oxygen-sensing systems are described in yeast (Hap1) and mammals (HIF-1α), but are not conserved [41], whereas hydrogenase-like genes are highly conserved.

Another possible explanation is that the oxygen sensitivity of nar1 mutants is merely a consequence of decreased Fe-S cluster assembly. Oxygen is known to damage Fe-S clusters, which then require a higher rate of *de novo* assembly compared with hypoxic conditions. In addition, decreased levels of cytosolic Fe-S proteins are more likely to cause lethality, unlike disrupted oxygen sensing. Further studies on the precise molecular function of the hydrogenase-like proteins, as well as on Fe-S cluster turnover in the presence and absence of oxygen, is needed to understand the evolution of hydrogenase-like proteins and their structural similarities to [FeFe]-hydrogenases.

**AUTHOR CONTRIBUTION**

James Godman designed and carried out all the experiments, Attila Molnár designed and generated RNA-silencing constructs and advised on selecting the silenced lines; David Baulcombe advised on the project and manuscript; Janneke Balk conceived the project, the overall experimental strategy and wrote the manuscript.

**ACKNOWLEDGEMENTS**

We thank Professor Peter J. Nixon for antibodies against HydA1, Camilla Lambertz and Thomas Happe for assistance with *in vitro* hydrogenase activity measurements, and Jean-David Rochaix, Sabeena Merchant and Antonio Pliork for general advice.
FUNDING
This work was supported by Downing College, Cambridge [E.E. Fritsch Fellowship (to J.E.G.)], the Royal Society [University Research Fellowship (to J.B.)] and Research Professorship (to D.C.B.); the Gatsby Charitable Foundation; and the European Union Sixth Framework Programme Integrated Project SIROCCO [grant number LSHG-CT-2006–037900].

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Received 28 June 2010/8 August 2010; accepted 28 August 2010
Published as BJ Immediate Publication 20 August 2010, doi:10.1042/BJ20100932

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SUPPLEMENTARY ONLINE DATA

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**Table S1** PCR primers used in the present study

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<td>RACK1 reverse</td>
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**Figure S1** Substrate-dependent formazan precipitation as a function of the amount of protein extract

The protein concentration in total cell extract (see the Experimental section in the main paper) was quantified using Bio-Rad Protein Assay reagent. Increasing amounts of protein per well were separated under native conditions. Gels were incubated with the synthetic AO substrates 1-naphthaldehyde and indole-3-carboxyaldehyde (1 mM each), in the presence of phenazine methosulfate and methylthiazolyl-diphenyl tetrazolium bromide (MTT), generating a purple formazan precipitate. The amount of formazan was quantified using ImageJ software, showing a linear correlation with the amount of protein extract.

Received 28 June 2010/9 August 2010; accepted 20 August 2010
Published as BJ Immediate Publication 20 August 2010, doi:10.1042/BJ20100932

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