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The iron–sulphur protein Ind1 is required for effective complex I assembly

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NADH:ubiquinone oxidoreductase (complex I) of the mitochondrial inner membrane is a multi-subunit protein complex containing eight iron–sulphur (Fe–S) clusters. Little is known about the assembly of complex I and its Fe–S clusters. Here, we report the identification of a mitochondrial protein with a nucleotide-binding domain, named Ind1, that is required specifically for the effective assembly of complex I. Deletion of the *IND1* open reading frame in the yeast *Yarrowia lipolytica* carrying an internal alternative NADH dehydrogenase resulted in slower growth and strongly decreased complex I activity, whereas the activities of other mitochondrial Fe–S enzymes, including aconitase and succinate dehydrogenase, were not affected. Two-dimensional gel electrophoresis, *in vitro* activity tests and electron paramagnetic resonance signals of Fe–S clusters showed that only a minor fraction (~20%) of complex I was assembled in the *ind1* deletion mutant. Using *in vivo* and *in vitro* approaches, we found that Ind1 can bind a [4Fe–4S] cluster that was readily transferred to an acceptor Fe–S protein. Our data suggest that Ind1 facilitates the assembly of Fe–S cofactors and subunits of complex I.

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Introduction

Iron–sulphur (Fe–S) clusters are ubiquitous and versatile cofactors of proteins mediating electron transfer, enzymatic catalysis and regulation of gene expression (Beinert, 2000). The most commonly found clusters are the rhombic [2Fe–2S] and the cubane [4Fe–4S] clusters. Despite the simple structure of Fe–S cofactors and spontaneous assembly of Fe–S proteins by chemical means, in living cells Fe–S protein maturation is an enzymatic process. Many of the genes involved have been identified over the past decade, and are markedly conserved from bacteria to higher eukaryotes (for reviews, see Balk and Lobréaux, 2005; Johnson *et al*, 2005; Lill and Mühlenhoff, 2008). At least three Fe–S cluster assembly systems can be distinguished. The NIF system is specialized for the maturation of nitrogenase in nitrogen-fixing bacteria. A second system called ISC (for iron–sulphur cluster assembly) is present in most bacteria, and is conserved in mitochondria. A third system termed SUF mediates Fe–S cluster assembly under iron-limiting and oxidative stress conditions. Each system, encoded by operons or gene clusters in bacteria but dispersed genes in eukaryotic genomes, consists of a cysteine desulphurase for the generation of sulphane sulphur (Nfs1 in Baker's yeast, *Saccharomyces cerevisiae*); a scaffold protein to which the sulphur is transferred and assembled with iron (Isu1 and Isu2 in *S. cerevisiae* mitochondria); and auxiliary proteins, the functions of which are less well understood, including frataxin that is thought to facilitate iron delivery.

The mitochondrial ISC machinery is also required for the assembly of Fe–S proteins in the cytosol and nucleus (Kispal *et al*, 1999; Lill and Mühlenhoff, 2008). An as yet unknown compound is exported through the ATP-binding cassette transporter of the mitochondria, Atm1, to assist iron–sulphur protein assembly in the cytosol and nucleus. The first known cytosolic Fe–S cluster assembly protein was the essential P-loop NTPase Cfd1, which was identified in a mutant screen in Baker's yeast (Roy *et al*, 2003). Two highly conserved cysteine residues in a CxxC motif (Cys201 and Cys204) were found to be important for its function. Cfd1 forms a stable complex with the related Nbp35 protein (Hausmann *et al*, 2005), and together they bind up to three [4Fe–4S] clusters. Rapid transfer of the labile Fe–S clusters to target Fe–S proteins *in vitro* and the requirement of Cfd1–Nbp35 for Fe–S cluster assembly *in vivo* demonstrated that the Cfd1–Nbp35 complex can function as a novel Fe–S scaffold in the yeast cytosol (Netz *et al*, 2007).

Cfd1 and Nbp35 are classified as P-loop GTPases based on a unique set of sequence and structural signatures (Leipe *et al*, 2002). The small subfamily of Mrp/NBP35-like proteins can be further divided into five groups based on sequence similarities and conserved domains. Nbp35 has a highly conserved N terminus that binds an Fe–S cluster (Hausmann *et al*, 2005), whereas Cfd1 lacks this domain (Figure 1A and Supplementary Figure S1). A third group of Mrp/NBP35-like proteins was identified in the model plant *Arabidopsis* in screens for photosynthesis mutants displaying high chlorophyll fluorescence. *hcf101* mutant alleles failed to accumulate photosystem I, which has three [4Fe–4S] clusters in its reaction centre (Lezhneva *et al*, 2004; Stöckel and

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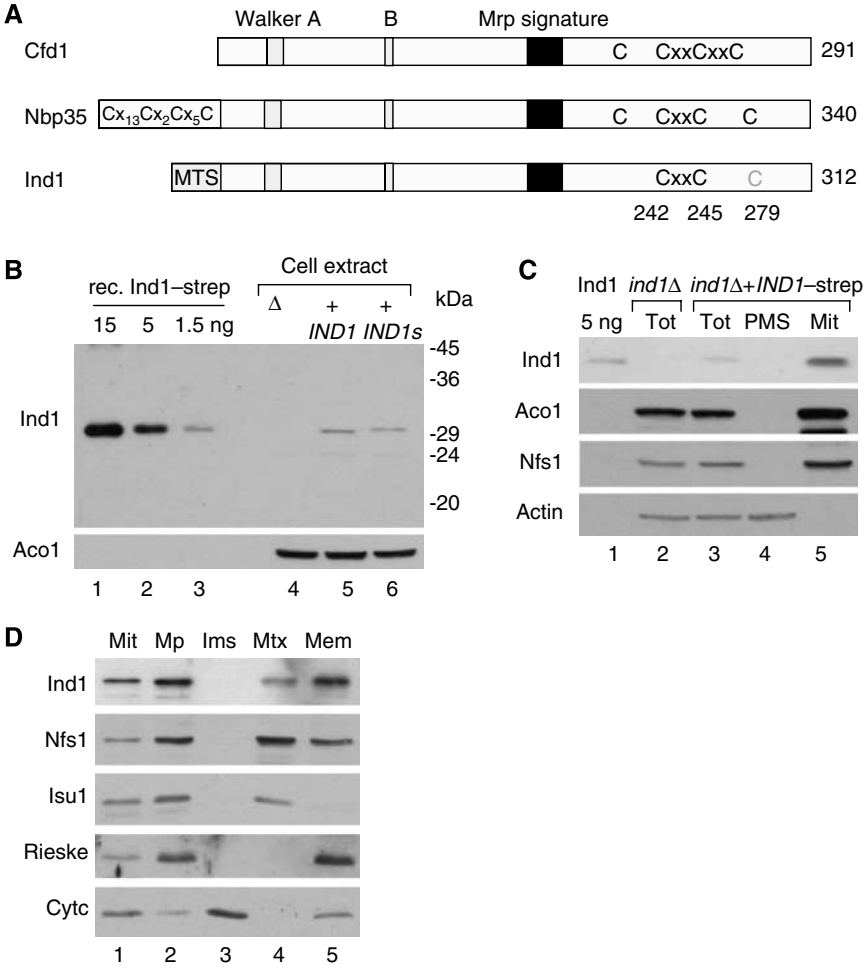

Figure 1 Ind1 is targeted to mitochondria. (**A**) Cartoon of Mrp/NBP35-like proteins found in *Yarrowia lipolytica*. Cfd1, YALI0E19074g; Nbp35, YALI0E02354g; Ind1, YALI0B18590g. The proteins are 40% similar in amino-acid sequence but differ in their N termini. MTS, mitochondrial targeting sequence. Conserved cysteine motifs are indicated in black. Cys279 in Ind1 is drawn in grey, as it is not conserved in the mitochondrial group. (**B**) Polyclonal antibodies raised against recombinant Ind1–strep recognized a 30–31 kDa protein in cell extracts (20 μg protein per lane) of *Y. lipolytica* expressing full-length *IND1* or *IND1*–strep (*IND1s*) under the control of its own promoter from plasmid pUB4. (**C**) Immunoblot showing the mitochondrial localization of Ind1. *Y. lipolytica* cells expressing *IND1*–strep were treated with zymolyase, broken in a Dounce homogenizer (Tot = total lysate) and fractionated in mitochondria (Mit) and post-mitochondrial supernatant (PMS). Protein (20 μg) was separated by SDS–PAGE, blotted and labelled with antibodies against Ind1, the mitochondrial proteins aconitase (Aco1) or cysteine desulphurase (Nfs1) or cytosolic actin. (**D**) Immunodetection of Ind1 in the mitochondrial matrix, associated with membranes. Mitochondria (50 μg protein) of *Y. lipolytica* expressing *IND1*–strep were incubated in hypotonic buffer to swell the organelles and break the outer membrane, followed by 10 min incubation and centrifugation in the presence of 150 mM KCl to separate soluble and membrane-bound proteins of the intermembrane space (Ims) from mitoplasts (Mp). The pellet (Mp) was resuspended in hypotonic buffer and subjected to three rounds of freeze–thawing, followed by centrifugation to separate soluble matrix proteins (Mtx) and membranes (Mem). The volume of each mitochondrial fraction was adjusted to 50 μl in 1 × gel loading buffer, and 20 μl of each fraction was analysed by SDS–PAGE and immunoblotting to visualize Ind1, Nfs1, the Fe–S scaffold protein Isu1 (soluble matrix protein), Rieske Fe–S protein (integral membrane protein of complex III) and cytochrome *c* (Cytc; protein of the intermembrane space). Note that the separation of cytochrome *c* from the mitoplasts was incomplete in this experiment. Similar results were obtained for cells expressing Ind1 without Strep tag (not shown).

Oelmüller, 2004). The mutants also had decreased levels of the [4Fe–4S] protein ferredoxin–thioredoxin reductase, suggesting a specific role of HCF101 in the assembly of Fe–S proteins (Lezhneva *et al*, 2004). The HCF101 group has a conserved N-terminal domain of unknown function, designated DUF59. The fourth group of Mrp/NBP35-like proteins has an N-terminal sequence that is predicted to target the protein to mitochondria. Although this protein is not found in Baker's yeast, it is present in most eukaryotes, including plants, mammals and fungi. The fifth group of Mrp/NBP35-like sequences is found in bacteria. Whereas the *Escherichia coli* Mrp (MetG-related protein) lends its name to the subfamily, only the ApbC orthologue in *Salmonella enterica* has been studied experimentally. Initially identified as a step in the alternative pyrimidine biosynthetic pathway (Petersen and Downs, 1996), the ApbC protein was later implicated in Fe–S cluster assembly. Deletion of *apbC* affected aconitase and succinate dehydrogenase activities by 30–35%, and this decrease was additive with mutations in the alternative Fe–S scaffold protein *iscA*, suggesting that the gene products function in the same biochemical pathway (Skovran and Downs, 2003).

Although *S. cerevisiae* has proved to be an excellent model organism for unravelling the biochemical pathways

of Fe–S protein maturation in the mitochondria and cytosol, it is lacking respiratory complex I (proton pumping NADH:ubiquinone oxidoreductase), a multi-subunit Fe–S-containing protein complex. Complex I contains 8 Fe–S clusters in mitochondria and 8–9 Fe–S clusters in bacteria. From X-ray crystallographic studies of the peripheral arm of complex I of the bacterium *Thermus thermophilus*, it was deduced that seven clusters form an 'electrical wire' transferring electrons from the substrate NADH, through FMN, to ubiquinone (Hinchliffe and Sazanov, 2005). Little is known about the assembly of complex I or the insertion of its Fe–S clusters. Only five assembly factors of complex I have been identified to date, of which three, CIA30, CIA84 and B12.7L, are conserved from fungi to man (Küffner *et al*, 1998; Ogilvie *et al*, 2005; Vogel *et al*, 2005, 2007; Dunning *et al*, 2007; Saada *et al*, 2008). These proteins have been classified as molecular chaperones, as they assist in the protein folding and/or assembly of intermediates but are not part of the fully assembled complex. Furthermore, it was shown that a functional sulphurtransferase associated with complex I in the yeast *Yarrowia lipolytica* was not required for Fe–S cluster assembly on complex I (Abdrakhmanova *et al*, 2006). It is therefore likely that the cysteine desulphurase Nfs1 and other ISC assembly components are involved in Fe–S insertion into complex I. In support of this, two recent reports described patients with strongly decreased levels of the mitochondrial scaffold protein ISCU who displayed a general deficiency in mitochondrial Fe–S proteins. The biochemical symptoms included a decrease in the activity and subunit abundance of complex I, although this effect was minor compared with the strongly affected succinate dehydrogenase and aconitase enzymes (Mochel *et al*, 2008; Olsson *et al*, 2008 and references therein). Also, frataxin may be involved in providing iron to complex I, as deletion of the bacterial frataxin homologue *cyaY* resulted in 30% less of the protein complex (Pohl *et al*, 2007).

The presence of a putative, mitochondrial Mrp/NBP35-like protein in eukaryotes with the exception of Baker's yeast led us to investigate the function of this protein in the yeast *Y. lipolytica*, with particular attention to complex I. Here, we describe a deletion mutant of the gene called *IND1* that lacked approximately 80% of complex I activity due to a similar decrease in the abundance of complex I. Survival of the mutant was dependent on expression of an alternative NADH dehydrogenase transgene. Moreover, aconitase and succinate dehydrogenase activities were not affected, indicating that Ind1 is specifically involved in the assembly of complex I. Ind1 has a CxxC motif that is essential for its function. Similar to the cytosolic scaffold proteins Cfd1–Nbp35, Ind1 bound an Fe–S cluster that could be transferred to an Fe–S apo-protein, suggesting that Ind1 performs a specific role in assembling Fe–S clusters in complex I.

Results

Y. lipolytica expresses an Mrp/NBP35-like protein targeted to mitochondria

The genome of the obligate respiratory yeast *Y. lipolytica* encodes three P-loop NTPases classified as Mrp/NBP35-like proteins (Leipe *et al*, 2002). Two of these, YALI0E02354 and YALI0E19074, bear high sequence similarity and domain organization to the *S. cerevisiae* proteins Nbp35 and Cfd1, respectively (Supplementary Figure S2). The third P-loop NTPase, YALI0B18590, carries the Mrp family signature and the conserved cysteine motif (CxxC) found in Nbp35 and Cfd1 (Figure 1A and Supplementary Figure S2). In addition, YALI0B18590, which we have named *IND1* for iron–sulphur protein required for NADH-dehydrogenase, encodes a protein with an N terminus comprising a putative mitochondrial targeting signal. To investigate expression of *IND1*, antibodies were raised against the recombinant Ind1 protein and applied to immunoblots of total extracts of *Y. lipolytica* cells expressing full-length *IND1*, with or without a C-terminal Strep tag, under the control of its own promoter. A protein of 30–31 kDa was detected in cells expressing *IND1*(–strep) (Figure 1B, lanes 5 and 6), which is smaller than expected from the full-length protein (34.365 kDa including Strep tag). Affinity purification of Ind1–strep from *Y. lipolytica* cell extract and N-terminal sequencing gave the sequence SxE(N), corresponding with cleavage after Arg34, thus giving a calculated molecular mass of 30.550 kDa including Strep tag. These results are consistent with the cleavage site predicted by TargetP (Emanuelsson *et al*, 2000), and with the observed molecular mass.

To determine the subcellular localization of Ind1, cells were fractionated in mitochondria and post-mitochondrial supernatant. Equal amounts of protein were separated by SDS–PAGE and subjected to immunoblotting. Ind1 was detected in the mitochondrial fraction but not in the post-mitochondrial supernatant of cells expressing Ind1–strep (Figure 1C, lanes 3–5). The purity of the mitochondrial fraction was confirmed by probing the same nitrocellulose membrane for the mitochondrial proteins aconitase (Aco1) and cysteine desulphurase (Nfs1), and for cytosolic actin. To establish in which mitochondrial subcompartment Ind1 is localized, mitochondria were further fractionated into mitoplasts, intermembrane space, matrix and membranes. Comparison with marker proteins showed that Ind1 is located in the matrix and is mostly membrane bound (Figure 1D).

Taken together, these data show that the predicted N-terminal targeting sequence of Ind1 is cleaved and that the mature protein is localized to the mitochondrial matrix with a large fraction bound to the inner membrane.

IND1 is implicated with complex I

What is the function of Ind1? Because of sequence similarity to Nbp35, Cfd1 and the bacterial Mrp/ApbC, Ind1 may have a role in Fe–S protein assembly. Moreover, the absence of Ind1 in *S. cerevisiae* suggested a specific mitochondrial target Fe–S protein that is not present in *S. cerevisiae*: complex I (NADH:ubiquinone oxidoreductase). We therefore examined the presence of *IND1* and complex I genes among 77 published eukaryotic genomes, using PSI-Blast searches to find homologues and phylogenetic analyses to determine orthology relations (Gabaldón *et al*, 2005). There is a strong genomic link between *IND1* and complex I genes, as they occur together in 53 genomes, and are both absent from 18 genomes. Furthermore, assuming a standard eukaryotic phylogeny, complex I genes and *IND1* have clearly co-evolved, as they have been lost concertedly five times in evolution, whereas in *Schizosaccharomyces pombe IND1* has been lost with a subset of complex I genes (Figure 2A). This indicates a functional link between *IND1* and complex I. Nevertheless,

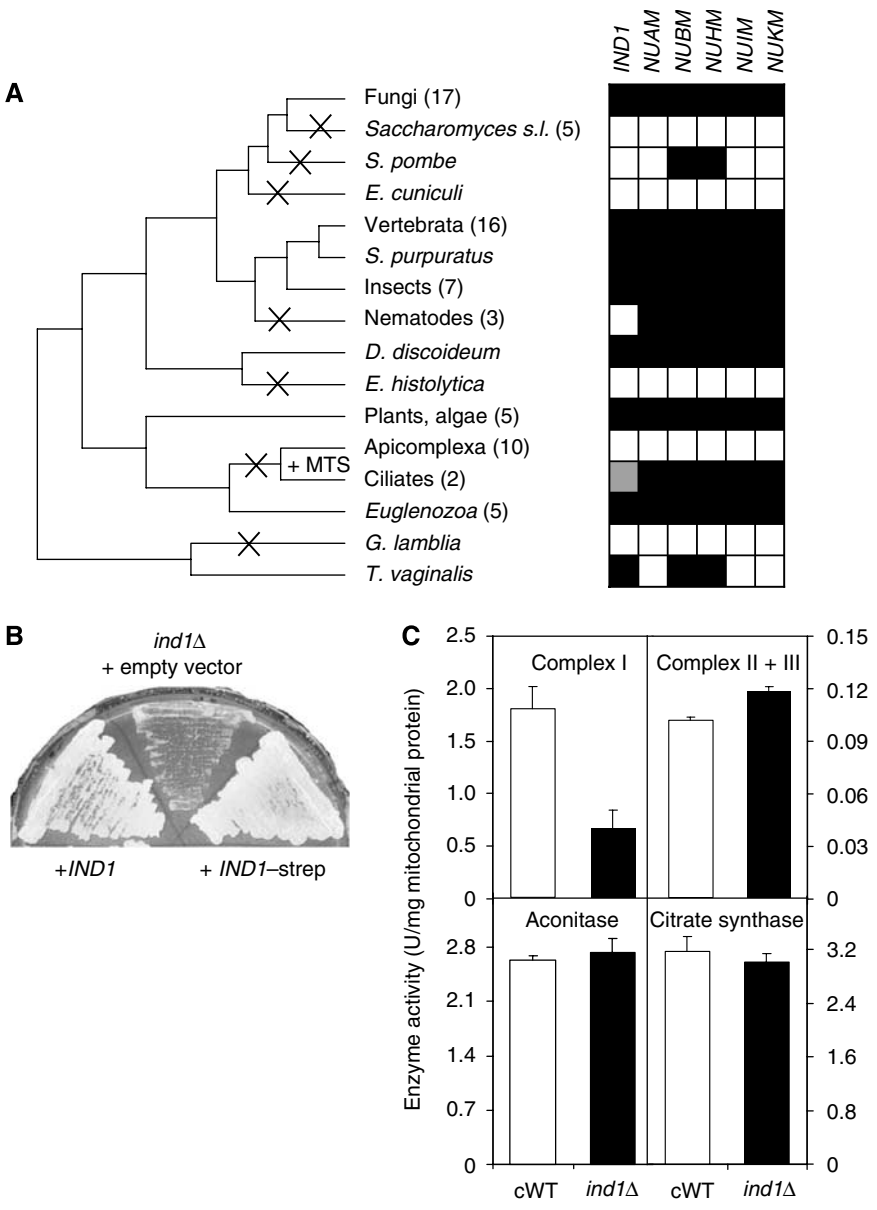

Figure 2 *IND1* is phylogenetically and functionally linked to complex I. (**A**) Coevolution of *IND1* and genes for Fe–S protein subunits of complex I. A cross indicates the loss of *IND1* in that lineage, and + MTS indicates the gain of a mitochondrial targeting signal in an *IND1* paralogue. White and black squares represent the absence or presence, respectively, of the gene indicated above each column. Grey indicates the presence of an *IND1* paralogue with a mitochondrial targeting signal. Abbreviated species names: *Saccharomyces* s.l. *Saccharomyces* sensu lato; *S.* (*Schizosaccharomyces*) *pombe*; *E.* (*Encephalitozoon*) *cuniculi*; *S.* (*Strongylocentrotus*) *purpuratus*; *D.* (*Dictyostelium*) *discoideum*; *E.* (*Entamoeba*) *histolytica; G.* (*Giardia*) *lamblia; T.* (*Trichomonas*) *vaginalis*. (**B**) Growth of *Y. lipolytica* strain *ind1*Δ in which the *IND1* gene was deleted (centre, transformed with an empty plasmid) and the *ind1*Δ strain expressing the wild-type *IND1* gene (left), or *IND1* fused to the Strep tag coding sequence (right), expressed under the control of the *IND1* promoter from the pUB4 plasmid. (**C**) Activities of Fe–S enzymes in purified mitochondria from *ind1*Δ cells (black bars) or the *IND1*-complemented wild type (cWT, white bars). Complementation with *IND1* or *IND1*–strep gave similar results (not shown). Complex I was measured as NADH:HAR oxidoreductase activity in alamethicin-permeabilized mitochondria. Complex II plus III activity was measured following the electron transfer from succinate to cytochrome *c* in intact mitochondria. Aconitase activity was assayed following cis-aconitate consumption. Citrate synthase activity served as a non-Fe–S enzyme control. Error bars represent the standard deviation, $n = 3$.

there are a few exceptions to the pattern: two ciliates appear to have replaced *IND1* with an *IND1* paralogue that has gained a mitochondrial targeting signal (Figure 2A and Supplementary Figures S1 and S4). Furthermore, the nematodes have a complete set of complex I genes without *IND1*, and *Trichomonas vaginalis* has an *IND1* with the same subset of complex I genes as *S. pombe*. The reverse, a genome that has *IND1* without having any complex I genes was not found, consistent with the hypothesis that Ind1 is required for the formation of complex I.

We then investigated whether Ind1 is functionally required for complex I activity. A deletion mutant was obtained in *Y. lipolytica* by replacing the entire coding sequence of *IND1* with a *URA3* marker using homologous recombination (Supplementary Figure S3). The haploid strain used contains the *Y. lipolytica NDH2* transgene fused to a mitochondrial

targeting sequence (*NDH2i*), resulting in the expression of a version of the alternative NADH dehydrogenase on the matrix side of the inner mitochondrial membrane, bypassing the absolute requirement for respiratory complex I (Kerscher *et al*, 2001). The absence of *IND1* and its expression in the deletion mutant was confirmed by PCR (Supplementary Figure S3) and immunoblotting (Figure 1B). The resulting *ind1*Δ (*NDH2i*) strain (in the following abbreviated as *ind1*Δ) was viable, but grew slowly. Growth of the *ind1*Δ strain was restored following complementation with a plasmid expressing the full-length *IND1* gene, with or without Strep tag, under the control of its own promoter (Figure 2B). Backcrossing the *ind1*Δ strain with a strain lacking *NDH2i* resulted in spores that had either *NDH2i*, or *IND1*, or both genes (Supplementary Figure S5). These data indicated that *IND1* is essential for viability and is required for NADH oxidation in the mitochondrial matrix.

Next, we assayed the activities of several key Fe–S enzymes in mitochondria purified from *ind1*Δ cells. No significant differences were found between *ind1*Δ and the complemented wild-type strain (cWT) for the enzyme activities of aconitase, succinate dehydrogenase (complex II) and cytochrome bc_1 complex (complex III), or the non-Fe–S enzyme citrate synthase (Figure 2C). In contrast, electron transfer from NADH to the artificial electron acceptor hexaammineruthenium(III) chloride (HAR), known to accept electrons from the 51-kDa NUBM subunit of complex I, was significantly decreased in the *ind1*Δ mutant. These results indicate that Ind1 is required for normal function specifically of complex I, as suggested by the co-evolution of Ind1 and complex I genes in eukaryotes.

Cys242 and Cys245 are essential for the function of Ind1

To analyse NADH oxidation by complex I in more detail, we isolated unsealed mitochondrial membranes and measured the electron transfer rate from NADH to HAR, as well as electron transfer from deamino-NADH (dNADH) to *n*-decylubiquinone (DBQ) that is sensitive to the complex-I-specific inhibitor 2-decyl-4-quinazolinyl amine (DQA). Whereas the NADH:HAR activity involves only the primary electron acceptor FMN bound to the 51-kDa subunit, the DQA-sensitive dNADH:DBQ oxidoreductase activity involves all cofactors of the peripheral arm, therefore reflecting the physiological NADH:ubiquinone oxidoreductase activity of complex I. Comparison of the two activities has been useful in the past to identify impaired electron transport due to mutations in the ubiquinone-binding pocket (Tocilescu *et al*, 2007). We found that both the NADH:HAR and the DQA-sensitive dNADH:DBQ activity of complex I were decreased by approximately 70% in mitochondrial membranes isolated from cells lacking Ind1 (Table I).

We then investigated the importance of the CxxC motif in Ind1 (Figure 1A) using site-directed mutagenesis of Cys242 and Cys245 to alanine, glutamate or serine. All single amino-acid changes resulted in a decrease in NADH:HAR and DQA-dependent dNADH:DBQ activity (Table I). The magnitude of the decrease was similar to that observed in the deletion mutant, demonstrating that the CxxC motif is essential for the function of Ind1. In contrast, mutations of Cys279, which is not evolutionary conserved (Supplementary Figure S4), to Ala or Ser had no effect on complex I activity. Changing Cys279 to Glu did abolish most of complex I activity, possibly because this change affected protein folding. Immunoblot analysis showed that all mutant Ind1 proteins were stably expressed (Figure 3A, lower panels).

We concluded from these enzyme assays that the function of Ind1 is required for full activity of complex I and that this function strictly depends on the cysteine residues of the conserved CxxC motif.

ind1 mutants have minor amounts of fully assembled complex I

The parallel decrease in NADH:HAR and dNADH:DBQ activities in the *ind1*Δ strain is indicative of a decrease in the amount, rather than a dysfunction, of complex I. To investigate this further, we analysed the amount and integrity of the 40-subunit protein complex using blue-native polyacrylamide gel electrophoresis (BN–PAGE) in combination with SDS–PAGE. First, mitochondrial membranes were subjected to BN–PAGE to separate the intact respiratory complexes, and stained using NADH and nitroblue tetrazolium (NBT) as a complex I-specific stain. The intensity of the major activity associated with monomeric, fully assembled complex I (Figure 3A, arrow) was strongly decreased in membranes from the *ind1*Δ strain and the C242/C245 cysteine mutants compared with the complemented wild type (cWT). However, a fraction of intact, active complex I was still present in the *ind1* mutants, in contrast to the mutant lacking

Table I Complex I activities in mitochondrial membranes from *Y. lipolytica IND1* mutants

Strain	NADH:HAR activity		DQA-sensitive dNADH:DBQ activity	
	U mg^{-1}	% of wild type	U mg^{-1}	% of wild type
*ind1*Δ + pUB4	0.43 ± 0.01	28	0.23 ± 0.01	28
*ind1*Δ + pUB4-*IND1*	1.56 ± 0.05	100	0.83 ± 0.02	100
*ind1*Δ + pUB4-*IND1*–strep	1.47 ± 0.08	94	0.82 ± 0.01	99
C242A	0.39 ± 0.02	25	0.19 ± 0.01	23
C242E	0.32 ± 0.02	21	0.17 ± 0.01	20
C242S	0.47 ± 0.03	30	0.21 ± 0.01	25
C245A	0.45 ± 0.02	29	0.23 ± 0.01	28
C245E	0.31 ± 0.02	20	0.15 ± 0.01	18
C245S	0.32 ± 0.02	21	0.17 ± 0.01	20
C279A	1.25 ± 0.06	80	0.67 ± 0.02	81
C279E	0.25 ± 0.01	16	0.12 ± 0.01	14
C279S	1.50 ± 0.07	96	0.83 ± 0.02	100

Activities are given as mean ± standard deviation ($n = 5$).

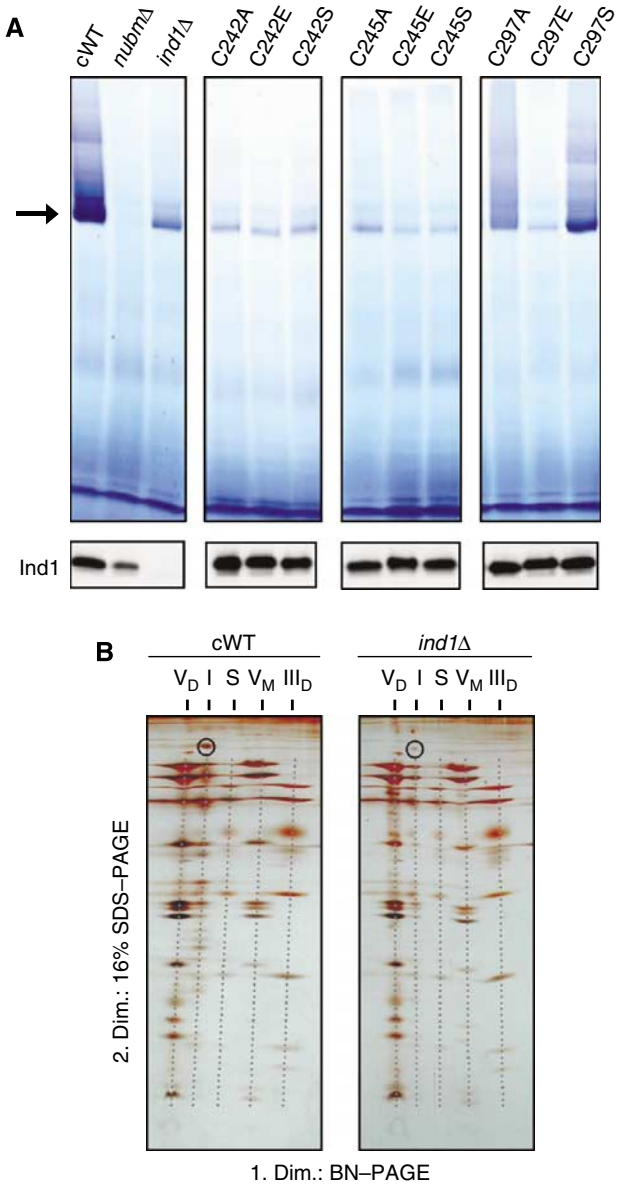

Figure 3 Deletion of *IND1* leads to a major decrease in fully assembled complex I. (**A**) In-gel staining of complex I in mitochondrial membranes (upper panels) and immunostaining of Ind1 protein (lower panels). Mitochondrial membrane proteins were separated by BN–PAGE and incubated with NADH and NBT to visualize NADH dehydrogenase activity. Monomeric complex I (with a molecular mass of 947 kDa) is marked by an arrow. Activity-stained bands of higher molecular mass represent supercomplexes containing complex I. cWT (*ind1*Δ + pUB4-*IND1*); *nubm*Δ, a strain lacking the gene encoding the 51-kDa NUBM subunit of complex I; *ind1*Δ carrying the pUB4 plasmid; C242A–C279S, point mutations that exchange cysteines at positions 242, 245 or 279 in pUB4-*IND1*. (**B**) Two-dimensional (BN/SDS) PAGE analysis of mitochondrial membranes from complemented wild-type (cWT) and *ind1*Δ cells, stained with silver. The following multiprotein assemblies are indicated by dashed grey lines: V_D and V_M, dimeric and monomeric forms of complex V; I, complex I; S, an incompletely characterized supercomplex that contains complex III, III_D, dimeric form of complex III. The 75-kDa NUAM subunit of complex I is circled.

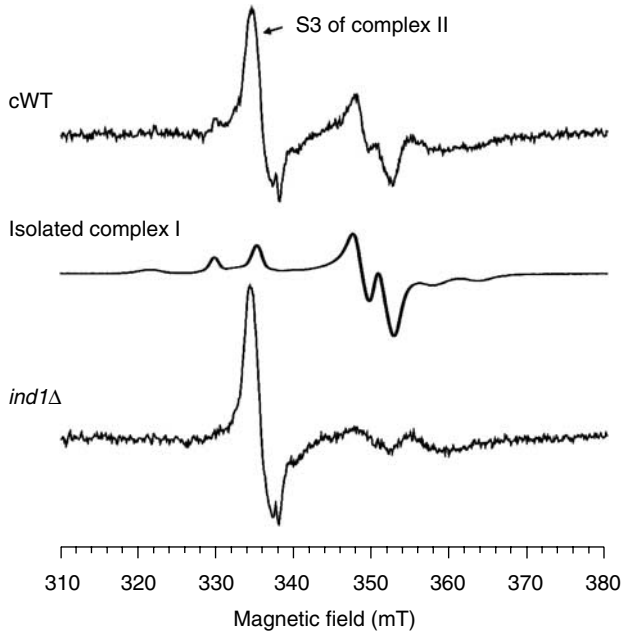

Figure 4 Deletion of *IND1* results in significantly diminished EPR signals of complex I. EPR spectra of mitochondrial membranes from complemented wild type (cWT, top) and *ind1*Δ cells (bottom). A spectrum of isolated complex I is shown for comparison (middle). Samples were treated with 2 mM NADH, frozen and EPR spectra were recorded at 12 K. Instrument parameters: microwave frequency 9.47 GHz, modulation amplitude 0.64 mT, modulation frequency 100 kHz, microwave power 5 mW.

the 51-kDa subunit (*nubm*Δ), which did not have any complex I staining at all. In the *IND1* mutants, no novel activities of smaller molecular mass were detected, indicating that subcomplexes containing a functional NBT reduction site (within the 51-kDa subunit) were absent.

Second, the assembly and subunit composition of complex I and other respiratory complexes were investigated by separating the individual subunits in the second dimension using SDS–PAGE and silver staining. This revealed that the abundance of all complex I subunits was strongly diminished in the *ind1*Δ mutant (Figure 3B). None of the other respiratory complexes were affected, in accordance with normal activities of complexes II and III (Figure 2C). Again, no subcomplexes of complex I were found. Two-dimensional gel electrophoresis of mitochondrial membranes of the C242 and C245 mutants showed similar results as for the deletion mutant (not shown). Semi-quantitative analysis of the complex I stain and the silver-stained polypeptides showed that the amount of fully assembled complex I was decreased by approximately 80% in agreement with the activity measurements (Table I).

The low levels of fully assembled complex I in the *ind1*Δ mutant strain could be confirmed using electron paramagnetic resonance (EPR) spectroscopy (Figure 4). The typical EPR signals of the Fe–S clusters of complex I, mainly appearing in the region of 350 mT ($g \sim 1.93$) and the characteristic g_z component of the cluster N2 spectrum at 330 mT ($g \sim 2.05$), were largely absent in the *ind1*Δ mutant strain. On the other hand, the prominent EPR signal of the S3 $[3Fe\text{–}4S]^{1+}$ cluster of complex II was unaffected. By comparing cluster N2 signal intensities of the two samples, complex I content in the mutant was estimated to be ~20% of the WT sample. A similar result was obtained by comparison of cluster N1 signal intensities at 40 K, conditions that allow the detection

of [2Fe–2S] clusters only (data not shown). Addition of the stronger reductant sodium dithionite clearly diminished the S3 signal intensity in both samples due to reduction of S3 to the $[3Fe–4S]^0$ state, which is EPR silent. In contrast, complex-I EPR signals did not alter in the *ind1*Δ mutant sample, indicating that there was no shift in redox midpoint potentials that prevented complete reduction of the Fe–S clusters by NADH (data not shown).

Taken together, these results show that in the absence of Ind1, the amount of fully assembled complex I was decreased by ~80%, and that no subcomplexes were formed. Thus, the presence of Ind1 significantly facilitates complex I assembly.

Ind1 binds a transferable Fe–S cluster

Earlier we showed that the CxxC motif is essential for the function of Ind1 (Table I and Figure 3A). As this motif is frequently found in Fe–S-binding sequences, we investigated whether Ind1 can bind an Fe–S cluster. First, Fe–S binding to the recombinant Ind1–strep was analysed. The protein was isolated from *E. coli* extracts as a colourless, non-[Fe–S]-containing protein following Strep-Tactin affinity purification. Metal ions or a cluster could have been lost due to inherent lability, especially as the purification was carried out in the presence of normal oxygen levels. Therefore, chemical reconstitution of Ind1 was attempted under strictly anaerobic conditions. Reconstituted and desalted Ind1 was brown in colour and had a non-structured UV–Vis spectrum with a shoulder around 400 nm (Figure 5A). Dithionite reduction led to 40–50% bleaching of the visible chromophore. These features are common for [4Fe–4S] cluster-containing proteins. The [2Fe–2S] content was low, as typical absorbance peaks of 410, 460 and 560 nm were absent (Orme-Johnson and Orme-Johnson, 1982). Upon air exposure, absorbance bands at 415, 532 and 606 nm became detectable before onset of protein precipitation and loss of visible chromophores (Supplementary Figure S6A), indicating the formation of an unknown cluster species during breakdown of the [4Fe–4S] cluster. The UV–Vis data were corroborated by a weak EPR signal for dithionite-reduced Ind1 in the $g = 2$ region, with similar g values as reconstituted Cfd1 from *S. cerevisiae* (Supplementary Figure S6B). Our observations suggest that under low oxygen conditions (i.e. inside the mitochondria) a [4Fe–4S] cluster can be loaded onto Ind1.

In a recent study (Netz *et al*, 2007), it has been shown that the cytosolic Cfd1–Nbp35 complex mediates transfer of transiently bound Fe–S clusters to the apoform of isopropylmalate isomerase (Leu1), a target Fe–S protein. As the apoform of complex I is not available as an *in vitro* target for Fe–S cluster transfer experiments, the Leu1 activation assay was used to assess the ability of Ind1 to transfer its Fe–S cluster to potential target proteins. Reconstituted and desalted Ind1 harbouring a [4Fe–4S] cluster was added to apo-Leu1 and cluster transfer was followed by the increase of enzymatic activity upon conversion of apo-Leu1 to the [4Fe–4S]-containing form. Cluster transfer from Ind1 occurred rapidly and efficiently *in vitro* (Figure 5B), and was comparable to the results with the Cfd1–Nbp35 complex (Netz *et al*, 2007). At the earliest time point that we were able to measure (1.5 min), more than 80% of the maximal Leu1 activity was obtained. In contrast, chemical reconstitution with the same concentration of ferric iron and sulphide hardly led to activation of apo-Leu1 (Figure 5B). These *in vitro* data suggest a scenario in which Ind1 assists with Fe–S cluster transfer to complex I.

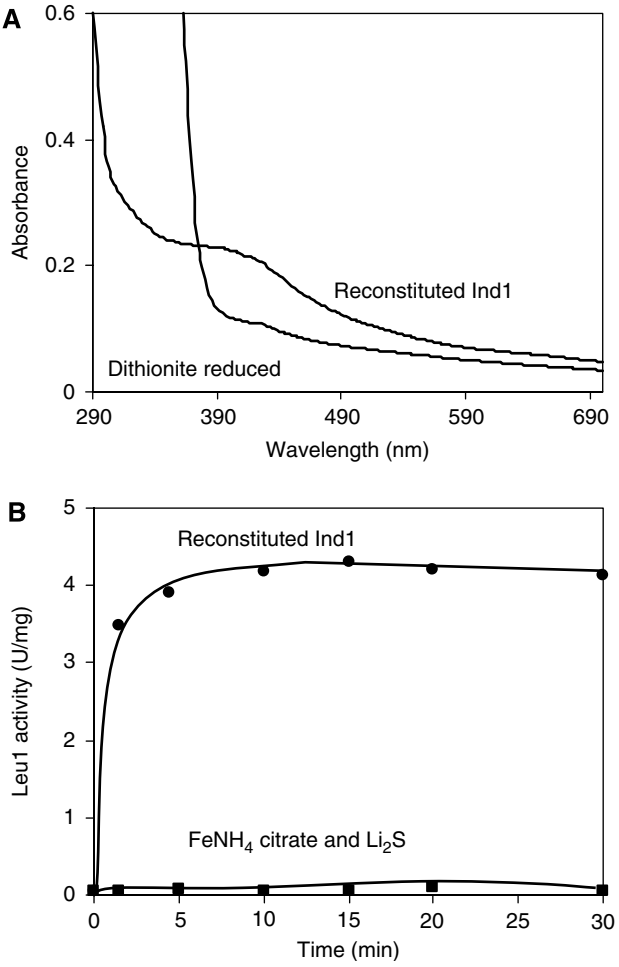

Figure 5 Ind1 binds a labile Fe–S cluster *in vitro*. (**A**) Binding of an [4Fe–4S] cluster to Ind1 *in vitro* as revealed by UV–Vis spectroscopy. Recombinant Ind1–strep (0.85 mg/ml, 26 μM) was reconstituted and desalted as described in Materials and methods. UV–Vis spectra were recorded in 100 mM Tris–HCl pH 9.0, 100 mM NaCl in the presence and absence of 2 mM sodium dithionite (as indicated). (**B**) Ind1 can efficiently transfer an Fe–S cluster to a model substrate, apo-isopropylmalate isomerase (Leu1). Reconstituted Ind1 (4 μM) was mixed with reduced apo-Leu1 (4 μM) under anaerobic conditions and Leu1 enzyme activity was measured at regular intervals (circles). As a control, activation of 4 μM apo-Leu1 with ferric ammonium citrate and Li_2S at concentrations identical to those present in Ind1 was carried out (squares).

In vivo *^{55}Fe binding to Ind1 requires Nfs1 and Isu1 in S. cerevisiae*

To test whether the *in vitro* studies are relevant for the function of Ind1 in the living cell, we investigated whether Ind1 binds Fe–S *in vivo*. For this purpose, we exploited a previously developed radiolabelling assay (Kispal *et al*, 1999). As the expression level of Ind1 in *Y. lipolytica* was too low for the detection limits of this assay, full-length Ind1–strep protein was overproduced under the control of an intermediate or strong promoter in *S. cerevisiae*. The protein was correctly targeted to mitochondria (not shown) and processed to its mature size, although processing

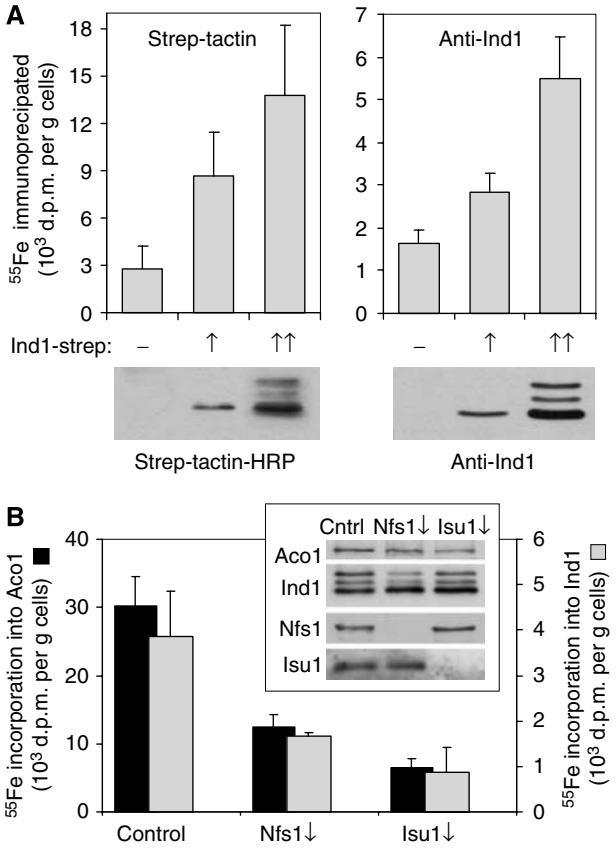

Figure 6 Ind1 binds an Fe–S cluster *in vivo*. (**A**) Wild-type *S. cerevisiae* cells carrying an empty plasmid (−) or plasmids for intermediate (↑) or high (↑↑) expression of *Y. lipolytica IND1*–strep were radiolabelled with ^{55}Fe. Cells were harvested after 2 h and, following preparation of a cell extract, Ind1–strep was immunoprecipitated with either Strep-Tactin or Ind1 antibodies coupled to sepharose A. The ^{55}Fe associated with the protein was quantified by scintillation counting. The expression levels of Ind1–strep were confirmed by immunoblotting (lower panels). (**B**) Iron-55 labelling and immunoprecipitation of Ind1 or endogenous aconitase (Aco1) upon depletion of the cysteine desulphurase Nfs1 (Nfs1↓) or the scaffold protein Isu1 (Isu1↓). Wild-type *S. cerevisiae* (control), Gal-NFS1 or Gal-ISU1/*isu2*Δ strains were transformed with a plasmid to overexpress *Y. lipolytica IND1*–strep, and grown on glucose to downregulate the expression of *NFS1* or *ISU1* as indicated. Expression of Aco1, Ind1, Nfs1 and Isu1 was visualized by immunoblotting (inset). The scintillation values were corrected for the average background signal obtained from experiments with cells containing an empty plasmid (for Ind1; 1.6×10^3 d.p.m./g cells, see A), or with *aco1*Δ cells (for aconitase; 1.3×10^3 d.p.m./g cells). Error bars represent the standard deviation, $n = 3$–5.

intermediates were observed at high expression levels (Figure 6A, lower panels). Cells were labelled with ^{55}Fe, followed by immunoprecipitation of Ind1–strep protein from the cell extract using either Strep-Tactin or Ind1 antibodies coupled to protein A-sepharose. Immunoprecipitation was confirmed by immunoblotting (not shown). The amounts of ^{55}Fe co-precipitated from cell extracts increased significantly with the amounts of Ind1–strep in the cell extract (Figure 6A), indicating that Ind1 bound iron *in vivo*.

To investigate whether ^{55}Fe binding to Ind1 was dependent on the cysteine desulphurase Nfs1 and the scaffold proteins Isu1/Isu2 and thus represents binding of an Fe–S cluster, we expressed Ind1–strep at high levels in *S. cerevisiae* cells in which the expression of *NFS1* or *ISU1* can be regulated (Kispal *et al*, 1999; Gerber *et al*, 2003). Ind1 antibodies were used for immunoprecipitation, because, for unknown reasons, the C-terminal Strep tag was cleaved off upon downregulation of *NFS1* or *ISU1*, but the Ind1 protein itself was stable (Figure 6B and data not shown). Upon depletion of either Nfs1 or Isu1, the amount of ^{55}Fe associated with Ind1 was significantly decreased. A similar decline in ^{55}Fe incorporation was observed under these conditions for mitochondrial aconitase (Aco1), a control Fe–S protein known to be dependent on Nfs1 and Isu1 (Figure 6B). These data show that ^{55}Fe binding to Ind1 is dependent on two central components of the ISC assembly system in the *S. cerevisiae* mitochondrial environment, thus indicating the presence of an Fe–S cluster on Ind1 *in vivo*.

Discussion

We have investigated the function of a mitochondrial P-loop NTPase, called Ind1, in the obligate respiratory yeast *Y. lipolytica*. Our data showed that Ind1 is specifically required for effective assembly of complex I, NADH:ubiquinone oxidoreductase. We first identified Ind1 as a protein sequence with high sequence similarity to the *S. cerevisiae* P-loop NTPases Cfd1 and Nbp35, which form a heterotetramer and mediate the transfer of Fe–S clusters to cytosolic Fe–S proteins (Roy *et al*, 2003; Hausmann *et al*, 2005; Netz *et al*, 2007). Most eukaryotes possess a Cfd1-like protein with an N-terminal sequence predicted to target the protein to mitochondria (Figure 2A and Supplementary Figure S1). Indeed, we confirmed that the *Y. lipolytica* Ind1 protein was localized exclusively in mitochondria (Figure 1C). Co-evolution of Ind1 and complex I (Figure 2A) suggested a role for Ind1 in the assembly and/or function of complex I.

The impact of *IND1* deletion on complex I was investigated at different levels, including *in vitro* enzyme assays, complex integrity in BN–PAGE gels, subunit abundance and composition in two-dimensional gel electrophoresis, as well as EPR spectroscopy of the Fe–S cofactors (Figures 2C, 3, 4 and Table I). All methods concurrently revealed a decrease in complex I content of approximately 80% in the *ind1*Δ mutant compared with the cWT strain. The deleterious effect was specific for complex I, as none of the other respiratory complexes were affected, either at the level of protein assembly (complexes III and V; Figure 3B), Fe–S cluster assembly (complex II; Figure 4) or activity (complexes II and III; Figure 2C). This specificity is different from that observed for the bacterial homologue ApbC in *Salmonella enterica*, in that lesions in the latter decreased complex II (succinate dehydrogenase) activity by 34% (Skovran and Downs, 2003). The NADH:ubiquinone oxidoreductase activity was not analysed in the *apbC* mutant and it will be interesting to do so to establish whether the ApbC and Ind1 have overlapping functions.

Our data indicate that Ind1 mediates an ATP or GTP-dependent, Fe–S-requiring step in complex I assembly, but the details remain to be investigated. Ind1 has not been identified previously as a protein associated with complex I, despite thorough proteomics studies (reviewed in Hirst *et al*, 2003; Brandt, 2006). Therefore, Ind1 would qualify as a true assembly factor, which is consistent with its low abundance

(Figure 1B). Future protein-binding studies, as well as immunodetection of Ind1 in assembly intermediates may establish the precise role of Ind1 in complex I assembly. It is interesting to note that small amounts of complex I (~20% of cWT) were still assembled in the absence of Ind1. Clearly, Ind1 facilitates a particular step in the assembly process that can be either bypassed or is also carried out by other proteins at low efficiency. The remaining complex I activity was not sufficient for survival during sporulation in the absence of *ND2i* (Supplementary Figure S5), suggesting that a certain threshold of complex I activity is required during this developmental stage.

Our *in vitro* and *in vivo* data established the ability of Ind1 to bind a [4Fe–4S] cluster (Figures 5 and 6). Cys242 and Cys245 were demonstrated to be essential for the function of Ind1 using site-directed mutagenesis (Figure 3A and Table I), and may form the ligands of the Fe–S cluster. It remains to be investigated whether other, non-cysteine residues function as additional ligands, or whether the cubane cluster is coordinated by ligands from two Ind1 polypeptides forming a homodimer, as is the case for NifH (Georgiadis *et al*, 1992). Further *in vitro* data showed that the Fe–S cluster is oxygen labile and can readily be transferred to an Fe–S apoprotein (Figure 5B). Although these properties suggest a scaffold function of Ind1, we found that it could not replace the scaffold protein Isu1 and vice versa. First of all, Fe–S cluster loading on Ind1 occurred downstream of Isu1 in *S. cerevisiae* mitochondria (Figure 6B). It needs to be investigated, however, whether the same is true in *Y. lipolytica*. Second, overexpression of *IND1* could not rescue growth of Isu1-depleted Gal-*ISU1/isu2*Δ *S. cerevisiae* cells on glucose (data not shown). Third, overexpression of *Y. lipolytica ISU1* using the inducible *POX2* promoter did not increase the levels of assembled complex I in the *ind1*Δ mutant (data not shown). These results suggested that Ind1 performs a molecular function that is clearly different from the generic Fe–S scaffold protein Isu1.

The identification of a novel assembly factor of complex I is of major clinical importance, as mutations in numerous genes encoding complex I subunits are causative of several human diseases. Only a handful of complex I assembly factors have been identified thus far and many complex I deficiencies remain to be elucidated at the molecular level (Janssen *et al*, 2006). We have preliminary evidence that the human orthologue of Ind1 (GeneID: 80224) could partially rescue the growth and complex I activity of the *Y. lipolytica ind1*Δ strain (data not shown). Moreover, RNAi-mediated downregulation of human *IND1* expression in HeLa cells resulted in a ~70% decrease of complex I activity (A Sheftel *et al*, manuscript in preparation). Thus, further molecular characterization of Ind1 in the model organism *Y. lipolytica* or in human cell lines will be of vital importance for our understanding of the assembly process of complex I, and the numerous mitochondrial diseases associated with complex I deficiency.

Materials and methods

Yeast strains and growth

The haploid *Y. lipolytica* GB10 strain (*ura3-302*, *leu2-270*, *lys11-23*, *NUGM*-Htg2, *NDH2i*, *MatB*) was used to generate an *IND1* deletion mutant, *ind1*Δ. The *IND1* gene (systematic name YALI0B18590g, GeneID: 2907193) is intron-less and is predicted to encode an open reading frame of 312 amino-acid residues. The open reading frame plus 27 bp upstream and 60 bp downstream were replaced by the *URA3* marker gene using the homologous recombination strategy described in Abdrakhmanova *et al* (2006). More than 440 transformants were screened and the correct gene replacement was confirmed by PCR. The *IND1* gene was reintroduced in the *ind1*Δ mutant to obtain the cWT. For this purpose, the entire *IND1* gene, including 1329 nucleotides upstream of the start codon containing the endogenous promoter, was cloned into the pUB4 plasmid (Kerscher *et al*, 2001), generating pUB4-*IND1*. A C-terminal Strep tag was added to the *IND1* open reading frame, creating pUB4-*IND1*–strep. pUB4-*IND1* was used for site-directed mutagenesis using PCR. *Y. lipolytica* cells were grown in rich medium containing 1% (w/v) glucose (YPD). Hygromycin B (50 μg/ml) was added for the initial selection of cells transformed with the pUB4 plasmid.

S. cerevisiae strain W303-1A (*MATa*, *ura3-1*, *ade2-1*, *trp1-1*, *his3-11,15*, *leu2-3112*) served as WT. The Gal-NFS1 and Gal-ISU1/*isu2*Δ strains and conditions for depletion of the respective proteins were described previously (Kispal *et al*, 1999; Gerber *et al*, 2003). The following yeast plasmids were used: pRS416 containing the *MET25* promoter for intermediate expression of *IND1*–strep, and pRS426 with the *TDH3* promoter for high expression (Mumberg *et al*, 1995).

Gel electrophoresis

Separation of the components of the respiratory chain in mitochondrial membranes of *Y. lipolytica* was carried out using BN–PAGE (Schägger, 2003). Total protein (500 μg) was solubilized with 3.0 g/g digitonin and 500 mM amino caproic acid. The resulting solubilized mitochondrial membranes were separated on non-denaturing 4–13% gradient gels. Individual lanes were cut out, incubated for 30 min in 1% SDS and applied on denaturing 16% polyacrylamide Tricine-SDS gels for resolution in the second dimension. Gels were stained with silver. For in-gel detection of the NADH dehydrogenase activity of complex I, unfixed BN–PAGE gels were incubated for about 5 min in a solution containing 3 mM NBT and 120 μM NADH. To stop the reaction, gels were incubated in 50% methanol and 10% acetic acid.

EPR spectroscopy

X-band EPR spectra were recorded with a Bruker ESP 300E spectrometer equipped with a HP 53159A frequency counter (Hewlett Packard), an ER 035 M NMR gaussmeter (Bruker, BioSpin) and a liquid helium continuous flow cryostat (Oxford Instruments). Mitochondrial membranes, at a final concentration of 25 mg protein/ml, were treated with 2 mM NADH. Samples were frozen in cold isopentane/methylcyclohexane (5:1, ~120 K) and stored in liquid nitrogen. Typically, spectra were recorded at temperatures of 40 K to analyse binuclear clusters only or at 12 K to analyse both binuclear and tetranuclear clusters. A highly concentrated sample of isolated complex I (~86 mg/ml) reduced by NADH was used as a reference.

Chemical reconstitution and transfer of Fe–S clusters

The *IND1*–strep open reading frame, excluding the first 36 codons and starting with a Met^{AUG}, was cloned into the pET15b vector (Novagen) for overexpression of the protein following the manufacturer's instructions. The protein was purified using Strep-Tactin (IBA, Göttingen, Germany) and eluted in 100 mM Tris–HCl pH 9.0, 100 mM NaCl (buffer A), 2.5 mM desthiobiotin. Freshly prepared, recombinant apo-Ind1–strep was chemically reconstituted in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). In a typical experiment, 20 μM of Ind1 in buffer A was treated with 5 mM DTT (final concentration) for 1 h at 15°C. The reconstitution was started by addition of 10 molar equivalents of ferric ammonium citrate and Li_2S and the mixture was incubated for 3 h at 15°C. To remove non-bound iron and sulphide, reconstituted Ind1 was passed through a Sephadex G25 gel filtration column using buffer A containing 1 mM DTT. The assembly of an Fe–S cluster on apo-Ind1 was ascertained by UV–Vis spectroscopy (Jasco V-550).

Chemically reconstituted Ind1 harbouring an [Fe–S] cluster was tested for the ability to transfer its cluster to the apoform of yeast isopropylmalate isomerase (apo-Leu1) *in vitro*, as described by Netz *et al* (2007).

Miscellaneous methods

The following published methods were used: isolation of intact mitochondria (Galkin *et al*, 2006) and unsealed mitochondrial membranes (Kerscher *et al*, 1999); enzyme assays of aconitase, succinate dehydrogenase and citrate synthase (Kispal *et al*, 1999); complex I activity assays (Tocilescu *et al*, 2007). Polyclonal antibodies were raised against Ind1–strep purified from *E. coli* (Harlow and Lane, 1998). Antisera against Aco1, Nfs1 and Isu1 have been described previously (Kispal *et al*, 1999; Gerber *et al*, 2003; Gelling *et al*, 2008). The monoclonal antibody against *Arabidopsis* actin was from Affinity BioReagents, Golden, CO, USA. Labelling with Strep-Tactin horse radish peroxidase (IBA) was carried out according to the manufacturer's instructions. Radiolabelling, cell lysis and immunoprecipitation of the Fe–S reporter proteins of interest were as described previously (Kispal *et al*, 1999). Error bars represent the standard deviation of the mean value.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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