Mimicking a **SURF1** allele reveals uncoupling of cytochrome *c* oxidase assembly from translational regulation in yeast

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Received January 28, 2011; Revised and Accepted March 29, 2011

Defects in mitochondrial energy metabolism lead to severe human disorders, mainly affecting tissues especially dependent on oxidative phosphorylation, such as muscle and brain. Leigh Syndrome describes a severe encephalomyopathy in infancy, frequently caused by mutations in **SURF1**. **SURF1**, termed Shy1 in *Saccharomyces cerevisiae*, is a conserved assembly factor for the terminal enzyme of the respiratory chain, cytochrome *c* oxidase. Although the molecular function of **SURF1/Shy1** is still enigmatic, loss of function leads to cytochrome *c* oxidase deficiency and reduced expression of the central subunit Cox1 in yeast. Here, we provide insights into the molecular mechanisms leading to disease through missense mutations in codons of the most conserved amino acids in **SURF1**. Mutations affecting G124 do not compromise import of the **SURF1** precursor protein but lead to fast turnover of the mature protein within the mitochondria. Interestingly, an Y274D exchange neither affects stability nor localization of the protein. Instead, **SURF1**Y274D accumulates in a 200 kDa cytochrome *c* oxidase assembly intermediate. Using yeast as a model, we demonstrate that the corresponding Shy1Y344D is able to overcome the stage where cytochrome *c* oxidase assembly links to the feedback regulation of mitochondrial Cox1 expression. However, Shy1Y344D impairs the assembly at later steps, most apparent at low temperature and exhibits a dominant-negative phenotype upon overexpression. Thus, exchanging the conserved tyrosine (Y344) with aspartate in yeast uncouples translational regulation of Cox1 from cytochrome *c* oxidase assembly and provides evidence for the dual functionality of Shy1.

**INTRODUCTION**

Oxidative phosphorylation plays a central role in energy metabolism within prokaryotic and eukaryotic cells. The principle of using energy in the form of reducing equivalents for asymmetric proton pumping across a lipid bilayer to generate a gradient that is used to drive ATP synthesis is a highly conserved process (1,2).

In humans, defects in mitochondrial energy metabolism lead to severe disorders, mostly affecting energy-demanding tissues, the so-called mitochondrial encephalomyopathies (3). These disorders lead to defects in brain and muscle tissues, affecting patients in early infancy (4). The most frequent causes for these disorders are defects in oxidative phosphorylation. The enzyme complexes of the oxidative phosphorylation system reside in the inner mitochondrial membrane and exist as multi-protein assemblies, which associate with each other into higher oligomers, so-called supercomplexes (5,6). Complexes I, III, IV and V are built from polypeptides of dual genetic origin. The small mitochondrial genome encodes for 13 hydrophobic proteins in humans that are subunits of these complexes (7–9). They usually represent the initial subunits of respiratory chain complex assembly (10–12). Consequently, misregulation of mitochondrial protein synthesis is among the main causes for mitochondrialopathies (13–15). However, most mitochondrial proteins are

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encoded by nuclear DNA, synthesized in the cytosol and imported into the organelle by sophisticated transport machineries (16–20). Protein translocases in the outer and inner membranes direct these proteins across and into the inner mitochondrial membrane. This holds true for the majority of subunits of the oxidative phosphorylation system. While loss of translocase function is probably lethal, defects in the import process of individual proteins have been reported to cause their mislocalization and can thus be associated with human disorders (21).

Assembly of respiratory chain complexes from individual subunits requires specific assembly factors. The best-studied example of complex assembly is the cytochrome c oxidase, for which more than 20 assembly factors have been characterized (9,12,22). In agreement with their critical role for building a functional enzyme complex, defects in assembly factors cause selective cytochrome c oxidase deficiency (OMIM 220110), associated with various diseases (23,24). The etiologies behind these disorders are as diverse as the assembly of the enzyme is complex. However, many of the assembly factors involved have been implicated in the biogenesis of the central subunit of the cytochrome c oxidase, COX1. COX1 carries the heme co-factors and one copper ion in its active center (25). Patients have been described to display mutations in genes encoding assembly factors that are associated with copper metabolism, such as SCO1, SCO2, COX11 and COX17 (26–29), or heme biosynthesis, such as COX10 and COX15 (30–32). However, mutations in the SURF1 gene (33,34) are the main reason for Leigh Syndrome with isolated cytochrome c oxidase deficiency (OMIM 256000; (35)), which is the most frequent mitochondrial disorder in infancy.

SURF1 is a highly conserved cytochrome c oxidase assembly factor from respiring bacteria to man (36–38). Findings, originally made in bacteria, suggest that SURF1 acts as a heme insertase, or as a chaperone that maintains COX1 competent for the insertion of heme (38–42). Patients lacking functional SURF1 accumulate cytochrome c oxidase assembly intermediates (33,43,44). The SURF1 homolog in S. cerevisiae, Shy1, serves as a model to analyze cytochrome c oxidase assembly and the molecular function of the protein itself (39,40,45–48). However, despite all efforts, the molecular function of SURF1 is still ill-defined. Genetic suppressor screens of shy1 mutants as well as biochemical isolation of Shy1-containing protein complexes have identified a number of novel Shy1-associated proteins that are part of the assembly intermediates of cytochrome c oxidase and link Shy1 functionally with the regulation of Cox1 expression (39,40,45,48,49).

In yeast, expression of Cox1 depends on Mss51 (45,50). Mss51 has two functions: (i) it acts as a Cox1-specific translational activator and (ii) it represents a Cox1-specific chaperone, which binds to unassembled Cox1. Cox14 and Coa3, two small integral membrane proteins, bind to unassembled Cox1 and stabilize the interaction with Mss51 (48,51–53). This sequestration of Mss51 into the quaternary complex is a prerequisite for the downregulation of Cox1 synthesis. Cox14, originally identified as a link between Cox1 translational regulation and Shy1, associates with the Cox1–Cox14–Coa3–Mss51 complex and is necessary to stall Cox1 synthesis (39,40,48). While this regulatory system is well-established in yeast, in humans, the regulation of Cox1 expression is less well understood and translational regulators have only recently been defined (15,54).

In this study, we have analyzed the effect of mutations originally identified in Leigh Syndrome patients, which affect two of the most conserved amino acids of SURF1, G124 and Y274 (G137 and Y344 in Shy1, respectively), to gain insight into the molecular basis for the observed defects. Mutations affecting the amino acid G124 lead to low abundance of the SURF1 protein in mitochondria (55,56). We show that SURF1/Shy1 import into mitochondria remains unaffected by the amino acid exchange, but that the mature mutant protein is effectively turned over by the mitochondrial proteolytic system. Similarly, an Y274D exchange does not affect protein transport into the mitochondrial inner membrane. However, the protein remains stable and integrates efficiently into early assembly intermediates of the cytochrome c oxidase, where it appears to accumulate in human mitochondria. In a yeast model, we find that the association of mutant Shy1Y344D into assembly intermediates releases the translational activator Mss51 from the sequestered inactive state, allowing for wild-type-like Cox1 synthesis in mitochondria. Yet, cytochrome c oxidase assembly is impaired owing to Shy1 dysfunction. Hence, the Y344D transition uncouples translational regulation of the Cox1 polypeptide from complex assembly, indicating that it represents a selective assembly-deficient allele of SURF1/Shy1.

RESULTS

Mutations mimicking patient SURF1 lead to cytochrome c oxidase deficiency in yeast

SURF1 encodes a highly conserved assembly factor, which is an integral protein of the inner mitochondrial membrane and is involved in the early steps of the COX1 biogenesis. To gain insight into the mechanisms underlying cytochrome c oxidase deficiencies owing to defects in SURF1, we analyzed reported mutations that lead to single amino acid exchanges in highly conserved amino acids (34,37). The invariant G124 (G137 in S. cerevisiae) is located in an intermembrane space (IMS) loop between the two transmembrane spans of SURF1 and has been reported to be exchanged with either E or R residues (55,56), replacing the small amino acid with a bigger, charged residue (Fig. 1A). The invariant Y274 (Y344 in S. cerevisiae) is also located on the IMS side of the protein in close proximity to the second predicted transmembrane span and was found to be mutated to a D (57), leading to altered charge distribution at the membrane interface (Fig. 1A). To analyze the effect of these amino acid exchanges, we cloned the Saccharomyces cerevisiae SHY1 gene, introduced the mutations by site-directed mutagenesis and transformed the facultative anaerobe yeast S. cerevisiae lacking the SHY1 open reading frame with plasmids encoding either wild-type Shy1 (Shy1W), or the corresponding mutant versions Shy1G137R, Shy1G137E and Shy1Y344D, or an empty plasmid as a control. The strains were tested for growth on media with fermentable and non-fermentable carbon sources at various temperatures (Fig. 1B). While all strains showed wild-type-like growth on fermentable medium, cells
expressing Shy1<sub>G137E</sub> did not grow on non-fermentable carbon sources at all temperatures tested (Fig. 1B), as reported previously for <i>shy1</i>Δ cells (36,39). This finding agrees with the previous analysis by Bestwick <i>et al.</i> (47). However, cells expressing Shy1<sub>G137R</sub> displayed a temperature-conditional growth phenotype and were selectively unable to grow at 30°C, but did grow at lower temperatures (Fig. 1B). Interestingly, cells expressing Shy1<sub>Y344D</sub> displayed a cold-sensitive growth phenotype and thus only slightly reduced growth at 30°C compared with wild-type, but lost viability at low temperatures (Fig. 1B).

To address the reason for the observed growth phenotype, we isolated mitochondria from these strains grown at 30°C, and analyzed respiratory chain complexes by blue-native polyacrylamide gel electrophoresis (BN-PAGE) and western blotting (Fig. 1C). Respiratory chain complexes associate into higher oligomers. In yeast one or two copies of the cytochrome<sub>c</sub> oxidase can be found in association with the cytochrome<sub>bc1</sub> complex dimer (III<sub>2</sub>IV, III<sub>2</sub>IV<sub>2</sub>). In wild-type cells, most of the <i>bc1</i> complex is found in these so-called supercomplexes (Fig. 1C, lane 12), however, in <i>shy1</i> mutant mitochondria, it is mainly present in the dimeric form (III<sub>2</sub>) owing to a lack of cytochrome<sub>c</sub> oxidase (Fig. 1C, lanes 11, 13–15). Antibodies against the mitochondria-encoded subunit Cox1 and the nucleus-encoded Cox4 did not detect higher molecular weight cytochrome<sub>c</sub> oxidase complexes in mitochondria with Shy1<sub>G137E</sub> or Shy1<sub>G137R</sub> similar to mitochondria lacking Shy1 (Fig. 1C). In contrast, shy1<sub>Y344D</sub> mitochondria displayed residual, but strongly reduced signals for cytochrome<sub>c</sub> oxidase complexes. As a control, we analyzed the F<sub>1</f>Fo-ATP synthase complexes, which were similar between the tested mitochondria. In order to investigate the temperature-sensitive growth phenotype of the mutants, we isolated mitochondria from cells grown at 19°C and analyzed respiratory chain complexes by BN-PAGE and western blotting. As expected from the growth phenotype, the amount of cytochrome<sub>c</sub>oxidase complexes at 19°C was similar between wild-type and the <i>shy1G137R</i> mutant. However, mitochondria isolated from the cold-sensitive <i>shy1Y344D</i> strain had severely reduced levels of cytochrome<sub>c</sub> oxidase at 19°C, as was seen in the shy1<sub>G137E</sub> mutant that did not grow at this temperature (Fig. 1D). Thus, we concluded that the growth phenotypes of the mutant strains reflect the observed cytochrome<sub>c</sub> oxidase deficiency.

**Figure 1.** Mutations in <i>SHY1</i> lead to respiratory deficiency and lack of cytochrome<sub>c</sub> oxidase in yeast. (A) Partial alignment of SURF1/Shy1 homolog proteins from different species using ClustalW2 with Blosum62 score matrix: Black boxes, 100% similar amino acids; dark gray, 80–100% similarity; light gray, 60–80% similarity. Shown are the areas surrounding the invariant amino acids G124 and Y274 (arrowheads). Predicted second transmembrane segment is indicated by a black bar (TM2). (B) Serial dilutions of shy1Δ cells expressing Shy1<sub>WT</sub>, Shy1<sub>Y344D</sub>, Shy1<sub>G137R</sub> and Shy1<sub>G137E</sub> or containing empty vector (−) were spotted on fermentable (YPD) or non-fermentable (YPG) medium and incubated at indicated temperatures. (C) Isolated mitochondria from indicated strains were solubilized and respiratory chain complexes analyzed by BN-PAGE and western blot analysis. (D) Isolated mitochondria from cells grown at 30°C or 19°C were analyzed as in (C). Western blots were decorated with anti-Cox1 antiserum.
Mutant forms of Shy1 are efficiently imported into isolated mitochondria

Studies on human mitochondria showed a reduced steady-state amount of SURF1 in patient cells (58–60). Thus, we analyzed the steady-state levels of the mutant Shy1 proteins in yeast mitochondria. Although control proteins of the different mitochondrial subcompartments as well as a number of early assembly factors, such as Cox14, Coa1, Coa3 and Mss51, were unaltered in the tested mitochondria, the mitochondria-encoded core subunit of the cytochrome c oxidase, Cox1, was strongly reduced in all mutants (Fig. 2A). Similar to a recent study, we also find Cox2 levels reduced (47), an effect that is probably secondary to the reduction of Cox1 (48,53). In shy1G137E and shy1G137R mitochondria, Cox1 and Cox2 levels were reduced to levels similar to shy1Δ mitochondria. In contrast, shy1Y344D mitochondria retained residual Cox1 and Cox2, confirming the results of the BN-PAGE analysis (Fig. 1C). Interestingly, the nucleus-encoded structural subunit Cox5a was hardly affected and Cox4 was present at wild-type levels, as it has been reported for the shy1Δ mutant previously (39). However, using antibodies directed against the native Shy1, we found that the amount of Shy1 was different between the various mutants. Although shy1Y344D mutant mitochondria showed wild-type levels of Shy1Y344D, only minute amounts of Shy1 were detectable in shy1G137E and shy1G137R mitochondria. It should be noted that antibodies directed against full-length Shy1 as well as against a C-terminal peptide, unaffected by the mutations, gave identical results (Fig. 2A). We considered two explanations for this finding: (i) exchanging G137 could destabilize the protein and lead to rapid intramitochondrial turnover; (ii) the exchange could alter the structure of the precursor protein in a manner that affects translocation of the precursor protein into mitochondria. To address these hypotheses, we synthesized radiolabeled wild-type and mutant Shy1 precursors in reticulocyte lysate and performed in vitro import analyses into isolated, energized mitochondria. All Shy1 proteins were processed to the faster migrating, mature form in the presence of a membrane potential (Δψ) (Fig. 2B, lanes 1–6). While the precursors were accessible to externally added protease, the mature forms remained stable, indicating translocation into mitochondria (Fig. 2B, lanes 7–11). A quantitative assessment of the import reactions showed that the different Shy1 proteins imported with similar kinetics and efficiency (Fig. 2C). Accordingly, mitochondrial import and Δψ-dependent transport across the inner membrane were not affected in the tested mutant versions of Shy1, making mislocalization an unlikely reason for low protein abundance. Additionally, in whole cell extracts prepared from the different mutants, Shy1 levels gave similar results as the steady-state protein level analysis of isolated mitochondria and no precursor form of the proteins was detected (Fig. 2A and D). Hence, all Shy1 mutant proteins were imported into mitochondria and did not display cytosolic accumulation.

Mature Shy1G137E and Shy1G137R undergo rapid turnover in mitochondria

To assess intramitochondrial protein turnover, we directly analyzed the stability of the imported proteins by pulse-chase analysis. After import of Shy1, the non-imported precursors
were digested by protease treatment. Subsequently, samples were taken at different time points of incubation (chase) and analyzed for the amount of mature Shy1 (Fig. 3A). Only a slight decrease in signal intensity was observed for Shy1 WT and Shy1 Y344D over time. In contrast, the Shy1 G137E and Shy1 G137R signals strongly decreased during the chase period (Fig. 3B, lanes 4–10). Quantification revealed a half-life of about 4 h for both Shy1 WT and Shy1Y344D. In contrast, both Shy1 G137E and Shy1 G137R displayed severely reduced stability and a half-life of less than 30 min (Fig. 3C). Therefore, we concluded that the reason for the observed reduction in steady-state levels of Shy1G137E and Shy1G137R is reduced stability of the mature proteins in mitochondria owing to degradation by the mitochondrial quality control system (61).

As Shy1 Y344D remained stable in mitochondria, but was apparently not fully functional, we considered that an amino acid substitution close to the second transmembrane span could affect its membrane insertion and topology. Therefore, we analyzed the topology of Shy1Y344D by protease protection analyses. When mitochondria were incubated with increasing amounts of protease, Shy1 WT and Shy1Y344D remained protected by the outer mitochondrial membrane (Fig. 3D, top

Figure 3. Shy1G137E and Shy1G137R are rapidly degraded in yeast mitochondria. (A) Experimental setup and procedure of the stability assay shown in (B). (B) 35S-labeled Shy1 precursors were imported into mitochondria for 5 min (import). After stopping the reaction, unimported precursor protein (p) was degraded, mitochondria re-isolated, resuspended in energized buffer and further incubated (chase). Samples were taken at indicated steps and chase times, separated by SDS–PAGE and analyzed by digital autoradiography. Mature, m. (C) Imported, mature Shy1 from (B) was quantified using the ImageQuant TL software (GE Healthcare), and plotted against the chase time. Values show the means of the Shy1 signals after the indicated time of incubation relative to 0 min chase (= 100%). SEM (n = 3). (D) Mitochondria or mitoplasts generated by osmotic swelling from indicated strains were treated with increasing concentrations of proteinase K (Prot. K) for 10 min, and analyzed by SDS–PAGE and western blotting. Asterisks, cross-reactive signals; Shy1’, C-terminal Shy1 fragments.
Upon generation of mitoplasts by osmotic swelling, Shy1WT and Shy1 Y344D became accessible to the protease. For detection of the Shy1 proteins, we used an antiserum directed against the C-terminus of Shy1, which is exposed to the mitochondrial matrix when the second transmembrane segment is inserted into the inner membrane. After protease treatment in mitoplasts, stable protease-resistant C-terminal fragments were observed for Shy1 WT and Shy1 Y344D (Fig. 3D, bottom panel). Thus, we conclude that Shy1 Y344D retains the physiologically correct topology despite the amino acid exchange.

Mutant SURF1 proteins are unstable after import into mitochondria

The mammalian cytochrome c oxidase assembly process differs in some aspects from the situation in yeast (9,12). In yeast, mitochondrial RNA molecules are transcribed with long untranslated regions. Thus, transcription and translational regulation were assumed to differ between higher eukaryotes and yeast. Only recent analyses have identified translational regulators in human mitochondria (15,54), suggesting that regulation of mitochondrial protein expression might occur to a similar extent. Moreover, several assembly factors have so far only been identified in yeast and it is unclear if this is because of a limited homology that prevents identification \textit{in silico} or loss of these proteins during evolution. Owing to these differences and because some of the yeast shy1 mutants displayed temperature-sensitive phenotypes with varying effects for cell viability, we wanted to confirm that, the mutant versions of human SURF1 were efficiently imported into human mitochondria but were then prone to degradation, as was seen in yeast. Therefore, we cloned SURF1 and introduced point mutations by site-directed mutagenesis encoding SURF1G124E, SURF1G124R and SURF1Y274D. After synthesis of radiolabeled precursors, we performed \textit{in vitro} import experiments into isolated HEK293T cell mitochondria. Similar to the yeast model, the precursors were processed to faster migrating forms in the presence of a membrane potential and accumulated in a protease-protected location over time (Fig. 4A). To confirm that the faster migrating form corresponded to the mature, processed SURF1 protein, we identified the authentic SURF1 protein by western blotting (Fig. 4A, lane 1). As expected, the imported and processed SURF1 proteins co-migrated with the authentic SURF1 protein. Moreover, all tested
about 60% of the imported SURF1WT or SURF1Y274D were detectable after 4 h of chase, SURF1G124E and SURF1G124R were rapidly turned over with half-lives of about 10 min (Fig. 4C). Hence, the mutations of the highly conserved G124 in SURF1 (G137 in Shy1) destabilized the protein, leading to rapid degradation within mitochondria by the organelar quality control system.

**SURF1Y274D accumulates in cytochrome c oxidase assembly intermediate**

While the reduced stability of SURF1G124E and SURF1G124R fully explains the cytochrome c oxidase deficiency of patients, the SURF1Y274D allele gave rise to a stable product that is properly located within mitochondria. To assess why this form of SURF1 is affected, we analyzed the assembly of SURF1Y274D into protein complexes. Cytochrome c oxidase assembly in mammals has been mainly studied by using dodecylmaltoside as a detergent for complex solubilization. So far, no physical interaction of SURF1 has been found using this detergent. Similarly, physical interaction partners of Shy1 were identified only when a mild detergent (digitonin) was used (39,40,42). Hence, we established conditions to separate digitonin-solubilized SURF1 protein complexes by BN-PAGE and identified them by western blotting. Antiserum directed against human SURF1 detected several protein complexes of different sizes, with two dominant complexes at approximately 100 and 200 kDa (Fig. 5A, lane 2). In contrast, the majority of mature cytochrome c oxidase complex runs at 400 kDa under these conditions (Fig. 5A, lanes 3–5). Interestingly, the antibody directed against human COX1 recognized an additional protein complex at about 200 kDa, which did not co-migrate with the SURF1 complex (Fig. 5A lanes 2 and 3). Since we had no access to SURF1 patient cell lines, we addressed the ability of SURF1Y274D to incorporate into the observed complexes by importing the radiolabeled precursor into isolated mitochondria. Radiolabeled wild-type SURF1 was incorporated, in a membrane potential-dependent manner, into 100 and 200 kDa complexes similar in size to those detected by western blotting (Fig. 5B). After long import times, a faint band at about 400 kDa was detected, which corresponded in size to the mature cytochrome c oxidase. In agreement with this conclusion, an assembly of radiolabeled COX6A1 revealed a complex of similar size (62) (Fig. 5B, lanes 3 and 5). In contrast, SURF1Y274D assembled preferentially into the 200 kDa complex, and neither the 400 kDa nor the more dominant 100 kDa SURF1 complex were detected (Fig. 5C). In a direct comparison, we found that under conditions where similar amounts of SURF1WT and SURF1Y274D were imported, the import of both proteins was virtually indistinguishable (Fig. 5D, bottom panel). However, compared with the wild-type form, an increased amount of the mutant form accumulated in the 200 kDa complex in wild-type mitochondria (Fig. 5D, lane 2 versus 6, and Supplementary Material, Fig. S1A).

To address the nature of the 200 kDa SURF1 complex, we treated cells with antibiotics affecting cellular translation before isolation of mitochondria. Chloramphenicol (CAP) specifically inhibits mitochondrial translation, thereby depleting the pool of mitochondrial translation products that are
consumed either by being assembled into mature complexes or by degradation. Cycloheximide (CHX) inhibits cytosolic translation and stops the supply of nucleus-encoded cytochrome c oxidase subunits. Consequently, assembly intermediates containing mitochondria-encoded subunits can be accumulated, as it has been shown for the F1Fo-ATP synthase and cytochrome c oxidase in yeast (48,63) or NADH dehydrogenase and cytochrome c oxidase in mammals (64,65). In mitochondria isolated from CAP-treated cells, less of both SURF1WT and SURF1 Y274D incorporate into the 200 kDa complex (Fig. 5D, lanes 2 and 3, 6 and 7), indicating that the formation of this complex is dependent on mitochondrial translation, most likely newly synthesized COX1. Thus, this complex is likely to represent a novel assembly intermediate containing SURF1.

In mitochondria from CHX-pretreated cells, SURF1WT associates with this complex to the same extent as in untreated cells (Fig. 5D, lanes 2 and 4), whereas SURF1 Y274D accumulates in the 200 kDa form (Fig. 5D, lanes 6 and 8). This indicates that association of SURF1 Y274D to this intermediate is promoted, either owing to a higher affinity of SURF1 Y274D trapping this intermediate form, or to a reduced off-rate.

### The Y344D exchange uncouples Cox1 translational regulation from assembly

SURF1 Y274D appears to be stalled in the assembly process in an intermediate complex that depends on mitochondrial translation products. In yeast, defects in cytochrome c oxidase assembly shut down translation of the central subunit Cox1 in a negative feedback-regulation mechanism, involving the translational activator Mss51. As no robust homolog of Mss51 has been identified and translational regulation of Cox1 synthesis is barely studied in mammals, we decided to study the effects on translational regulation by mimicking SURF1 mutations in the yeast model.

First, we asked if Cox1 synthesis was affected by the different SHY1 alleles, as reported for shy1Δ cells. Therefore, we performed labeling experiments of mitochondria-encoded translation products in intact yeast cells, in the presence of CHX that inhibits
cytosolic translation. Whereas the shy1G137E and shy1G137R mutants displayed reduced Cox1 synthesis, similar to the shy1Δ mutant, cells expressing Shy1Y344D showed wild-type-like Cox1 expression (Fig. 6A). This was also the case when cells were grown at non-permissive temperature (Supplementary Material, Fig. S1B). Nevertheless, the steady-state levels of Cox1 were reduced in shy1Y344D mitochondria (Fig. 2A). How can this finding be explained? In yeast, defects in cytochrome c oxidase assembly inactivate the translational activator Mss51 in a complex consisting of newly synthesized Cox1, and the assembly factors Cox14, Cox3 and Coa1. This inactivation leads to a reduction of Cox1 synthesis (48,51,66). The negative feedback regulation mechanism is active in the absence of Shy1 and thus Cox1 expression is reduced in shy1Δ, shy1G137E and shy1G137R mitochondria (Fig. 6A). However, Shy1Y344D fails to downregulate Cox1 synthesis, although cytochrome c oxidase assembly is defective. To assess the reason for this unexpected effect, we analyzed the interactions of the assembly factors that inactivate Mss51 by co-immunoprecipitation experiments in shy1Y344D, shy1Δ and wild-type mitochondria. Antibodies directed against Coa1 efficiently precipitated Coa1 in all tested mitochondria (Fig. 6B). Compared with wild-type, significantly more Mss51 and Cox1 were co-precipitated with Coa1 in shy1Δ mitochondria (Fig. 6B, lanes 5 versus 7). Apparently, assembly intermediates containing Cox1 accumulate in this mutant. However, in case of shy1Y344D, the amount of Mss51 and Cox1 that co-precipitated with Coa1 was similar to wild-type (Fig. 6B, lanes 7 versus 9). Since Coa1 is important for the inactivation of Mss51 in a late step of the regulatory cycle, we performed co-immunoprecipitation experiments using antibodies directed against Coa3 that is involved from an early step in the regulatory cycle until later steps (48,53). Similar to Coa1, Coa3 was efficiently precipitated by the antibodies in all mutants (Fig. 6C). In agreement with an accumulation of assembly intermediates, in shy1Δ mitochondria more Mss51, Coa1 and Cox1 were co-precipitated with Coa3 compared with wild-type (Fig. 6C, lanes 5 versus 7). However, in the shy1Y344D mutant, Mss51 and Cox1 were co-precipitated at wild-type levels. These observations indicate that unlike shy1Δ mutant mitochondria, Mss51 is not efficiently sequestered and inactivated in shy1Y344D mitochondria. This finding fully explains the wild-type-like Cox1 expression in the mutant (Fig. 6A).

This observation was unexpected considering that the Cox1 protein levels were reduced in Shy1Y344D mitochondria at steady state (Fig. 2A). We speculated that the newly synthesized Cox1 was degraded in shy1Y344D mitochondria by the mitochondrial quality control system. Thus, we performed pulse-chase experiments of mitochondrial translation products. In wild-type mitochondria, the translation products remained stable over the time course of the experiment (Fig. 6D, lanes 1–5), while the Cox1 protein as well as Cox2 and Cox3 were destabilized in shy1Δ and coa1Δ mutants as signals decreased significantly over time (Fig. 6D). In a quantification of the stability assays, it became apparent that the stability of Cox1 in shy1Y344D mutant mitochondria was less affected than in the other mutants (Fig. 6E). This finding explains the observed differences in Cox1 levels at steady state (Fig. 2A).

Shy1Y344D exerts a dominant-negative effect on Cox1 stability

Since the stability of newly synthesized Cox1 was reduced in the shy1Y344D background, we wondered if this effect was dominant over the wild-type allele. Therefore, we overexpressed the SHY1Y344D allele in wild-type and shy1Δ cells, and performed pulse-chase experiments of mitochondrial translation products. It should be noted that overexpression of the SHY1Y344D allele did not impair the growth of the resulting mutant at conditions tested (data not shown). However, wild-type-like growth has been reported to occur even when respiratory chain activity is reduced to below 10% (67–69). Thus, we compared the expression and stability of newly synthesized Cox1 in the presence and absence of a Shy1WT copy. As expected from the mutant analyses, expression of Cox1 was not affected by increased levels of Shy1Y344D (Fig. 7A, pulse). However, overexpression of the SHY1Y344D allele in a wild-type background resulted in the destabilization of

Figure 7. Shy1Y344D overexpression shows a dominant-negative effect on Cox1 stability in yeast. (A) Quantification of Cox1 synthesis (pulse, 15 min) and stability (chase, 135 min) as described in Fig. 6A in indicated strains (SEM, n = 3). Given in brackets are the proteins expressed from transformed plasmids. Overexpression is indicated by arrows (↑). (B) Whole cell extracts of indicated yeast strains were separated by SDS–PAGE and analyzed by western blotting. The strains expressed Shy1 (wild-type or the Shy1Y344D) from CEN/ARS or 2μ (†) plasmids, or contained empty plasmid (−) in a shy1Δ or wild-type (WT) background as indicated.
newly synthesized Cox1 (Fig. 7A, chase). Thus, raising the level of Shy1Y344D led to a dominant-negative effect on Cox1 stability. Consistently, the analysis of the steady-state protein levels of Cox1 in different mutants showed that co-expression of Shy1WT and Shy1Y344D at similar levels had little effect on the Cox1 levels (Fig. 7B, lane 5). However, by overexpressing the Shy1Y344D allele, the amount of Cox1 dropped significantly (Fig. 7B, lane 6). This finding illustrates that the Shy1Y344D mutant displays a dominant-negative effect on Cox1 stability and Cox1 levels when overexpressed, but escapes the negative feedback-regulation mechanism of Cox1 synthesis.

**DISCUSSION**

In this study, we analyzed three mutations affecting conserved amino acids in SURF1 and its yeast homolog Shy1 in order to gain insight into the role of SURF1/Shy1 in the cytochrome c oxidase assembly process and into the molecular pathologies caused by the described mutations. Missense mutations that lead to these exchanges have been reported in Leigh Syndrome patients and thus are known to affect cytochrome c oxidase biogenesis in a yet unresolved manner (55–57).

In cases of mitochondrial disorders, previous studies reported that in addition to direct effects on protein function, single amino acid exchanges may lead to mislocalization of mitochondrial proteins. Examples for a role of mitochondrial protein mistargeting in the molecular pathology of a human disorder have been reported, e.g. for tafazzin, a protein involved in Barth Syndrome (70,71). In the case of SURF1/Shy1, we demonstrate that the single amino acid exchanges do not affect mitochondrial transport or maturation of the mutant proteins. Thus, defective transport does not explain the low mitochondrial amounts of the G124E/R SURF1 mutants or of their yeast counterparts. However, whereas SURF1Y274D (Shy1Y344D) represents a stable protein, exchanging G124 (G137 in yeast) to bulkier, charged amino acids, destabilizes the protein after import, leading to rapid degradation by the mitochondrial quality control system in human and yeast mitochondria. Thus, Shy1G137E and Shy1G137R are barely detectable in mutant mitochondria resulting in the deletion-like phenotype at normal temperatures. Hence, it is not surprising that patients bearing mutations affecting G124 are phenotypically indistinguishable from patients who have more severe alterations in the SURF1 gene (55,56).

Absence of Shy1 from yeast mitochondria causes the accumulation of cytochrome c oxidase assembly intermediate complexes, which contain the COX1-specific translational activator Mss51. In these complexes, Mss51 is considered to be inactive; consequently reduced synthesis of Cox1 is observed. Certain MSS51 mutations have been found to suppress the shy1△ phenotype (45), but it is unclear how they affect the activity cycle of Mss51. Some unassigned Cox1 species act as pro-oxidants, generating reactive oxygen species (72). Therefore, this negative feedback regulation is an important mechanism to prevent the accumulation of misfolded or otherwise deleterious Cox1 species when the cytochrome c oxidase assembly process is defective (51,66). We find that yeast strains expressing Shy1G137E or Shy1G137R display functional negative feedback regulation. Owing to the defect in cytochrome c oxidase assembly, Cox1 expression is reduced. In contrast, in the case of Shy1Y344D, translation of Cox1 is indistinguishable from wild-type mitochondria. In agreement with this finding, assembly intermediates containing Mss51 do not accumulate in this mutant, unlike in shy1△ mitochondria. However, genetic analyses indicate that Shy1Y344D allows for the Cox1 assembly to progress to the pro-oxidant state that requires heme a insertion (42,47). Apparently, Shy1 displays dual functionality in the biogenesis process: on the one hand, Shy1Y344D releases the translational block and thus promotes wild-type-like synthesis of Cox1; on the other hand, it is not able to fulfill its physiologic function in cytochrome c oxidase assembly, despite the successful insertion of heme a into Cox1. This defect appears more severe at reduced temperatures (19°C) in yeast, but leads to only slight growth reduction at 30°C on non-fermentable carbon sources. Thus, the shy1Y344D mutant displays uncoupling of the two Shy1 functions, in the translational regulation of the central subunit Cox1 and in later steps of cytochrome c oxidase biogenesis. Interestingly, upon overexpression, Shy1Y344D exhibits a dominant-negative effect on the assembly process, suggesting that it generates a block or bottleneck in the biogenesis pathway after heme insertion into Cox1. Thus, the shy1Y344D mutant could represent an ideal tool for further analyses of both processes, independent of each other.

Recent studies used the yeast model to analyze early steps of cytochrome c oxidase assembly, and the involvement of Shy1 in this process. Shy1 is associated with different assembly intermediate complexes (39,40,42,47,48). Although a lack of functional SURF1 in cells derived from Leigh Syndrome patients revealed potential assembly intermediates (43,44), these studies did not identify an association of SURF1 with any of these complexes. Here, we utilized mild solubilization conditions to separate SURF1-containing complexes by BN-PAGE. Similar to Shy1, SURF1 is present in several protein complexes, with apparent sizes of about 100, 200 and 400 kDa on BN-PAGE. The 400 kDa complex co-migrated with mature cytochrome c oxidase and was only visible after long import times. This finding suggests that a subgroup of SURF1 associates with mature cytochrome c oxidase complexes. Whereas protein associations with respiratory chain complexes have been well-characterized in yeast (39,48,73,74), they await further evidence in mammalian mitochondria. In the case of SURF1/Shy1, the exact function of this association is still unknown; however, it could be speculated that SURF1 can fulfill its function on an assembled cytochrome c oxidase or is involved in repair processes. Such a repair function for mature membrane complexes is not without precedence and has, for example, been described for the photosystem II in chloroplasts, where part of the complex has a high turnover rate (75), as it is exposed to a high amount of electrons, similar to the cytochrome c oxidase. We propose that the 200 kDa SURF1 complex is a bona fide assembly intermediate, as its formation was strongly reduced when cells were depleted of mitochondria-encoded subunits. Interestingly, the SURF1Y274D protein assembles predominantly into this complex, and appears to accumulate when assembly intermediates are enriched by blocking cytosolic translation. Obviously, the association of the mutant protein
to this intermediate is altered when compared with the wild-type protein, as identical wild-type mitochondria have been used for the import and assembly analysis of both proteins. If SURF1\(^{Y244D}\) has a higher affinity to the intermediate or a reduced off-rate from the complex cannot be judged by our analyses. However, the mutant protein generates a bottleneck that becomes apparent at the stage of this intermediate and affects later steps in the assembly pathway. This specific effect on the assembly function might explain the dominant-negative effect of the corresponding Shy1 mutant upon over-expression in yeast. Under these conditions, Shy1\(^{Y344D}\) might titrate the wild-type copy from intermediates and consequently leads to release of the translational regulator, Miss51. However, it cannot fulfill its assembly function and Cox1 is trapped in this assembly intermediate containing Shy1\(^{Y344D}\). Eventually, Cox1 is degraded by the mitochondrial quality control system. Similar to Shy1\(^{Y344D}\), the SURF1\(^{Y274D}\) mutant did not have this dominant effect when co-expressed with wild-type SURF1 at similar levels. This could explain why a heterozygous SURF1\(^{Y274D}\) allele does not become apparent, as was seen in the father of the first case where this mutation was described (57).

As a result of the defective assembly process, the shy1\(^{Y344D}\) mutant displays reduced levels of mature cytochrome c oxidase complex and a severe reduction in mitochondria-encoded cytochrome c oxidase subunits, such as Cox1 and Cox2. The observation that Cox1 is synthesized with similar rates as in wild-type supports the conclusion that newly synthesized Cox1 is degraded in the mutant. Interestingly, the rate of Cox1 degradation is slightly lower than in the case of cells lacking Shy1 or other assembly factors. Mitochondrial proteases of the AAA family in the inner mitochondrial membrane have been reported to be involved in degrading misfolded membrane proteins in mitochondria, including mitochondria-encoded proteins, such as Cox1 (76,77). Recently, the metalloproteinase Oma1 (78) was reported to contribute to the degradation of Cox1 as well (79). It is tempting to speculate that either Oma1 or the AAA proteases are also responsible for the degradation of Cox1 in shy1 mutants. Moreover, they are likely to be involved in the degradation of mutant, unstable Shy1. Interestingly, defective cytochrome c oxidase assembly, as in the case of Shy1 malfunction, could be partially overcome by mutations affecting the mitochondrial quality control system (49,79). Moreover, increasing Cox1 stability by overexpressing nucleus-encoded subunits that assemble early with Cox1 could overcome the lack of Shy1 in yeast mutants (46). If such strategies can be considered for therapeutic approaches remains speculative. However, the residual cytochrome c oxidase activity of up to 20% (33,36) in the absence of either SURF1 or Shy1 shows that assembly of the cytochrome c oxidase complex—although being impaired—is possible, and further stabilization of Cox1 might be a key to further improve cytochrome c oxidase assembly and function.

Taken together, our analyses explain the molecular mechanisms leading to the development of Leigh Syndrome in patients bearing the described missense mutations in the SURF1 gene. Mutations of the invariant G\(^{124}\) produce an instable protein that is rapidly degraded within mitochondria.

### Table 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Backbone</th>
<th>Insert</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS416 (-)</td>
<td>pRS416</td>
<td>SHY1(^{+})</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pDaM20 (Shy1(^{WT}))</td>
<td>pRS416</td>
<td>SHY1-(G410A)(^{-})</td>
<td>This study</td>
</tr>
<tr>
<td>pDaM21 (Shy1(^{G137E}))</td>
<td>pRS416</td>
<td>SHY1-(G410A)(^{-})</td>
<td>This study</td>
</tr>
<tr>
<td>pDaM22 (Shy1(^{Y344D}))</td>
<td>pRS416</td>
<td>SHY1-(T1030G)(^{-})</td>
<td>This study</td>
</tr>
<tr>
<td>pDaM23 (Shy1(^{Y344D}))</td>
<td>pRS416</td>
<td>SHY1-(T1030G)(^{-})</td>
<td>This study</td>
</tr>
<tr>
<td>pDaM24 (Shy1(^{Y344D}))</td>
<td>pRS426</td>
<td>SHY1(^{+})</td>
<td>This study</td>
</tr>
<tr>
<td>pDaM27 (Shy1(^{Y344D}))</td>
<td>pRS426</td>
<td>SHY1-(T1030G)(^{-})</td>
<td>This study</td>
</tr>
<tr>
<td>pDaM10 (Shy1(^{WT}))</td>
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<td>SHY1(^{+})</td>
<td>Mck et al. (2007)</td>
</tr>
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<td>This study</td>
</tr>
<tr>
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<td>pGEM4Z</td>
<td>SHY1-(G410A)(^{-})</td>
<td>This study</td>
</tr>
<tr>
<td>pDaM13 (Shy1(^{Y344D}))</td>
<td>pGEM4Z</td>
<td>SHY1-(T1030G)(^{-})</td>
<td>This study</td>
</tr>
<tr>
<td>pRR7 (SURF1(^{WT}))</td>
<td>pGEM4Z</td>
<td>SURFI(^{+})</td>
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<td>pRR13 (SURF1(^{Y344D}))</td>
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<td>This study</td>
</tr>
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<td>pRR14 (SURF1(^{Y274D}))</td>
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<td>SURFI-(T280G)(^{-})</td>
<td>This study</td>
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</tbody>
</table>

\(^{+}\)Including 327 bp upstream and 363 bp downstream of the ORF.

In contrast, the Y\(^{274D}\) mutant protein (Y\(^{344D}\) in yeast) remains stable and does not affect synthesis of the central cytochrome c oxidase subunit, Cox1, in yeast. However, it exhibits defects in the subsequent assembly events that lead to the accumulation of SURF1\(^{Y274D}\) in assembly intermediates that depend on mitochondrial translation, such as newly synthesized COX1. Inevitably, these products are degraded and cytochrome c oxidase assembly fails. A better understanding of the principles that underlie mammalian mitochondrial quality control may enable researchers to alter the degradation of potential fruitful intermediates, which could improve the fate of proteins and patients alike.

### MATERIALS AND METHODS

#### Molecular cloning

**SHY1** with flanking promoter and terminator sequences was cloned from yeast (YPH499) genomic DNA into pRS416 and pRS426 (Stratagene). For *in vitro* transcription/translation, the open reading frames of SHY1 and SURF1 (from a cDNA Clone, Imagenes IRATp970C0445D) were cloned under transcription/translation, using the SP6-promoter into pGEM4Z (Promega). All plasmids were verified by sequencing (see Table 1 for details).

#### Yeast culturing, isolation of mitochondria and sample preparation

Saccharomyces cerevisiae strains used in this study were derived from YPH499 (80). shy1Δ and coa1Δ strains (DaMY21 and DaMY22) were described previously (39). Plasmids were transformed into yeast as described (81). Yeast cultures were grown at 30°C (unless indicated otherwise) in rich medium: 1% yeast extract, 2% peptone and 2% dextrose (YPD) or 3% glycerol (YPG). For *in vivo* labeling and growth of plasmid-containing yeast cells, synthetic medium was used: 0.67% yeast nitrogen base (Difco, BD) and 2% glucose (SD) or galactose (SGal). Medium was
supplemented with 0.7 g/l drop-out mix of CSM–Ura (MP Biomedicals) and 20 μg/ml of uracil was added if required. For growth tests, serial dilutions (1:10) of overnight yeast cultures were spotted on YPD- and YPG-plates and incubated for 3–5 days at indicated temperatures. Yeast whole-cell extracts were obtained from cultures in log-phase as described, with the exception that the acetone wash was omitted (82). Yeast mitochondria were isolated from cells grown at 30°C or 19°C in YPG or SGal-Ura as described (83).

Isolation of mitochondria from cultured human cells

HEK293T cells were cultured at 37°C under 5% CO₂ atmosphere in Dulbecco’s Modified Eagle Medium containing 10% fetal bovine serum (Gibco, Invitrogen). For inhibition of mitochondrial or cytosolic translation, the medium was supplemented with 50 μg/ml of CAP for 16 h or 100 μg/ml of CHX for 2 h, respectively. Cells were harvested and mitochondria were isolated after cell homogenization by differential centrifugation as described (62).

In vitro import and assembly, stability assay of imported proteins

Precursor proteins were radiolabeled with [35S]methionine by in vitro translation: Shy1 and its mutant derivatives were translated from plasmids using the TNT SP6 Quick Coupled Transcription/Translation system (Promega). SURF1 and its mutant forms were PCR-amplified from plasmids (using SP6 and T7 Primers). Subsequently, mRNA was transcribed and purified using the mMessage mMachine SP6 system and the MEGAclear kit (Ambion). The SURF1 precursor proteins were subsequently translated using the Flexi Rabbit Reticulocyte Lysate system (Promega).

Precursor proteins were imported into the isolated yeast or human mitochondria as described previously (62,84). After in vitro import, samples were processed for SDS (sodium dodecyl sulfate)– or BN-PAGE analysis and radiolabeled proteins detected by digital autoradiography.

To monitor the stability of imported proteins, an in vitro import-pulse/chase study was performed according to Röttgers et al. (85). In brief, in vitro import into isolated mitochondria was performed for 5 min at 25°C (yeast) or 37°C (human). The import was stopped by the addition of 1% AVO mix (100 μM valinomycin, 1 mM antimycin A and 2 mM oligomycin in ethanol) and samples were taken before and after the precursor protein was degraded by incubation with proteinase K (40 μg/ml) for 10 min on ice. Mitochondria were re-isolated and resuspended in pre-warmed, energized import buffer. Samples were taken after incubation at 30°C (yeast) or 37°C (human).

In vivo labeling and stability assay for mitochondrial translation products

Labeling of mitochondrial translation products was performed in whole cells essentially as described (48). In brief, mitochondrial translation products were radiolabeled using logarithmically growing yeast cells. SGal-Ura or SGal cultures were grown to OD₆₀₀ of 1.0. From these cultures, 1.0 OD₆₀₀ equivalents were harvested by centrifugation and washed with labeling buffer (40 mM potassium phosphate pH 6.0, 2% galactose). Subsequently, cells were resuspended in the labeling buffer and incubated at 30°C or 19°C. After 10 min, CHX was added to a concentration of 150 μg/ml to stop cytoplasmic translation. After 5 min, labeling was started by the addition of 40 μCi [35S]methionine. After labeling for 5 or 15 min at 30°C (10 or 30 min at 19°C), samples were taken, excess methionine added and after further incubation, translation stopped on ice. To analyze the stability of mitochondrial translation products, labeling was performed with 1.0 OD₆₀₀ equivalents of cells in labeling buffer for 15 min at 30°C. Mitochondrial translation was stopped by the addition of CAP and methionine. After further 2 min incubation, a pulse sample was taken. Chase samples were taken after further incubation at 30°C. To visualize radiolabeled proteins, whole-cell extracts were prepared as described above and analyzed by SDS–PAGE and digital autoradiography.

Co-immunoprecipitation

Mitochondria were solubilized in buffer (1% digitonin, 60 mM NaCl, 5 mM EDTA, 10% (w/v) glycerol, 20 mM Tris–HCl, pH 7.4) for 30 min at 4°C with mild agitation. After a clarifying spin, a loading control was taken, the supernatant divided and incubated with antibody-coated beads for 60 min at 4°C. Therefore, polyclonal antibodies raised against Coa1, Coa3 or control serum were coupled with Protein A-Sepharose (GE Healthcare) by chemical cross-linking with dimethyl pimelimidate. After extensive washing of the beads with buffer, bound proteins were eluted with 0.1 M glycine, pH 2.5 and neutralized with 1 M Tris base. Samples were analyzed by SDS–PAGE and western blotting.

Miscellaneous

SDS–PAGE and western blotting of polyvinylidene fluoride membranes were performed using standard methods. Proteins of interest were probed using primary antibodies raised in rabbit and horseradish-peroxidase- or fluorophore-conjugated secondary anybodies. Signals were detected using the ECL enhanced chemiluminescence detection system and X-ray films (GE Healthcare) or by laser scanning (FLA-9000, Fuji-film). For BN-PAGE, mitochondria were solubilized in 1% digitonin, 20 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% (w/v) glycerol, 1 mM phenylmethylsulfonyl fluoride for 30 min at 4°C and the unsoluble material was sedimented at 20 000g and 4°C for 15 min. After addition of 10X loading dye (5% Coomassie brilliant blue G-250, 500 mM 8-aminon caproic acid, 100 mM Bis–Tris, pH 7.0), the supernatant was separated on a 4–13% polyacrylamide gradient gel as previously described (86). Storage Phosphor Screens (GE-Healthcare) were used to detect radioactively labeled proteins. The signals were digitalized using a scanner (Storm820, GE Healthcare) and quantified using ImageQuant TL software (GE Healthcare).
SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS
We are grateful to Drs M. Deckers, M. van der Laan, C. Meisinger and N. Wiedemann for helpful discussions. We thank C. Schulz for critical comments during the course of the project and J. Melin for comments on the manuscript.

Conflict of Interest statement. The authors declare that they have no conflict of interest.

FUNDING
This work was supported by grants of the Deutsche Forschungsgemeinschaft, the Niedersächsisches VW Vorab and the Max-Planck-Society.

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