

DEFINING THE ROLE OF Hem25 IN MITOCHONDRIAL
FUNCTION: IMPLICATIONS FOR CONGENITAL
SIDEROBLASTIC ANEMIA

by

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Abstract

Congenital sideroblastic anemia (CSA) is a hematological disease caused by mutations in genes that result in a defect in heme/hemoglobin biosynthesis. Mutations in *SLC25A38* cause CSA. I determined that SLC25A38, and its budding yeast *Saccharomyces cerevisiae* homologue Hem25, are mitochondrial glycine transporters with the imported glycine used in the first enzymatic step in heme synthesis. I also performed genetic interaction analyses and determined that loss of function of a small subset of the SLC25 members found in yeast results in decreased fitness for cells lacking Hem25 function. The genetic interaction between *FLX1*, encoding a flavin transporter, and *HEM25* was studied in further detail. My results showed that the abundance in electron transport chain subunits was decreased in *flx1* Δ *hem25* Δ cells. My genetic screen may help discover new pathways and processes that affect cell fitness when Hem25 function/heme synthesis is deficient.

List of Abbreviations and Symbols Used

5-Ala 5-aminolevulinic acid.

Aac3 mitochondrial ADP/ATP translocator.

ADP adenosine diphosphate.

ALAD aminolevulinic acid dehydratase.

ALAS aminolevulinic acid synthases.

Arg arginine.

ATP adenosine triphosphate.

BACH1 Btb and Cnc Homology 1.

CH₂-THF 5,10-methylene-tetrahydrofolate.

CH-THF 5,10-methenyl-tetrahydrofolate.

CHO-THF 10-formyl-tetrahydrofolate.

Cor2 subunit 2 of ubiquinol cytochrome-c reductase.

Cox2 subunit 2 of cytochrome c oxidase.

Cox4 subunit 4 of cytochrome c oxidase.

CPOX coproporphyrinogen oxidase.

CSA congenital sideroblastic anemia.

Cyc2 ferric reductase.

Cyt1 cytochrome c1.

DMT1 transmembrane iron transporter.

EDTA ethylenediaminetetraacetic acid.

eIF2 α eukaryotic translation initiation factor α subunit.

ETC electron transport chain.

FAD flavin adenine dinucleotide.

Fe-S iron-sulfur.

FECH ferrochelatase.

Flx1 mitochondrial flavin adenine dinucleotide transporter.

Fre1 ferric reductase.

G418 geneticin aminoglycoside antibiotic.

GCV glycine cleavage system.

Gly1 threonine aldolase.

Hap1 heme activator protein 1.

Hb hemoglobin.

Hem1 5-aminolevulinate synthase.

Hem14 protoporphyrinogen oxidase.

His histidine.

HMC higher-order complex.

Hph hygromycin.

HRE heme responsive element.

HRI heme-regulated inhibitor.

HRM heme regulatory motif.

IDT integrated DNA technologies.

IRE iron responsive element.

IRP iron responsive protein.

KGD ketoglutarate dehydrogenase complex.

LCR locus control region.

Lpd1 dihydrolipoamide dehydrogenase.

MAREs Maf recognition elements.

MATa mating type protein a.

mM milli molar.

Mtm1 Mitochondrial pyridoxal 5'-phosphate (PLP) transporter.

n.s. not significant.

NatMX4 nourseothricin acetyltransferase.

Ndi1 NADH ubiquinone oxidoreductase.

NF-E2 nuclear factor erythroid 2.

NGS Next Generation Sequencing.

OMIM online mendelian inheritance in man.

ORF open reading frame.

Ort1 mitochondrial ornithine transporter.

PBGD porphobilinogen deaminase.

Pet8 mitochondrial S-adenosylmethionine transporter.

PLP pyridoxal 5-phosphate.

PMPS Pearson marrow-pancreas syndrome.

PMSF phenylmethanesulfonylfluoride.

Por1 mitochondrial porin.

PPIX protoporphyrin IX.

PPO protoporphyrinogen oxidase.

rpm rotations per minute.

RSA random spore analysis.

SAM S-adenosylmethionine.

SC synthetic complete.

SC-ura SC without uracil.

SD synthetic defined.

SD-ura SD medium without uracil.

Sdh1 subunit 1 of succinate dehydrogenase.

Sdh2 subunit 2 of succinate dehydrogenase.

SDS sodium dodecyl sulfate.

SDS-page sodium dodecyl sulfate polyacrylamide gel electrophoresis.

SEM standard error of the mean.

Ser1 3-phosphoserine aminotransferase.

Sfc1 mitochondrial succinate-fumarate transporter.

SGA synthetic genetic array.

SGD *Saccharomyces* Genome Database.

Shm1 mitochondrial glycine hydroxymethyltransferase.

Shm2 cytosolic glycine hydroxymethyltransferase.

sMaf small Maf proteins.

Sro9 cytoplasmic RNA-binding protein.

succinyl-CoA succinyl coenzyme A.

TCA tricarboxylic acid.

TEMED tetramethylethylenediamine.

TfR1 transferrin receptor protein 1.

THF tetrahydrofolate.

TRMA thiamine-responsive megaloblastic anemia.

Ura uracil.

UROD uroporphyrinogen III decarboxylase.

UROS uroporphyrinogen III synthase.

WES whole exome sequencing.

WGS whole genome sequencing.

WT wild type.

XLSA X-linked recessive sideroblastic anemia.

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Chapter 1

Introduction

Congenital sideroblastic anemia is a rare disease affecting 1:50,000 infants born [10], that can be caused by mutations in several genes. Although a substantial number of mutations in several genes and mechanisms responsible for CSA are known to date, there are many cases that are presumed to be of a genetic nature where their molecular basis is still unknown. The most common form of CSA is X-linked recessive sideroblastic anemia (XLSA), which is caused by mutations in *ALAS2* [8]. This gene codes for a mitochondrial erythroid-specific 5-aminolevulinic acid (5-Ala) synthase that requires pyridoxal 5-phosphate (PLP) as a cofactor. More than 50 different mutations have been identified in *ALAS2*, and a considerable number of them can be cured by supplementation with pyridoxine [58]. The second most common form of CSA is due to mutations in *SLC25A38* [8, 37, 42]. Unlike *ALAS2*, the function of the SLC25A38 is not known, and patients with mutations in *SLC25A38* are pyridoxine refractive and are awaiting a cure.

The main objective of this thesis was to study the function of SLC25A38 to give a molecular explanation for the cause of the disease, and to find a possible therapy for patients that suffer CSA due to mutations in *SLC25A38*. In order to study this protein, all the experiments contained in this thesis were carried out in the budding yeast *Saccharomyces cerevisiae*, where *HEM25* is the yeast homologue of *SLC25A38*.

1.1 Gene Identification for Rare Diseases

Rare diseases are defined as such because they affect a very small percentage of the population, normally less than 1 in 2,000 persons [11]. However, collectively they affect millions of individuals worldwide. The severity of rare diseases can vary from individuals that have quite a good life quality due to the existence of a therapy, to individuals that suffer and even die at an early age due to a lack of therapy. Unfortunately, 75% of affected individuals are children who have high morbidity and

many of them die at an early age [10]. It is hard to provide an exact number of rare diseases caused by mutations in a single gene, but there is an estimation of 6,000 to 7,000 based on the online mendelian inheritance in man (OMIM) and Orphanet (inventory of rare diseases) databases. The etiology of just 3,500 rare diseases is known.

Some of the genes that cause rare diseases were determined primarily based on linkage mapping and candidate gene analysis. However, since 2010, more than 180 novel genes were discovered using Next Generation Sequencing (NGS) with the first significant increase occurring in 2011 and 2012 [11]. NGS is a revolutionary and powerful tool for the identification of novel gene mutations that cause rare diseases and cancer. NGS has enabled whole genome sequencing (WGS), and whole exome sequencing (WES), which is the protein coding portion (1% of the genome). The sequences can be compared with those in databases to distinguish disease-associated variants from the variants that typically exist among individuals. Disease-associated variants can be inherited or *de novo* and the rate-limiting step in the discovery of disease-causing genes is the validation of these variants. For this validation, it is necessary to analyze the same gene in unrelated individuals or families, but when there is only one family affected a functional study of the gene is often required. However, the success of NGS in identifying a disease-causing gene is hard to measure because negative studies are not published. The disease-causing gene for many rare diseases has not been found yet and it is suggested that this could be due to the presence of mutations outside of the exome, i.e. in the non-coding region. To identify these mutations would require WGS.

Rare diseases can be caused by mutation in genes by several mechanisms, but this section is focused on inherited and *de novo* mutations in a single gene. An inherited mutation is when there is a recurrence of a rare disease in a single family or when there is high degree of parental consanguinity, whereas a *de novo* mutation is when the disease has an isolated occurrence. The approach to study these mutations depends on the mode of inheritance and the availability of patients.

There are different strategies to study a rare disease [11]. When there is a familial recurrence of a rare phenotype or consanguinity, it is likely that the rare disease is monogenic. The selection and number of individuals for sequencing depends on the

mode of inheritance of the disease. However, when the disease-causing gene is not known or there is a lack of understanding of the gene function, additional unrelated families for genetic analyses are required for the study. Autosomal recessive disorders have been over-represented with the use of NGS, with more than 115 genes identified. For recessive diseases, in the absence of consanguinity or a founder population, the disease is often due to heterozygous compound mutations where the identification is straightforward because of the small number of affected individuals. In the case of autosomal dominant genes, linkage analysis of a large pedigree is required. However, when the mutation is *de novo* autosomal dominant, it is easier to identify because the affected individual carries a variant that is not present in the parents.

The use of NGS will allow identification of disease-causing genes that will advance the care of patients with rare diseases and allow genetic-testing for diagnostic purposes. Global organizations have formed an International Rare Disease Research Consortium, whose main objective is to create an atlas of rare diseases and to provide diagnostic tools for all rare diseases by the year 2020 [11]. My work focuses on the rare inherited disease CSA.

1.2 Heme and Erythropoiesis

Erythropoiesis is a biological process that consumes large amounts of iron for heme biosynthesis [22]. Heme is the part of the hemoglobin molecule that contains iron. This iron is in charge of binding oxygen reversibly, hence giving hemoglobin the ability to transport oxygen. Heme biosynthesis is a complex process that occurs in all eukaryotic cells. However, most heme biosynthesis occurs during differentiation of erythroid progenitors in the marrow, and there is also a small amount of daily production that takes place in the liver for the synthesis of heme-containing enzymes. The regulatory mechanisms for controlling heme synthesis are different in these two cell types.

Heme is not only a structural component of hemoglobin, but it also plays multiple regulatory roles during the differentiation of erythroid precursors since it controls its own synthesis and regulates the expression of several erythroid-specific genes. Defects of heme synthesis in the erythroid lineage result in sideroblastic anemias or in erythropoietic porphyrias, which are detailed in Section 1.4. The current section

details heme biosynthesis, heme and heme precursor transporters, and the regulatory roles of heme.

1.2.1 Heme Biosynthesis

Heme biosynthesis is carried out by a series of reactions [26], which are catalyzed by eight enzymes located in the cytosol and mitochondrial compartments (Figure 1.1). These enzymes are nuclear-encoded and translated in the cytoplasm. The heme biosynthetic pathway can be divided into four basic processes:

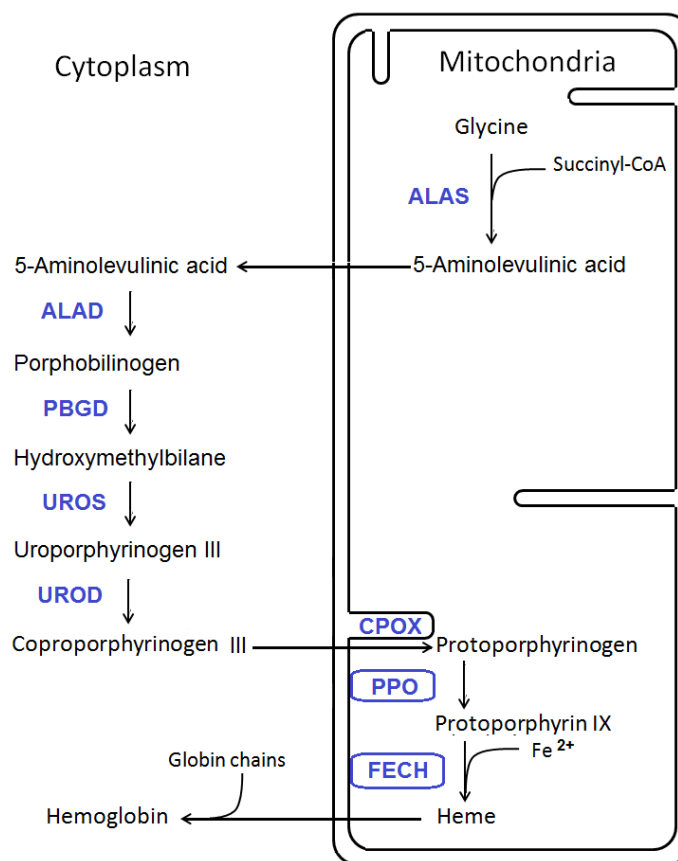


Figure 1.1: **Heme biosynthesis pathway** [48]

Formation of the pyrrole: the first reaction in the pathway takes place in the mitochondria and is the condensation reaction between glycine and succinyl coenzyme A (succinyl-CoA) to form 5-aminolevulinic acid (5-Ala). The reaction is catalyzed by two different aminolevulinic acid synthases (ALAS), one

expressed ubiquitously (ALAS1) and the other expressed only in erythroid precursors (ALAS2). ALAS requires pyridoxal 5-phosphate (PLP) to catalyze the reaction. Then, 5-Ala is exported out of the mitochondria and the second reaction is catalyzed by aminolevulinic acid dehydratase (ALAD). This enzyme condenses two molecules of 5-Ala to generate one molecule of monopyrrol porphobilinogen in the cytoplasm.

Assembly of the tetrapyrrole: the first reaction in this process is catalyzed by porphobilinogen deaminase (PBGD) in the cytoplasm to form a linear tetrapyrrol hydroxymethylbilane by assembling four molecules of monopyrrol porphobilinogen. Then hydroxymethylbilane is converted to uroporphyrinogen III by a reaction catalyzed by uroporphyrinogen III synthase (UROS), which closes the tetrapyrrole macrocycle. All of this process occurs in the cytosol.

Modification of the tetrapyrrole side chains: in this process the first side chain modification is the conversion of uroporphyrinogen III to coproporphyrinogen III. This is a sequential decarboxylation of the four carboxylic groups of the acetic acid side chains catalyzed by uroporphyrinogen III decarboxylase (UROD), which is the last cytoplasmic enzyme in the pathway. The next side chain modification is the conversion of coproporphyrinogen III to protoporphyrinogen IX. It is a sequential oxidative decarboxylation of the propionate groups and the reaction is catalyzed by coproporphyrinogen oxidase (CPOX), which is located in the mitochondrial intermembrane space.

Oxidation of protoporphyrinogen IX to protoporphyrin IX with the insertion of iron: this process is catalyzed by the two final enzymes in the pathway. One is protoporphyrinogen oxidase (PPO) and the other is ferrochelatase (FECH). In the penultimate reaction protoporphyrinogen IX is oxidized to protoporphyrin IX by protoporphyrinogen oxidase. The last reaction of the pathway is catalyzed by FECH and it is the insertion of ferrous iron into protoporphyrin IX. The final product of the heme biosynthesis pathway is heme. Protoporphyrinogen oxidase and FECH are located in the inner mitochondrial membrane.

The reactions catalyzed by ALAS and FECH are the rate-limiting reactions of the heme biosynthesis [26]. FECH and ALAS2 expression are increased during erythroid differentiation and are regulated by transcription factors.

1.2.2 Transport of Heme and Heme Precursors in the Mitochondria

After heme is synthesized, it must be transported across the mitochondrial membrane for normal erythropoiesis. Although all the enzymatic reactions and the compartmentalization are well known, it is not known how 5-Ala, heme and other precursors are transported across the mitochondrial membranes [22].

The exporter of 5-Ala out of the mitochondria has not been determined. It has been hypothesized that the transporter could be SLC25A38 or ABCB10 [85, 20, 22], which are in the mitochondrial inner membrane. SLC25A38 is a member of solute carrier SLC25 family of transporters and is highly expressed in erythrocytes and will be discussed in more detail in Section 1.6. Knock-down of *SLC25A38* gene expression in zebrafish causes anemia and deletion of the *SLC25A38* homologue gene (*HEM25*) in yeast causes defects in 5-Ala biosynthesis [37]. ABCB10 is a member of ABC transporters and has an important role during erythroid differentiation and its over expression promotes hemoglobin synthesis.

The mitochondrial importer of coproporphyrinogen III has not been determined. It has been proposed that ABCB6, which is in the outer membrane and binds porphyrins and heme, could be the transporter [49]. Moreover, ABCB6 expression is positively regulated by the stimulation of erythroid differentiation and by heme levels.

The heme mitochondrial exporter has also not been determined, but there are several lines of evidence that suggest that it is FLVCR1b [21, 29]. One of several studies showed that the over expression of *FLVCR1b* promotes heme biosynthesis, whereas when it is silenced, it causes heme accumulation in mitochondria. Moreover, FLVCR1b is essential for erythroid differentiation. FLVCR1a is a heme exporter in the plasma membrane [85]. Finally, there are several transporters in the cytoplasmic membrane that export heme out of the cell for detoxification [21]. The subcellular trafficking of heme is carried out by chaperones, which bind and transport heme in the cell to incorporate heme into hemoproteins [105].

1.2.3 Regulatory Roles of Heme

Heme is a prosthetic group of proteins, and it also has regulatory roles during erythroid differentiation [22]. Heme regulates the transcription of several genes by binding to specific transcription factors that bind to heme responsive element (HRE) in gene promoters. The regulatory roles of heme during the erythropoiesis are three-fold: to regulate its own synthesis, to control the transcription of erythroid-specific genes, and to regulate protein synthesis.

Heme synthesis is regulated by heme in erythroid and non-erythroid cells

In non-erythroid cells, heme synthesis depends on ALAS1 and this is directly controlled by heme levels. Heme negatively regulates the transcription, translation and stability of ALAS1 [103]. The import of ALAS1 into the mitochondria is also regulated by heme through the interaction with heme regulatory motif (HRM) [62]. When heme binds the HRM present in ALAS1, mitochondrial import of ALAS1 is inhibited. In contrast, in erythroid cells, high heme levels are required for differentiation, and *ALAS2*, which is expressed in erythroid cells, is not regulated negatively by heme. Recent data suggest that heme could increase its own synthesis by the regulation of *ALAS2* expression through the iron responsive element (IRE)–iron responsive protein (IRP) system [101, 38]. *ALAS2* contains a 5' IRE that interacts with IRPs. IRP2 senses the intracellular iron and regulates the expression of *ALAS2* mRNAs by binding to IRE.

ALAS2 is not the only gene involved in iron metabolism that is regulated by the IRE–IRP system [38]. H-ferritin, L-ferritin, m-aconitase and ferroportin mRNAs contain a 5' IRE. The binding of IRPs to the 5' IRE prevents translation. In contrast, the transmembrane iron transporter (DMT1) and the transferrin receptor protein 1 (TfR1) transcripts have a 3' IRE. The binding of IRPs to the 3' IRE protects the mRNA from degradation.

FECH expression is also increased during erythroid differentiation and it is regulated by the transcription factors Sp1 and NF-E2 by binding to GATA elements [53]. At the post-transcriptional level, the expression of FECH is controlled by the availability of iron-sulfur (Fe–S) clusters because FECH is an Fe-S cluster protein [22].

Transcription of erythroid-specific genes is controlled by heme

The transcriptional repressor named Btb and Cnc Homology 1 (BACH1) binds heme. BACH1 senses cellular heme levels and antagonizes the activity on small Maf proteins (sMaf). sMaf bind to Maf recognition elements (MAREs) to activate the transcription of specific genes [65]. Heme inhibits binding of BACH1 with the MARE site of the locus control region (LCR) of globin genes. This allows globin gene expression when heme levels are high, ensuring a balanced synthesis of all constituents of hemoglobin. Furthermore, heme controls the transcription of ubiquitous genes such as heme-oxidase 1, ferritin and ferroportin [57].

Protein synthesis during erythroid differentiation is regulated by heme

The protein synthesis in erythroid precursor cells is dependent on heme levels, which is sensed by a member of a family of protein kinases named HRI. This kinase phosphorylates the α -subunit of the eukaryotic translation initiation factor α subunit (eIF2 α) and inhibits protein synthesis [25]. When heme binds HRI, eIF2 α phosphorylation is decreased and the translation of α - and β -globin mRNAs is increased. In contrast, when heme is low, HRI is activated and causes inhibition of protein synthesis.

1.3 Hemoglobin

Appropriate oxygen levels are vital for cell respiration, playing an important role in the metabolism of human beings. In the human body, oxygen is absorbed by erythrocytes in blood vessels in the lungs and then transported to all the cells in the body. Hemoglobin is responsible for carrying oxygen inside the erythrocyte.

Hemoglobin is a tetramer with a molecular weight of 64.5 kDa. Most of the hemoglobins consist of two α -like and two β -like globin polypeptide chains. Each chain is covalently bound to a heme group. Each heme molecule is composed of an iron atom bound to the protoporphyrin IX ring, which carries the oxygen [99].

In humans, there are seven different globin chains, three α -like globin chains ($\alpha 1$, $\alpha 2$ and ζ), and four β -like globin chains (β , γ , δ and ϵ) [74]. These chains form different types of hemoglobin (Hb), which when mutated are associated with different types of hemoglobinopathies depending on which gene is affected by a mutation: α -

thalassemia, β -thalassemia and sickle cell disease. Globin chain synthesis is encoded by two gene clusters located on chromosome 16p13.3 (α -gene), and 11p15.5 (β -like-genes). The globin gene sequences are only 7 kb in the β -gene region, and the 53 kb flanking sequences contain regulatory elements. These regulatory regions include the LCR, enhancer sequences and the promoter regions of the globin genes [99].

1.4 Diseases Related to Defects in Heme/Hemoglobin Biosynthesis and Iron Overload

As previously mentioned in Section 1.3, several diseases are associated with defects in heme/hemoglobin biosynthesis and iron overload. This section will give a brief overview of diseases associated with defects in heme biosynthesis, such as CSA and porphyrias, and diseases associated with defects in hemoglobin synthesis, such as thalassemias and sickle cell disease. Finally, some diseases associated with iron overload are explained, such as hemochromatosis, disorders associated with ineffective erythropoiesis, defects in iron transporter and iron overload in specific tissues. All the mutations found for these diseases can be found at <http://www.genecards.org/>.

1.4.1 Defects in Heme Biosynthesis

Sideroblastic Anemias

Sideroblastic anemias are a group of anemias defined by the presence of pathologic iron deposits within the mitochondria of erythroid precursors [9]. In these types of anemias, the body has iron available but it cannot be incorporated into heme, and as a consequence oxygen cannot be transported efficiently.

Precursors of red blood cells are named erythroblasts. Normally, erythroblasts are localized in the bone marrow, where they mature by means of a process called erythropoiesis [16]. The last step of this process occurs in the normoblast where the nucleus is expelled and cytoplasmic granules are eliminated to finally become a reticulocyte. Erythropoiesis, non-nucleated cells are liberated to the systemic circulation. In contrast, in sideroblastic anemias erythropoiesis is ineffective. Abnormal nucleated normoblasts with granules of iron accumulated in perinuclear mitochondria are found in the systemic circulation. These nucleated cells, which contain such granules, are

named sideroblasts. Sideroblastic anemia is named after the presence of sideroblasts.

The causes of this disorder can be congenital or acquired. Both cases involve dysfunctional heme synthesis. The former is caused by different genetic defects in genes required for heme synthesis. The molecular basis of this type of anemia will be described in Section 1.5. In contrast, one of the most common reasons for acquired sideroblastic anemia is nutritional deficiencies of copper or vitamin B-6 (pyridoxal 5'-phosphate or PLP), but it can also be due to alcohol abuse, lead poisoning, excess zinc or antimicrobial drugs. Moreover, acquired sideroblastic anemia can occur indirectly as part of myelodysplastic syndrome, which can evolve into hematological malignancies, such as myelogenous leukemia.

Among the most common symptoms of sideroblastic anemia are skin paleness, fatigue and dizziness. Also, a person with this disorder can have an enlarged spleen and liver. In severe cases, heart disease, liver damage, and kidney failure can result from systemic iron overload in these organs [9].

The diagnosis of anemia is based on several parameters. At first, a group of parameters have to be measured in peripheral blood. These are: mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); serum iron (SI); total iron binding capacity (TIBC); transferrin saturation (TS); free erythrocyte protoporphyrin (Epp) and serum ferritin (SF). All these parameters help to determine if there is iron deficiency [70]. Then anemia can be classified as macrocytic when the erythrocytes are too large compared with average, or in contrast microcytic when the erythrocytes are smaller than normal. Also it can be classified as hypochromic when erythrocytes are paler than normal, or normochromic when the color is normal.

When the anemia is sideroblastic, it usually presents as microcytic and hypochromic. However, the sideroblastic anemias associated with Pearson marrow-pancreas syndrome, mitochondrial myopathy and sideroblastic anemia, or thiamine-responsive megaloblastic anemia present as macrocytic anemias.

The most important parameter to classify sideroblastic anemia is the presence of sideroblasts [16]. They are found with a specific test, which is the Prussian blue stain of red blood cells. This test involves the reaction of ferrous iron with ferrocyanide-forming blue granules and sideroblasts with iron granules in the mitochondria forming a ring around the nucleus that can be seen by light microscopy.

Also, molecular analyses can be used to identify the mutation that may cause the anemia. In this case, DNA from the patient is sequenced looking for a mutation in genes that could be responsible for CSA. These genes are explained in detail in Section 1.5. The importance of knowing the specific mutation that cause the anemia is that, depending on the gene mutated and the nature of the mutation, the patient may be able to receive a treatment for the disease.

Erythropoietic Porphyrrias

The erythropoietic porphyrias are another group of diseases characterized by a defect in heme biosynthesis. This defect is due to mutations in genes that encode enzymes that are part of the heme biosynthesis pathway. These mutations lead to accumulation of toxic heme precursors and iron. The consequences of the accumulation of these metabolites are hepatic and hematopoietic alterations, neurological and/or cutaneous symptoms.

There are different classifications for these disorders based on the symptoms associated with the porphyria or based on the enzyme that is defective [78]. Some of the erythropoietic forms, which are inherited as autosomal recessive, will be explain briefly here [22]. See Figure 1.1 for steps in the heme biosynthetic pathway.

Erythropoietic protoporphyria is due to specific mutations in the *FECH* gene. The reduced FECH activity leads to abnormal high levels of free protoporphyrin IX (PPIX) in erythrocytes, bone marrow, plasma and liver. The PPIX accumulation induces tissue damage through reactions with free radicals. The X-linked erythropoietic protoporphyria is caused by gain-of-function mutation in the *ALAS2* gene. These affected persons show high levels of zinc-protoporphyrin in erythrocytes. The congenital erythropoietic porphyria is due to mutations in the *UROS* gene. Reduced activity of UROS causes incomplete metabolism of hydroxymethylbilane and accumulation of porphyrins in erythrocytes, bone marrow and other organs. The hepatoerythropoietic porphyria is very rare and is due to mutations in the *UROD* gene. This leads to the accumulation of uroporphyrin and other metabolites in erythrocytes and liver. The last disorder is harderoporphria, which is caused by mutations in the *CPOX* gene. It is characterized by neonatal hemolytic anemia, sometimes accompanied by skin lesions, and massive excretion of harderoporphyrin in feces.

1.4.2 Defects in Hemoglobin Synthesis

The hemoglobinopathies are a group of disorders with defects in the structure of the hemoglobin protein. There are several hemoglobinopathies such as Sickle Cell disease and thalassemias. These diseases are inherited in an autosomal recessive manner and common mutant alleles originated mostly in Asia and the Mediterranean.

Sickle Cell anemia is a disease caused by a point mutation in β -chain gene [41], which produces an abnormal type of hemoglobin named Hemoglobin S [99]. Homozygosity for the sickle mutation (i.e., HbSS disease) is responsible for the most common and most severe variant of Sickle Cell disease. Several other genetic variants of Sickle Cell disease result from the interaction of different mutations of the human β -globin genes [31].

The thalassemia syndromes constitute the most common of all single gene disorders and are spread around the world [99]. The thalassemias are a group of disorders characterized by abnormal production of the globin chains, which are part of the hemoglobin molecule. This disease is classified into α - and β -thalassemia. β -thalassemia is caused by reduced or absent synthesis of the β -chains of hemoglobin [34]. In contrast, α -thalassemia is caused by a defect in the synthesis of the α -globin chains [74]. The most severe cases of α -thalassemias include Hemoglobin Barts ($\gamma\gamma$) and Hemoglobin H ($\beta\delta$).

1.4.3 Iron Overload Disorders

Several disorders are characterized by iron overload, some of which are quite common, such as hereditary hemochromatosis and β -thalassemia, whereas others are exceedingly rare. They can be categorized according to their pathophysiological effects: disorders of the hepcidin-ferroportin axis, disorders associated with ineffective erythropoiesis and disorders associated with defects in iron transport [30].

The six disorders of the hepcidin-ferroportin axis include specific types of hemochromatosis. They show ineffective down regulation of ferroportin (enterocyte transporter) mediated by hepcidin (hormone involved in iron metabolism). The most common disorder is hereditary hemochromatosis due to mutations in *HFE*, which codes for a protein that regulates the interaction of the transferrin receptor and transferrin (protein that controls the level of free iron in biological fluids). However,

hemochromatosis can be also caused by mutations in *TFR2* (gene that codes for the transferrin receptor 2) and the ferroportin encoding gene. Disorders associated with ineffective erythropoiesis are anemias caused by mutations in genes required for production of heme precursors (*SLC25A38*, *ALAS1*, *GRLX5* and *ABCB7*) and disorders such as the α -thalassemias and β -thalassemias. The disorders of iron transport have a common pathophysiological feature, which is the insufficient delivery of transferrin-bound iron required for heme biosynthesis. Hypotransferrinemia is a condition with reduced functional transferrin. Aceruloplasminemia is due to loss of activity of the enzyme that loads iron on transferrin and mutations in the DMT1 that prevent normal delivery of transferrin-bound iron. There are some disorders that have iron overload localized in specific tissues. One example is the neurodegeneration with brain iron accumulation (NBIA), which can be caused by mutations in different genes [30].

1.5 Molecular Basis of CSA

CSA is a rare and heterogeneous disease that can be caused by mutations in different genes. There are mutations that directly affect heme biosynthesis such as mutations in *ALAS2*, or indirectly, such as mutations in *ABCB7*, *SLC25A38*, *GLRX5*, *SLC19A2*, *PUS1* and *YARS2*. There are other mutations in pleiotropic genes that affect overall mitochondrial function, including mutations in the mitochondrial genome.

This section lists all the genes that have been found to be causative of CSA, but it is mainly focused on CSA due to mutations in *SLC25A38*, which is the gene studied in this thesis. Mutations in *ALAS2* and *SLC25A38* are the most common cause of CSA. The different mutations that lead to CSA are summarized in Figure 1.2.

The mode of inheritance can be different depending on the gene that is affected by the mutation. There are three modes of inheritance for sideroblastic anemias: autosomal recessive, X-linked recessive and maternal mode of inheritance.

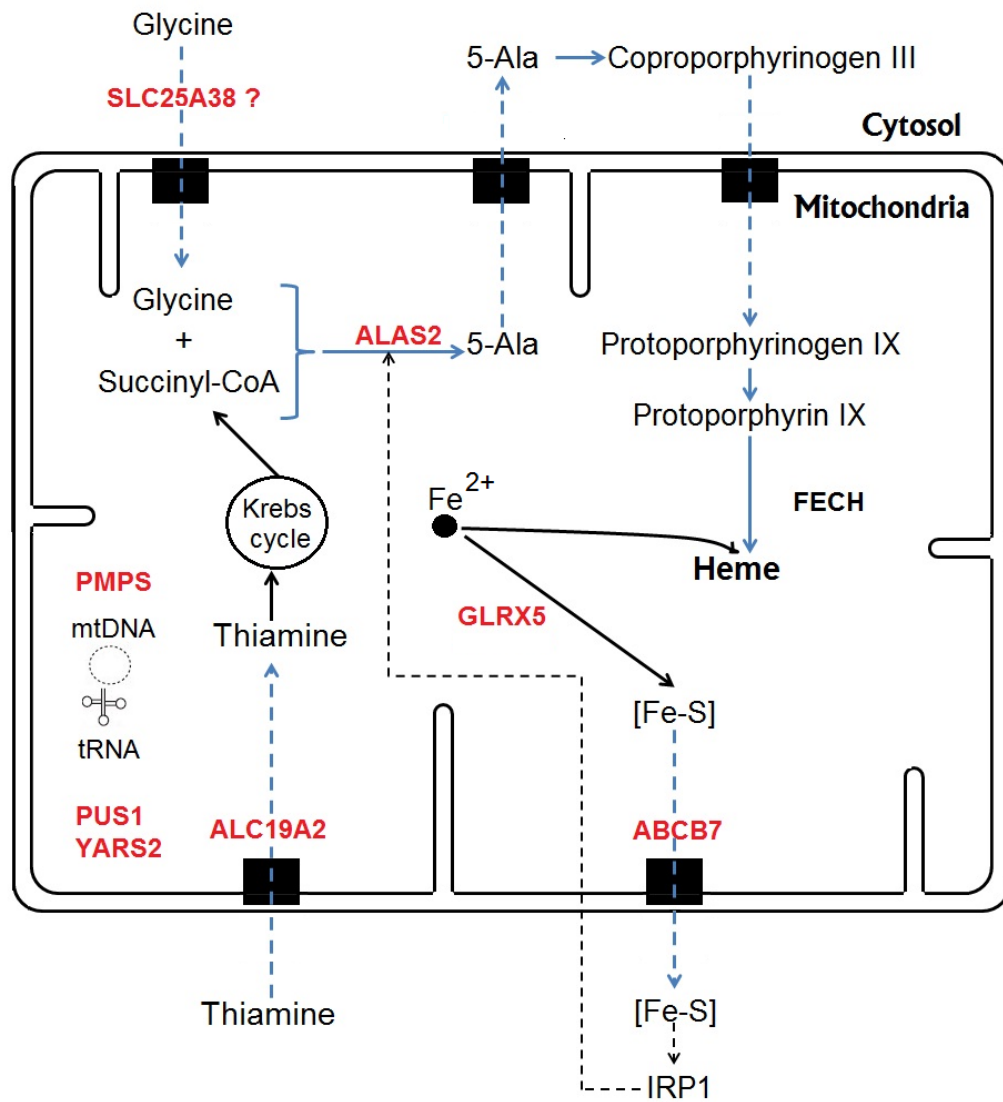


Figure 1.2: Diagram of the different proteins that can lead to CSA [32]. Responsible proteins are in red.

1.5.1 Autosomal Recessive Sideroblastic Anemia

Autosomal Recessive Sideroblastic Anemia Caused by Mutations in the *SLC25A38* Gene

A recent study showed a new form of autosomal recessive sideroblastic anemia was due to a mutation in the *SLC25A38* gene that codes for the mitochondrial carrier protein SLC25A38 [37, 42]. This autosomal recessive form is typically severe in patients and causes short life expectancy. Three families with one child affected with this form of CSA were found in the Canadian Maritime provinces [37]. One affected individual was negative for any mutation in *ALAS2*. Moreover, this CSA was not autosomal dominant or X-linked recessive because the patient was a female without evidence of skewed X inactivation and neither of the parents were affected. Furthermore, affected individuals were refractory to pyridoxine therapy. To find the gene associated with the disease, single nucleotide polymorphism-based genome-wide scans were done for eleven individuals including the three affected patients, their parents and one unaffected sibling from two of the families. The results showed that there was a homozygous haplotype region in chromosome 3. The region linked with the disease contained 32 genes and all of them were sequenced. A homozygous stop codon mutation in exon 4 of the *SLC25A38* gene was found in the affected individuals. The mutation was heterozygous in the parents. The results suggested an identity by descent possibly due to a founder effect.

In this same publication, additional studies were done of another 41 individuals with familial or sporadic CSA, who were negative for any mutation in *ALAS2*. They found multiple mutations in the *SLC25A38* gene in some of the individuals. In total they found 11 variants: 3 stop codon, 2 frameshift mutations, 1 splice acceptor site mutation, 1 in a presumptive stop codon and 4 missense mutations. The mutations were found in unrelated families with different haplotypes. These studies also supported the linkage between *SLC25A38* and the disease.

In order to determine the role of *SLC25A38* in erythropoiesis, several experiments were conducted by Guernsey et al [37] in zebrafish and the budding yeast *Saccharomyces cerevisiae*. The first experiment was done in zebrafish, which has two *SLC25A38* orthologs that are called *slc25a38a* and *slc25a38b*. Both genes were

knocked down by injecting antisense morpholinos, and the zebrafish embryos showed anemic phenotypes with a reduction in hemoglobin levels. These genetic and functional data demonstrated that loss-of-function mutations in *SLC25A38* cause an autosomal recessive form of CSA in humans. The phenotypic similarity between affected individuals with mutations in *SLC25A38* and *ALAS2* suggests that the former might also be associated with defect in heme biosynthesis.

The rest of the experiments were done in *S. cerevisiae* in which the *SLC25A38* ortholog is *HEM25*. They observed that under respiratory conditions *ydl119cΔ* cells grew poorly. This result indicated a defect in respiration probably due to a defect in heme synthesis. Moreover, this mutant strain was unable to reduce sodium nitroprusside. This also indicated a likely defect in heme synthesis since heme is required to catalyze the the reduction of sodium nitroprusside. The phenotype was rescued by supplementation with glycine or 5-Ala, which are a precursor and a product of the reaction catalyzed by ALAS2, respectively. They also measured total cellular glycine and 5-Ala levels by mass spectrometry and showed a significant reduction of 5-Ala content in *HEM25Δ* cells.

In 2011, Kannengiesser *et al.* [42] also found that patients with autosomal recessive sideroblastic anemia were homozygous or heterozygous compound for ten different mutations in *SLC25A38* that cause premature terminations of translation, splicing alterations or missense substitutions.

Taken together the results demonstrated that SLC25A38 is required for heme biosynthesis, that the defect is present upstream of 5-Ala synthesis and that there was a reduction in 5-Ala concentration, and that the yeast homologue HEM25 might perform a similar function. Based on these analyses, we decided to study SLC25A38 using yeast as the model system.

Autosomal Recessive Sideroblastic Anemia Caused by Mutations in *GLRX5*, *PUS1*, *YARS2* and *SLC19A2* Genes

The first case of congenital sideroblastic anemia caused by mutation in glutaredoxin 5 gene (*GRLX5*) was reported in 2007 [14]. GLRX5 is a mitochondrial protein essential for Fe-S cluster synthesis and it is highly expressed in erythroid cells. *GLRX5* was demonstrated to be essential for maintenance of normal mitochondrial and cytosolic

iron homeostasis in human cells [28]. When the GLRX5 protein is abnormal, there is an impairment in synthesizing Fe-S clusters, and as a result there might be a decrease of the clusters in the cytosol. IRP1 senses the depletion of Fe-S clusters in the cytosol, which activates IRP2. The activation of IRP2 produces the translational repression of *ALAS2* that results in CSA [104].

ALAS2 and *FECH* contain a labile Fe-S cluster that is required for function and stability. Thus, activity of the first and last steps of heme biosynthesis in erythroid cells can be governed by Fe-S cluster levels, either by determining activity of IRP1 or by directly contributing to the activity or stability of the enzymes [102, 104].

Mitochondrial myopathy and sideroblastic anemia (MLASA) is a rare type of mitochondrial disease. This disease can be caused by a mutation in the pseudouridine synthase 1 gene (*PUS1*) or in the mitochondrial tyrosyl-tRNA synthase gene (*YARS2*) [77, 28]. Patients with this disease present with abnormal mitochondrial protein synthesis and that appears to be the responsible for the sideroblastic anemia. The mechanism is not completely understood, but it seems that normal mitochondrial protein synthesis is required to have normal heme biosynthesis [32].

thiamine-responsive megaloblastic anemia (TRMA) is a rare disease caused by a mutation in the *SLC19A2* gene [64, 28], which encodes a thiamine transporter in the mitochondria. The link between the mutation in *SLC19A2* and CSA presented in patients with TRMA has not yet been found. Researchers suggested that the decrease of thiamine could impact the generation of succinyl-CoA, which is required for heme biosynthesis in the step catalyzed by *ALAS2* [32].

1.5.2 XLSA: X-linked Recessive Sideroblastic Anemia

Sideroblastic anemias with an X-linked mode of inheritance are called XLSA. This is because the gene that is affected by a mutation is located on the X chromosome. The X-chromosomal linkage of this hereditary sideroblastic anemia was first documented in 1946 [76]. XLSA is the most common of the inherited forms of sideroblastic anemia. Moreover, there are two different XLSAs as a result of mutations in two distinct genes. One XLSA is due to a mutation in the aminolevulinic acid synthase gene (*ALAS2*). The other XLSA is caused by a mutation in *ABCB7* gene that encodes the Fe-S transporter (ABCB7).

ALAS2 is the first enzyme in the pathway of heme synthesis and is a rate-limiting step in heme biosynthesis [26]. A large collection of distinct mutations for *ALAS2* have been discovered in XLSA. The mutations in *ALAS2* can affect the catalytic domain of the enzyme, the site of binding to PLP, or the gene promoter [17, 2]. A subset of CSA cases due to mutations in *ALAS2* result in decreased PLP binding to the ALAS2 protein. These patients can be treated with high levels of pyridoxine. Patients with mutations outside of the PLP binding region are refractory to pyridoxine treatment.

A less common type of XLSA is caused by mutations in the *ABCB7* gene. This gene codes for an Fe-S cluster transporter. This transporter is essential for the central nervous system and for hematopoiesis in erythroid cells. Patients with XLSA due to a mutation in *ABCB7* present with defects in heme biosynthesis [28]. The mechanism by which a mutation in *ABCB7* is related to heme biosynthesis is still not well known [32], although there is work that suggests that *ABCB7* may indirectly alter heme biosynthesis by the activity of IRP1 [102]. IRP1 senses cytosolic Fe-S clusters, and regulates mRNA stability and the translation of iron metabolism genes. Researchers found that the loss of the Fe-S cluster assembly in animals activated IRP1 to block ALAS2. By this same mechanism, the absence of the *GLRX5* gene, which encodes a protein required for Fe-S cluster synthesis, may affect heme biosynthesis.

1.5.3 Sideroblastic Anemia with Maternal Mode of Inheritance

Pearson marrow-pancreas syndrome (PMPS) is a rare disorder that presents as a sideroblastic anemia. The mechanism of ringed sideroblast formation in PMPS is still unclear. Patients with PMPS were studied and were found to have a deletion in the mitochondrial genome. These mutations lead to a deficiency of mitochondrial-encoded subunits of the respiratory complexes I, IV and V as well as a deficiency of the mitochondrial tRNA genes [32]. The mechanism by which this mutation produces sideroblastic anemia is not fully understood. However, it was suggested that the respiratory complexes are required to reduce iron, so that they can be incorporated into protoporphyrin IX by FECH in the last step of the biosynthesis.

1.6 SLC25 Family Carriers

Although the outer mitochondrial membrane is quite permeable to solutes up to 5 kDa due to the presence of voltage-dependent anion channels, the inner membrane is comparatively impermeable in order to maintain efficient oxidative phosphorylation. To overcome this barrier, members of a transporter family, called the SLC25 family, reside within the inner mitochondrial membrane and facilitate the transport of molecules required for normal cell metabolism. SLC stands for solute carrier family and 25 indicates that these SLC family members generally reside in the mitochondria. The 53 members of this family found in human cells share high identity, but when studied they have been found to transport different chemicals. There is a discrete common substrate-binding site that determines selectivity. However, some of the carriers can transport more than one solute [54].

The SLC25 proteins are nuclear encoded and then targeted to the mitochondria by means of a mitochondrial targeting sequence, which can be anywhere within the 6 transmembrane helices. This sequence is recognized by a translocase of the mitochondrial outer membrane complex. Then, the protein is translocated through the Tom40 complex pore and inserted into the inner membrane by Tim22 complex. Although there are abundant functional data for many of these carriers, the only 3D structures available are from UCP2 and ANT [7]. These structures show that the carriers are integral membrane proteins, with six transmembrane helices that form homodimers.

The majority of the carriers are in the inner mitochondrial membrane. However, SLC25A17 is also present in peroxisomes, and SLC25A49 and SLC25A50 are located in the outer mitochondrial membrane. All these carriers play fundamental roles during physiological processes. For this reason the proper expression, targeting and transport mechanism is critical as defective carriers can lead to pathological conditions or diseases [54].

As described in Section 1.5.1, SLC25A38 is one of the carriers that causes a disease when mutated. Mutations in *SLC25A38* cause CSA with increased ferritin levels and immature erythroid precursor cells with iron deposits [37]. The knockdown and knockout in zebrafish and yeast produce a decrease in hemoglobin and heme levels, respectively.

Other SLC25 transporters are also important for iron and heme metabolism.

SLC25A28 and SLC25A37 named mitoferrin-2 and mitoferrin-1 [87], transport iron into the mitochondria matrix that is required for the formation of Fe–S clusters. The clusters are then incorporated into many mitochondrial enzymes or are exported out of the mitochondria. Iron is also required for the last step of heme biosynthesis, which occurs in the mitochondrial matrix. Interestingly, SLC25A28 knockdown has a greater effect on heme synthesis, whereas SLC25A37 depletion has a greater effect on Fe–S cluster synthesis. The import of iron by SLC25A37 is enhanced by the interaction with the transporter Abcb10. There is no pathogenesis associated with this transporter yet.

SLC25A32 is a folate transporter called MFT [92]. It carries folate into the mitochondrial matrix. The MFT tissue distribution and mechanism are still unknown. MFT appears to be essential for glycine synthesis because folate is required for the conversion of serine to glycine by mitochondrial serine hydroxymethyltransferase. Mitochondrial folate is also required for the glycine cleavage system, which produces formylmethionine and is necessary for mitochondrial translation initiation. It has also been suggested that MFT could transport FAD across the inner membrane [90].

SLC25A4, SLC25A5, SLC25A6 and SLC25A31 are adenine nucleotide translocases, called ANT1, ANT2, ANT3 and ANT4, respectively. Their role is as a main supplier of ADP to the mitochondria and ATP to the cytoplasm to confer metabolic control over many cellular processes. ANTs are some of the most highly expressed family of proteins in the inner mitochondrial membrane and can bind respiratory supercomplexes. In addition, the ANT transporters could transport of heme and heme precursors [5].

1.7 Specific Aims

The absence of some of these transporters is known to cause defects in the mitochondria, and may be involved in human diseases. Mutations in *SLC25A38* have now been found to lead to CSA disease. At the outset of my thesis work the function of SLC25A38 was not known. I went on to determine the function of SLC25A38 and its yeast homologue Hem25, how defects in SLC25A38 function could cause CSA, a potential treatment for CSA patients, and increased our knowledge of the contribution of Hem25 to mitochondrial fitness.

Chapter 2

Materials and Methods

2.1 Gene and Protein Nomenclature

Human and yeast genes are written in uppercase and italicized (e.g. *SLC25A38*, *HEM25*). Genes written in lowercase and italicized denote a recessive allele (*hem25*). The Delta symbol (Δ) indicates a deletion of the preceding gene. Human proteins are written in uppercase and not italicized (SLC25A38) and yeast proteins are written with first letter uppercase, the rest lowercase and not italicized (Hem25).

2.2 Materials

2.2.1 Yeast strains

Strains from the W303 background were used for the characterization of Hem25 in the first part of this thesis (Chapter 3). W303 has a wild type allele for the *HAP1* gene. *HAP1* is an important gene for this study because it encodes a transcription factor that regulates gene expression in response to levels of heme and oxygen. The experimentation showed in Chapters 4, 5, 6 where I studied genetic interactions with *HEM25* was done in BY4741/BY4742 background, which have a Ty1 insertion in the 3' end of *HAP1* that renders a non-functional protein product. For this reason all the diploids in BY4741/BY4742 background were transformed with pRS426-*HAP1* (named NDY 601) to obtain haploid strains expressing *HAP1*. The yeast strains used in this study are shown in Table 2.1. All the oligonucleotides used to amplify genes and confirm genotypes are shown in the Table 2.2.

Table 2.1: Yeast strains used in this study

Strain	Relevant genotypes	Parental strain
W3031-a	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	W303 [6]
W3031-α	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	W303 [6]
NDY 046	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hem25Δ::NatMX4</i>	W303 [6]
NDY 323	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 shm1Δ::KanMX4</i>	W303 [6]
NDY 252	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 shm2Δ::KanMX4</i>	W303 [6]
NDY 254	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 gly1Δ::KanMX4</i>	W303 [6]
NDY 086	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hem1Δ::NatMX4</i>	W303 [6]
NDY 263	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hem25::NatMX4 gly1Δ::KanMX4</i>	W303 [6]
NDY 264	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hem25::NatMX4 gly1Δ::KanMX4</i>	W303 [6]
NDY 265	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hem25::NatMX4 gly1Δ::KanMX4</i>	W303 [6]
NDY 387	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ser1Δ::HphMX4</i>	W303 [6]
NDY 432	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ser1Δ::HphMX4 hem25::NatMX4</i>	W303 [6]
NDY 466	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 lpd1Δ::KanMX4</i>	W303 [6]
Y2454	<i>MATα mfa1Δ::MFA1pr-HIS3 can1Δ0 his3Δ1,15 leu2Δ0 ura3Δ0 lys2Δ0</i>	Y2454 [93]
NDY 005	<i>MATα mfa1Δ::MFA1pr-HIS3 can1Δ0 his3Δ1,15 leu2Δ0 ura3Δ0 lys2Δ0 hem25Δ::NatMX4</i>	Y2454 [93]
BY 4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	
NDY 009	<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hem25Δ::KanMX4</i>	BY4742 [12]
NDY 296	<i>MATa leu2Δ0 ura3Δ0 can1Δ0</i>	BY4741 [12] and Y2454 [93]
NDY 297	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 487	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 aac3Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 210	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 aac3Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 301	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ymc2Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 212	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ymc2Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 493	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ctp1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 202	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ctp1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 495	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 yea6Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 213	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 yea6Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 300	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 pic2Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 214	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 pic2Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 305	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 yia62Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 216	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 yia6Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 497	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 oac1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]

Table 2.1 – Continued from previous page

Strain	Relevant genotypes	Parental strain
NDY 219	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 oac1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 480	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 mrs4Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 220	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 mrs4Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 500	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ggc1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 231	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ggc1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 307	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 tpc1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 215	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 tpc1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 492	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 mtm1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 230	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 mtm1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 490	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 mrs3Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 218	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 mrs3Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 491	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 mir1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 206	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 mir1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 485	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 sfc1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 207	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 sfc1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 298	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 dic1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 221	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 dic1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 486	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 pet8Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 224	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 pet8Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 498	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 sal1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 208	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 sal1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 494	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 crc1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 225	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 crc1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 489	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ort1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 226	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ort1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 303	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 odc2Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 227	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 odc2Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 306	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 odc1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 205	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 odc1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 302	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 agc1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 211	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 agc1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 483	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ymc1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 229	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ymc1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 499	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 flx1Δ::KanMX4</i>	BY4742 [12]
NDY 589	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 flx1Δ::KanMX4</i>	BY4742 [12]
NDY 596	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 flx1Δ::KanMX4</i>	BY4742 [12]
NDY 217	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 flx1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 585	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 flx1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 586	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 flx1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 484	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ypr011cΔ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 209	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ypr011cΔ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 482	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 yfr045wΔ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 203	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 yfr045wΔ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]

Table 2.1 – Continued from previous page

Strain	Relevant genotypes	Parental strain
NDY 304	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 yhm2Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 222	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 yhm2Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 481	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 aac1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 204	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 aac1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 496	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 pet9Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 232	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 pet9Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 299	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 mme1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 223	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 mme1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
DNY 488	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ant1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 228	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 ant1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 338	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 lpd1Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 339	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 lpd1Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 334	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 aim22Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 250	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 aim22Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 335	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 kgd2Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 249	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 kgd2Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]
NDY 342	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 htd2Δ::KanMX4</i>	BY4741 [12] and Y2454 [93]
NDY 343	<i>MATa leu2Δ0 ura3Δ0 can1Δ0 htd2Δ::KanMX4 hem25Δ::NatMX4</i>	BY4741 [12] and Y2454 [93]

Table 2.2: Oligonucleotides used in this study

Name	Sequence 5' → 3'
HEM25-NAT-for	AAA GCA AAT AGC ACA GCG GGA ACA ACT AGA AGG TAC ACA ATT TGT GAA GCA GCA TAC ATG ACA TGG AGG CCC AGA ATA CCC
HEM25-NAT-rev	ACG TAT TTA TAC CTT TTT TGA AGA ATG CGA GAT TGC AAT GAA ATA TGT GTC TAT TTT TTA CAG TAT AGC GAC CAG CAT TCA C
Conf-HEM25-NAT-for	TGT GGT GAC TGT TTC CTT TTG
Conf-HEM25-NAT-rev	TTT GAC CTT CTC AAT GGG GA
HEM1-NAT-for	CCT CAA TAA TCA TAA CAG TAC TTA GGT TTT TTT TTC AGT ATG CGT ACG CTG CAG GTC GAC
HEM1-NAT-rev	CTT GTA CCT CTA TCT CAG CCC ATG CAT ATA TTG GTT GTT TTA ATC GAT GAA TTC GAG CTC G
Conf-HEM1-NAT-for	TCC CTG CTT TTT TAC CAA CA
Conf-HEM1-NAT-rev	CCA AAC TTC TCC ATT CCG TCA
SHM1-KAN-for	TTT CTA TCC ACC AGG TCC TGT
SHM1-KAN-rev	CGA GAT GCG AAT ATT GCT TTG
conf-SHM1-KAN-for	AGT AGC ACA AAA TAC GGA GGA
conf-SHM1-KAN-rev	TCT TGA CAC CGA TGC ACA TT
SHM2-KAN-for	TAA TGC AGA ACT TGC TTC CC
SHM2-KAN-rev	CGT TAG AAA AAT CGT ACG CAC A
conf-SHM2-KAN-for	GCA CCG ATT TTT AAT GTA GAA A
conf-SHM2-KAN-rev	CCA AAG GAT TGT GGC AAA
GLY1-KAN-for	GTG TTG AGC TTG CCT GTC AAA T
GLY1-KAN-rev	GGT GTG AGA GTT GAA ATA TCC TTG
conf-GLY1-KAN-for	TTC TTT TTT CCC GCT GCT TC
conf-GLY1-KAN-rev	CCT TTT TTT CCT CCT TCC CT
SER1-HYG-for	CTC TTT CTA AGC ATC GGCG AA
SER1-HYG-rev	ATC GCT ATT TTT ATC TCA TCA CGG
conf-SER1-HYG-for	CGA AAA AGA AAT GGG TCC AC
conf-SER1-HYG-rev	GGT AGA CGC GAC AAA AAA AAG
LPD1-KAN-for	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GAT TCA GAA CTC ACG TCC G
LPD1-KAN-rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TCA GGA CTT CAG GCC CAT
conf-LPD1-KAN-for	GCG GAG CAA CTT AAT GGA AA
conf-LPD1-KAN-rev	TGC GCC TGA ATT TGA ATG GA
SLC25A38-for	GCC ACT GAC AAA CGT ATG CG
SLC25A38-rev	GCA AAC GTA GTG GTC ATC C

2.2.2 Reagents

The custom oligonucleotides were supplied by integrated DNA technologies (IDT), HIFI Taq DNA polymerase from Invitrogen was used to perform PCR amplifications, and all restriction enzymes and T4 DNA ligase used in subcloning procedures were purchased from New England BioLabs. Plasmids from bacteria were purified using the QIAprep Spin Miniprep Kit from Qiagen. Genomic DNA was purified from yeast with the Yeast DNA Extraction Reagent Kit from Pierce. DNA fragments and PCR amplicons used for ligation were cleaned with the GeneCleanII Kit from MP Biomedicals. Complete protease inhibitor tablets were from Roche. Nourseothricin (Nat) and ClonNAT were from Werner BioAgents. Most yeast and bacterial media were purchased from Difco except for the synthetic complete (SC) media and SC-Ura which were from Sunrise Science products. Heme determination was carried out with the Hemin Assay Kit from BioVision.

2.2.3 Media

Yeast were grown in different media depending on the background and the experiment. Synthetic defined (SD) medium or minimal medium was used for the W303 background and it contained 2% dextrose, 1.74 g/L yeast nitrogen base (without ammonium sulfate and amino acids), 5 g/L ammonium sulfate and only the supplements required by strain auxotrophies: 20 mg/L adenine sulfate, 20 mg/L L-histidine HCl, 100 mg/L L-leucine, 20 mg/L L-tryptophan and 20 mg/L uracil. SD medium without uracil (SD-ura) was used in BY background to select for yeast transformed with the plasmid encoding *HAP1* (named NDY 601) which had a *URA3* marker. SD composition was the same, but the amino acids added were: 20 mg/L L-histidine HCl, 100 mg/L L-leucine, 30 mg/L L-lysine and 20 mg/L L-methionine. SC and SC without uracil (SC-ura) were used normally to grow cells overnight. Yeast were cultured and media prepared by standard protocols [75].

In experiments in which yeast were made to undergo mitochondrial respiration, dextrose in SD and SD-Ura medium was substituted with 2% lactate (SD or SD-ura medium with lactate). Dextrose in SD-ura medium was substituted with 2% raffinose (SD or SD-Ura with raffinose) to grow cells to prepare mitochondrial extracts. In the experiments where glycine was used as the sole nitrogen source, the ammonium sulfate

in SD and SD-Ura was substituted with 30 g/L of glycine (SD or SD-ura with glycine).

Prior to transformation, yeast were grown in YPD medium (1 % yeast extract, 2 % peptone and 2 % dextrose) [75]. G418 and Nat were added to YPD at 200 mg/L and 100 mg/L concentration, respectively, to select for gene deletions. Bacteria used for cloning and propagation of yeast plasmids were grown in Luria Bertani (LB; contains 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) broth or LB plates (with 2% agar). Ampicillin (100 mg/L) was included in LB medium to select for colonies with plasmids.

2.3 Methods

2.3.1 DNA techniques

Yeast transformation

Yeast cells were transformed with plasmid DNA or PCR amplicons of genomic DNA as described previously [35] using the lithium acetate/polyethylene glycol method. Cells were grown in 5 ml of YPD medium at 30 °C until they reached mid-log phase (0.5 OD₆₀₀). Then, cells were harvested by centrifugation at 3000 rpm for 5 min. The pellet was then resuspended in 50 μ l of LiAc/TE (100 mM lithium acetate, 10 mM Tris-HCl pH 8.0 and 0.1 mM ethylenediaminetetraacetic acid (EDTA)). The DNA to be transformed (1–3 μ g) and heat denatured herring sperm DNA (50 μ g) were added to the resuspended pellet, mixed by inversion and then incubated at room temperature for 5 min. Then, 300 μ l of LiAc/TE containing 40% polyethyleneglycol 3400 was added to the above mixture, vortexed and transferred to a 14 ml culture tube (Bio-Rad). Then, the tubes were incubated in a water bath at 42 °C for 1 hr with intermittent gentle shaking. After the incubation period, the cells suspension was centrifugated for 30 sec at 14000 rpm and the resulting pellet was washed once with sterile water. The cells were resuspended in water and plated on appropriate solid medium: SC-Ura plates (for transformations with NDY 601 plasmid) or YPD medium containing G418 or Nat (for targeted gene disruptions). The plates were incubated at 25 °C for 2–4 days, except in case of targeted gene disruptions where cells were incubated overnight before they were replica plated onto appropriate selective medium.

Bacterial transformation

Chemically competent *Escherichia coli* strain DH5 α cells were transformed with plasmid DNA (0.1–0.2 μ g). Cells were incubated for 30 min on ice, heat-shocked at 42 °C for 30 sec, and incubated in 200 μ l SOC medium (0.5% yeast extract, 2.0% typtone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM MgSO₄, 20 mM dextrose) for 1 hr at 37°C. Cells were plated on the appropriate solid selection medium.

Electrocompetent cells DHB10 (Invitrogen), (10 μ l with 50 μ l water) were incubated with plasmid DNA (0.1–0.2 μ g) for 5 min on ice. Cells were transferred to 0.1 cm gap electroporation cuvette and electroporated at 25 μ F, 200 Ohms, 1.61 kVolts. LB medium was added and cells were incubated for 1 hr at 37°C and plated on the appropriate solid selection medium.

Plasmids

The plasmid p416-GPD-*SLC25A38* (named PM149), which expresses the human SLC25A38 protein in yeast under the control of glyceraldehyde-3-phosphate dehydrogenase promoter, was constructed by inserting a *SLC25A38* cDNA clone, received as a gift from Dr. Karen Bedard (Pathology Department, Dalhousie University), into the cloning site of the vector p416-GPD. This is a low copy number expression vector. Plasmid pRS426-*HAP1* (named NDY 601), which expresses the yeast *HAP1* gene in yeast, was a gift from Dr. Li Zhang [106]. This is a high copy number expression vector.

Yeast Strains Construction

The BY4741 strain with the *HEM25* gene deleted (*hem25* Δ ::*KanMX4*) was obtained from EUROSCARF [1]. The *KanMX4* marker gene was replaced with the nourseothricin acetyltransferase (*NatMX4*) gene resistance cassette by transformation of the corresponding strain with a linearized pAG25 plasmid, as described in [98]. Y2454 *hem25* Δ ::*NatMX4* and W303-a *hem25* Δ ::*NatMX4* were constructed by transferring the gene deletion allele from the BY4741 strains into the Y2454 and W303-a background. Briefly, an amplicon generated by PCR of the disrupted gene with $\tilde{0}$.2 Kb of flanking genomic sequence was transformed into Y2454 and W303-a

yeast cells. Cells were plated and grown in YPD medium for 1 day and after were replicated on YPD containing the appropriate antibiotic to select for the transformants with the corresponding selectable marker disrupted gene. Antibiotic resistant colonies were streaked for isolation of single cell colonies. Genotyping of the transformants was carried out by PCR amplification. Genomic DNA was isolated from the antibiotic-resistant colonies and PCR amplification was performed using flanking primers for both ends at 0.4 Kb of the marker disrupted gene. When unambiguous identification was not achievable by molecular size determination, restriction analyses of the amplicons were carried out.

The rest of the strains (W303- α *hem1* Δ ::*NatMX4*, W303- α *shm1* Δ ::*KanMX4*, W303- α *shm2* Δ ::*KanMX4*, W303- α *gly1* Δ ::*KanMX4* and W303- α *lpd1* Δ ::*KanMX4*) were constructed following essentially the same procedure. To construct the W303- α *ser1* Δ ::*HphMX4* strain, the *KanMX4* marker was replaced by the *Hph* gene resistance cassette using a linearized plasmid pAG32, as described in [98].

All yeast strains with two genes of interest deleted in the W303 background used in this study were constructed by standard yeast genetic crosses [26]. Those in the BY4741/BY4742 background were constructed by using single gene deletion mutant strains from EUROSCARF [1] with the Y2454 *hem25* Δ ::*NatMX4* strain (NDY 005). Heterozygous diploids were selected and then transformed with NDY 601 plasmid. Haploids with both genes deleted were obtained by random sporulation and selection on solid medium [93].

2.3.2 Synthetic Genetic Array (SGA) Analysis

In *S. cerevisiae*, there are about 4700 non-essential genes and deletion of any one of these genes in a haploid yeast cell does not affect its viability. At present, approximately 4700 mutants (deletion collection) each carrying a single non-essential gene deletion is available as an ordered array from EUROpean *Saccharomyces cerevisiae* archive for functional analysis (EUROSCARF) [1]. Each deletion collection mutant has the deleted gene replaced with a dominant kanamycin-resistance selectable marker (*KanMX4*). For SGA analysis, a query strain carrying a nourseothricin-resistance marker (*NatMX4*) that replaces the ORF of the gene of interest is also generated. SGA analysis is carried out with a robot and facilitates a large scale mating between

the query strain and each of the 4700 mutant strains in the EUROSCARF deletion strain collection, which generates an array of 4700 diploids cells. Diploid cells were sporulated. Since the disrupted genes are replaced with antibiotic-resistance markers, the *MATa* haploids with both the query gene and collection gene deleted can be easily selected on a medium with G418 (200 mg/L, Invitrogen) and ClonNAT (100 mg/L, Wener BioAgents). Among the 4700 double mutant haploids, some of them may not be viable or may grow slower than its normal growth, which indicates a synthetic lethal or synthetic sick interaction between genes [93].

The SGA analysis was conducted as previously described [93]: the query strain Y2454 *hem25* Δ ::*NatMX4* used in this study was generated as described in Section 2.3.1. This was robotically crossed with each of the *MATa* deletion collection mutants and the array of generated diploids was selected on plates with YPD medium containing G418 and ClonNAT. Meiosis was induced in these diploids by pinning them onto sporulation medium (2% agar, 1% potassium acetate, 0.1% yeast extract, 0.05% dextrose, supplemented with uracil, histidine and leucine). After 5 days of sporulation at 22°C, *MATa* haploid spores were specifically selected by incubating the spores in SD medium lacking histidine and arginine but containing canavanine (SD–His–Arg, canavanine 50 mg/L) for 2 days at 30 °C. The *MAT α* query and the *MATa* deletion collection mutants are histidine auxotrophs and they do not grow in the absence of histidine. The query strain carries the *HIS3* gene under the control of the *MATa*-activated promoter (*MFApr*) such that *HIS3* is only expressed in *MATa* cells. Therefore, growth in SD medium without histidine only supports the growth of *MATa* haploid spores that resulted from mating between the query and one of the deletion collection mutants. In addition, unlike the deletion mutants, the query strain has a defective arginine permease gene (*can1* Δ) and cannot take up arginine or its analogue canavanine from the medium. When canavanine instead of arginine is present in the medium, *CAN1* cells incorporate it into cells and this causing cell death. After another round of selection in haploid selection medium, the cells were pinned to SD medium with G418 (SD–His–Arg, G418 200 mg/L, canavanine 50 mg/L) for one day. Finally, cells were transferred to agar plates containing both G418 and ClonNAT (SD–His–Arg, G418 200 mg/L, ClonNAT 100 mg/L and canavanine 50 mg/L) for 2 days at 30 °C. Final pinning results in an ordered array of double-mutant

haploid strains whose growth rate is monitored by visual inspection or image analysis of colony size to look for synthetic lethal or sick interactions.

2.3.3 Random Sporulation Assay

The Y2454 *hem25*Δ::*NatMX4* strain was manually mated with *aim22*Δ::*KanMX4*, *kgd2*Δ::*KanMX4* or *yhm2*Δ::*KanMX4* mutants from the deletion collection. The resulting diploids were selected on YPD solid medium containing G418 and ClonNAT. The diploids were transformed with NDY 601 plasmid. Then, diploids were suspended at approximately 2×10^7 cells/ml in pre-sporulation broth (YM1: 1% succinic acid, 0.5% yeast extract, 0.5% peptone, 0.67% yeast nitrogen base without ammonium sulfate, 2% dextrose, 0.6% NaOH, supplemented with histidine, leucine, methionine and lysine) without uracil to keep the plasmid in the cells, and grown for 1 day. The cells were washed twice with distilled water and resuspended in sporulation medium without uracil (1% potassium acetate, 0.1% yeast extract, 0.05% dextrose, supplemented with histidine leucine, methionine and lysine). They were allowed to sporulate for 5–6 days. Cells were harvested, washed and resuspended at approximately 5×10^8 cells/ml in distilled water containing 100 μg/ml zymolase 100T and incubated for 25 min at 30 °C to completely digest the ascus wall. Aliquots of 0.5 ml were transferred to 1.5 ml Eppendorf tubes and pelleted at 14000 g for 30 sec. The supernatant was disposed of and the pellets were resuspended in 1 ml of distilled water, centrifuged as before and resuspended in 0.2 ml fresh distilled water. This mixture was vigorously vortexed for 2 min; since the spores are far more hydrophobic than vegetative cells, they clump together and also they adhere to the polypropylene walls of the 1.5 ml Eppendorf tubes. The vegetative cells were discarded by three successive washes with distilled water before the adhere spores were resuspended by the addition of 1 ml of water containing 0.01% Triton X-100, and sonicated for 1-3 min. The purified spores were diluted and about 200 spores were evenly plated on plates of four different media: containing SD-Ura-His-Arg (SD medium without uracil, histidine and arginine) with canavanine 50 mg/L, SD-Ura-His-Arg with canavanine 50 mg/L and G418 200 mg/L, SD-Ura-His-Arg with canavanine 50 mg/L and ClonNAT 100 mg/L and the last condition SD-Ura-His-Arg with canavanine 50 mg/L, G418 200 mg/L and ClonNAT 100 mg/L. The last condition was used to select for the double mutant haploids.

The plates were incubated at 30 °C and colony numbers and sizes in each of the plates were scored after 3-4 days to study whether there was any genetic interaction with deletion of *HEM25* gene.

2.3.4 Serial Dilution Growth

The growth potential of yeast strains used in this thesis was estimated using a growth assay. Cells were grown to early stationary phase (0.9–1 OD_{600nm}) at 30 °C in SC or SC-Ura medium; the cell density of the culture was adjusted to OD₆₀₀ 0.4 and then serially diluted (1:10) four times in a 96 well culture plate and spotted onto appropriate solid medium using a replica pinner. The plates were incubated at 30 °C for 3–7 days, after which they were imaged using a Bio-Rad VersaDoc.

2.3.5 Isolation of Yeast Mitochondria

Crude mitochondrial fractions were prepared by differential centrifugation as previously described [27]. Briefly, yeast strains were grown in SC-Ura medium to select cells with NDY 601 plasmid, at 30 °C until they reached mid-log phase. Cells were washed and reinoculated in 200 ml of SD-Ura with raffinose medium at OD₆₀₀ 0.2 and they grown until OD_{600nm} 1-1.5. Cells were washed and transferred to SD-Ura with lactate medium for 5 hr. The cells were collected by centrifugation at 2500 × *g* for 5 min at 4 °C. The resulting pellet was resuspended in ice-cold 1x SHP buffer (0.6 M sorbitol, 20 mM HEPES-KOH, 1 mM phenylmethanesulfonylfluoride) at density 0.5 g cells/ml and glass beads were added to occupy two-thirds of the final volume. Cells were broken by vortexing twice for 15 sec at maximum speed and put on ice after every vortexing. The resulting suspension was subjected to low-speed (10 sec at 600 × *g*) centrifugation and this suspension was collected in a different tube. The suspension in Eppendorf tubes were centrifuged for 5 min at 600 × *g* at 4 °C. The supernatant harbors mitochondria, other cellular membranes and the cytosol. This supernatant is removed carefully from the pellet with a pipette and centrifuged for 10 min at 10000 × *g* at 4 °C. The resulting pellet, which mostly contains mitochondria, was resuspended in 20 μl buffer I (Tris 50 mM pH 7 and sodium dodecyl sulfate (SDS) 1%) and protein content of the extracts was determined by a modified Lowry method. Then, 10 μl of buffer II (3% SDS, 132 mM Na₂CO₃ and 4% βmercaptoethanol) was

added to the above mentioned mitochondrial suspension. Finally, urea was added to 6M final concentration in the mitochondrial suspension [96].

2.3.6 Protein techniques

Protein Determination

Proteins concentrations were determined by a modified Lowry method [55]. This method is a modification of Lowry Assay because it utilizes the detergent deoxycholate. The use of this detergent makes this method suitable to measure hydrophobic proteins present in membranes.

SDS-PAGE and Western Blot Analysis

A Mini-PROTEAN3 unit (Bio-Rad) was utilized as per manufacturer's directions to perform both SDS-PAGE and protein transfer to nitrocellulose membranes (Bio-Rad). Briefly, the samples used in the analyses were mitochondrial extracts prepared as described in Section 2.3.5. To maintain equal protein loading between samples, protein concentration was determined in mitochondrial extracts before SDS-PAGE using a modified Lowry method. Proteins were denatured by the addition of loading buffer (0.05% bromophenol blue, 25% glycerol, 6% sodium SDS, 6 mM EDTA and 150 mM Tris-Cl pH 8.8 and 0.5% β -mercaptoethanol) and then incubated for one hr at 37°C. Samples were resolved by 12% SDS-PAGE (0.375 M Tris-HCl pH 8.8, 0.1% SDS, 12% acrylamide, 0.075% ammonium per sulphate and 10 μ l/10 ml TEMED) at a constant current 100 volts. The separated proteins were then wet-transferred to nitrocellulose membrane in 25 mM Tris-HCl, 200 mM glycine, 10% methanol at 20 volts and 90 mA overnight. The membranes were allowed to sit for 1 hr under constant shaking in Odyssey blocking buffer (LI-COR). The blots were then incubated with an appropriate dilution of primary antibody (see Table 2.3) in blocking buffer under constant shaking overnight at 4 °C. The following day, the membranes were washed three times (10 min wash each with 0.1% Tween-20 in phosphate-buffered saline (PBS)) before they were incubated with the appropriate dilution of secondary antibody (see Table 2.3) in blocking buffer for 1 hr. After this, the excess antibodies were washed off with 0.1% Tween-20 in PBS (three washes, 10 min each). Finally, proteins were detected

and imaged by using the Odyssey as directed by the manufacturer. The membranes were stripped with New Blot Nitro Stripping Buffer (LI-COR) for 10 min under constant shaking at room temperature, and then reprobed with anti-porin antisera (Por1) as a loading control.

Table 2.3: **Antibodies used in this study**

Immunogen	Dilution	Source
Primary antibodies		
anti cytochrome c oxidase, subunit 2 (Cox2)	1 500	Invitrogen
anti cytochrome c oxidase, subunit 4 (Cox4)	1 2,000	Abcam
anti cytochrome c1 (Cyt1)	1 1,000	received as a gift from Dr. Carla M. Koehler [23]
anti ubiquinol cytochrome-c reductase, subunit 2 (Cor2)	1 1,000	received as a gift from Dr. Carla M. Koehler [23]
anti Complex V (F1 α)	1 1,000	received as a gift from Dr. Carla M. Koehler [23]
anti complex V (F1 β)	1 1,000	received as a gift from Dr. Carla M. Koehler [23]
anti succinate dehydrogenase, subunit 1 (Sdh1)	1 5,000	received as a gift from Dr. Dennis R. Winge [45]
anti succinate dehydrogenase, subunit 2 (Sdh2)	1 5,000	received as a gift from Dr. Dennis R. Winge [45]
anti NADH:ubiquinone oxidoreductase (Ndi1)	1 125	received as a gift from Dr. Takao Yagi [86]
anti mitochondrial porin (Por1)	1 2,000	Abcam
Secondary antibodies		
Goat anti-mouse	1 15,000	LI-COR
Goat anti-rabbit	1 5,000	LI-COR

2.3.7 Glycine Mass Determination

Glycine determination was performed at the IWK Health Center, Halifax, NS. Free glycine content in yeast cells was estimated by the ninhydrin method [61]. Briefly, yeast cells grown to log phase in SD medium were harvested, washed with ice-cold water and resuspended at a density of 4×10^8 cells per ml of 10% sulphosalicylic acid containing 0.5 mM norleucine. Cells were broken using glass bead beater for 4 min and whole cell extracts were clarified by centrifugation at $18000 \times g$ for 30 min. Cell extracts were subjected to amino acid quantification using the Biochrom 30 Amino Acid Analyzer.

2.3.8 Heme Determination

Logarithmically-growing cells were harvested, washed in ice-cold water, and resuspended in 10 mM Tris-HCl, pH 8, 150 mM NaCl. Cells were lysed by vortexing with glass beads beating for two periods of 1 min intercalated with 1 min on ice. Cell debris was removed by centrifugation at $500 \times g$ for 3 min, and heme and protein content of the supernatant were assayed. Heme determination was carried out with the Hemin Assay Kit from BioVision. This kit is based on the reconstitution of the Apo-peroxidase activity in the presence of hemin [4]. Peroxidase activity causes the conversion of a colorless probe to a strongly-colored compound. A standard curve was made with different concentrations of a hemin standard. The color was measured (Abs=570 nm) at 30 min from the beginning of the reaction using a Thermo Labsystems Multiskan Ascent Microplate Reader. Protein concentration was determined by a modified Lowry method.

2.3.9 Comparison of Means

One-way ANOVA was used to calculate whether differences among mean values coming from different conditions in the experiments were significant or not. Depending on the experiments, means have been calculated using from three to nine samples per condition. Although we have not applied a goodness of fit test to check whether data seem to follow a Gaussian distribution, the one-way ANOVA is considered a robust test against the normality assumption [83]. Reported p -values would indicate the probability of being wrong when determining that means are different (type-I error).

Chapter 3

Characterization of Hem25 Function

Mutations in the human gene *SLC25A38* are known to cause congenital sideroblastic anemia (CSA) [37, 42]. SLC25A38 is a member of a mitochondrial carrier family called SLC25. Mitochondrial SLC family members are subdivided into three major groups: keto acid, amino acid and adenine nucleotide carriers [50, 67]. Phylogenetic analysis of the human SLC25 family grouped SLC25A38 with amino acid carriers (see Figure A.1 in the Appendix). This is consistent with the prediction that SLC25A38 could be a mitochondrial glycine or 5-Ala transporter, both of which contribute to the synthesis of heme [37]. Guernsey *et al.* [37] also showed that patients with mutations in *SLC25A38* had low levels of heme in their red blood cells. Moreover, it was shown that *S. cerevisiae* lacking the yeast homologue of *SLC25A38*, *YDL119c*, which we have named *HEM25* (Heme synthesis by SLC25 family member), the cellular content of 5-Ala was decreased.

The objective of this study was to investigate SLC25A38 function using a yeast genetic model to understand why mutations in the *SLC25A38* cause CSA. Based on the results of Guernsey *et al.* and the phylogenetic analysis, a model for Hem25 function is proposed. My hypothesis is that Hem25 is a mitochondrial glycine importer. The rationale behind this hypothesis is that glycine is a substrate of the first reaction of the heme synthesis pathway catalyzed by Hem1 that produces 5-Ala, which was shown to be decreased in *hem25* Δ cells.

3.1 Results

3.1.1 Human *SLC25A38* Complements Yeast Hem25 Deficiency for Heme Synthesis

The objective for this study was to characterize SLC25A38 function using *S. cerevisiae* as model system. In order to validate the yeast model, the first experiment carried

out was to study whether there was conservation of function between the human SLC25A28 and the yeast Hem25 proteins. The *HEM25* gene was deleted from the yeast genome. Cells lacking *HEM25* (*hem25* Δ) did not show any difference in growth compared to wild type cells when grown in dextrose-fermenting conditions. Whole cell extracts were prepared from wild type and *hem25* Δ cell cultures, and heme content was measured. Consistent with the notion that *HEM25* is the homologue of *SLC25A38*, *hem25* Δ cells exhibited a 50% decrease in heme levels compared with wild type cells (Figure 3.1). Human *SLC25A38* was expressed from a p416-GPD vector in *hem25* Δ cells in order to determine whether the human protein complements the absence of the yeast protein. The p416-GPD vector allows for constitutive expression of the SLC25A38 at a ‘moderate’ level. The expression of SLC25A38 human protein restored heme content to wild type levels in yeast cells lacking a *HEM25* gene. These results indicate conservation of function between the yeast and the human proteins (Figure 3.1). Furthermore, they validate the use of yeast as a model system to investigate the role of *SLC25A38*.

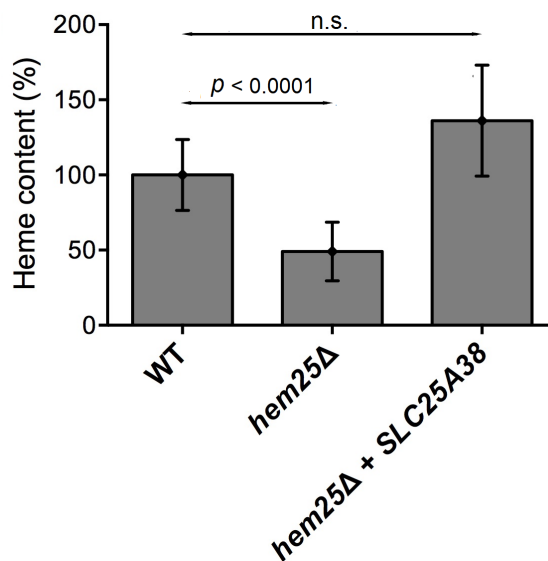


Figure 3.1: **Human SLC25A38 complements loss of yeast Hem25 function.** Yeast cells of the indicated genotypes were grown to log phase (OD_{600nm} 0.6-1) in SD medium. Cells were processed for heme determination. Wild type cell heme content was 31.1 fmol/ μ g protein. The numbers represented in the graph are the percentages relative to wild type (WT). Heme values are the mean \pm Standard Error of the Mean (SEM) of at least six independent determinations. n.s. stands for not significant.

3.1.2 Hem25 Facilitates Mitochondrial Glycine Metabolism

In order to test the hypothesis that Hem25 is a mitochondrial glycine importer, I used two different metabolic conditions where the efficient uptake of glycine into the mitochondria is required for yeast cells to grow. In the first condition, glycine is provided as the sole nitrogen source [88]. For yeast cells to grow using glycine as a nitrogen source, glycine has to be imported into the mitochondria where it becomes a substrate for the glycine cleavage system (GCV) generating NH_3 (Figure 3.2). My rationale is that if Hem25 is a glycine transporter, the absence of this protein would produce an impairment of cell growth when glycine is provided as the sole nitrogen source. To determine the ability of *hem25* Δ cells to grow with glycine as the sole source of nitrogen, growth over time was measured under this condition. As a negative control a strain carrying a deletion of *LPD1* was used. *LPD1* encodes dihydrolipoamide dehydrogenase, an essential component of the GCV. Inactivation of the *HEM25* gene impaired cell growth when glycine was the sole nitrogen source, although not to the extent observed for inactivation of *LPD1* (Figure 3.3). This result is consistent with the hypothesis that when Hem25 is absent, glycine import into the mitochondria is impaired.

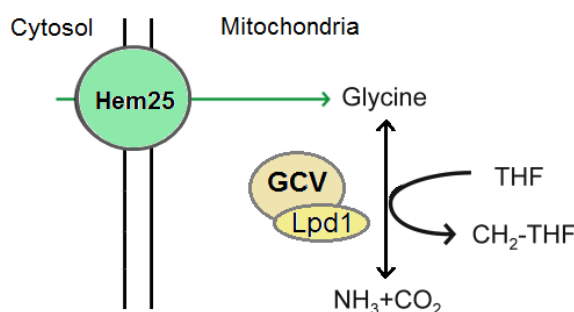


Figure 3.2: **The utilization of glycine as nitrogen source depends on the GCV.** 5,10-methylene-tetrahydrofolate ($\text{CH}_2\text{-THF}$), tetrahydrofolate (THF)

The second condition to test the hypothesis that Hem25 is a glycine importer is based on serine-glycine metabolism. For yeast cells growing on fermentable media, serine is synthesized from 3-phosphoglycerate, an intermediary of glycolysis. Serine, in addition to being required for protein, phospholipid and sphingolipid synthesis, is the main source of one-carbon units through the serine hydroxymethyltransferase enzymes Shm2 and Shm1 located in the cytosol and the mitochondria, respectively

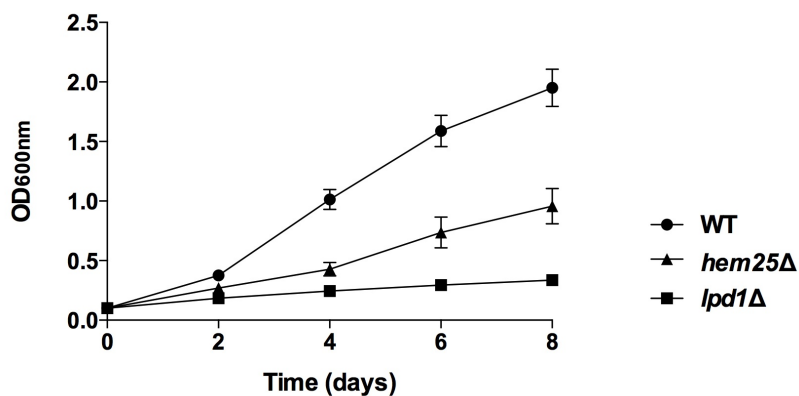


Figure 3.3: **Impairment of *hem25*Δ cells to grow in glycine as sole nitrogen source.** Cells of the indicated genotypes were grown into log phase at 30 °C in SD containing 1 g/L of ammonium sulfate. Cells were washed and reinoculated at OD_{600nm} 0.1 into SD medium containing 30 g/L glycine as sole nitrogen source. Growth at 30 °C was monitored by turbidity at 600 nm, for eight days. Data shown are the mean ± SEM for four replicates for wild type and *lpd1*Δ cells and nine replicates for *hem25*Δ cells. Growth rates for *hem25*Δ and *lpd1*Δ strains were both significantly different ($p < 0.0001$) to wild type (WT) growth rate.

(Figure 3.4). Yeast cells with an inactivated *SER1* gene are deprived of serine coming from glycolysis. Under this condition the mitochondrial metabolism of glycine can support the growth of *ser1*Δ cells but only if glycine can be efficiently imported into the mitochondria [59]. In this context, glycine becomes the major source of one-carbon units through its catabolism by the mitochondrial GCV. Subsequently, serine can be synthesized by the reverse reaction of the mitochondrial serine hydroxymethyltransferase consuming glycine and one-carbon units [59]. Based on this rationale, the absence of Hem25 would further impair the growth of *ser1*Δ cells when glycine was used to provide one-carbon units.

The ability of the *ser1*Δ, *hem25*Δ and *ser1*Δ *hem25*Δ cells to grow were compared and the effect of serine or glycine addition was examined (Figure 3.5). As mentioned above, *hem25*Δ cells grew as well as wild type cells in medium without any addition. As a positive growth condition 1 mM serine was added to the medium, allowing all the strains to grow (Figure 3.5). Consistent with the dependence on GCV for the generation of one-carbon units to synthesize serine, *ser1*Δ cells grew poorly on medium without additions. Importantly, the absence of Hem25 exacerbated this defect. Low concentration of glycine from 0.1 mM up to 1 mM slightly improved

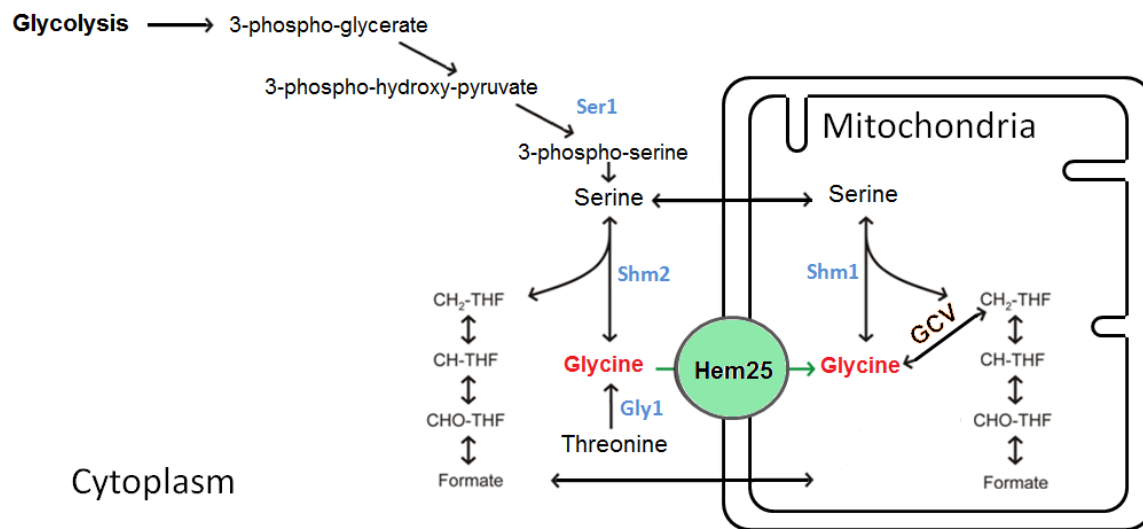


Figure 3.4: **Mitochondrial metabolism of glycine sustains serine synthesis.** Under dextrose-growth conditions the absence of Ser1 deprives the cells of the major pathway of serine synthesis. Therefore, there is a decrease of one-carbon units through Shm2. One carbon metabolism can be sustained in cells lacking Ser1 function through the import of exogenously supplied glycine, but only if that glycine can be imported into the mitochondria for metabolism by the GCV. Enzymes responsible for glycine and one-carbon units synthesis under dextrose-growth conditions are: cytosolic glycine hydroxymethyltransferase (Shm2) in the cytosol and mitochondrial glycine hydroxymethyltransferase (Shm1) in the mitochondria. Threonine aldolase (Gly1) synthesizes glycine from threonine in the cytosol. One-carbon units are: CH₂-THF, 5,10-methenyl-tetrahydrofolate (CH-THF) and 10-formyl-tetrahydrofolate (CHO-THF) [3]. Ser1 is the 3-phosphoserine aminotransferase. Transfer of glycine, serine and formate occurs between compartments and is modulated through mitochondrial carriers.

the growth of *ser1*Δ cells, without enhancing the growth of *ser1*Δ *hem25*Δ cells. These results are consistent with Hem25 being required for mitochondrial glycine metabolism. Interestingly, a higher concentration of glycine (5 mM) restored the growth of the double mutant cells, suggesting the presence of another glycine transporter.

The combined results strongly support the hypothesis that Hem25 is required for glycine import into the mitochondria.

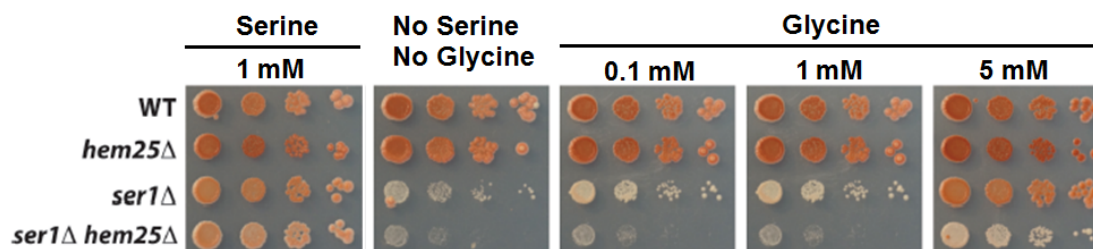


Figure 3.5: **Hem25 deficiency impairs *ser1* Δ cell growth.** Cells of the indicated genotypes were grown to mid-log phase in SD medium containing 1 mM serine, washed and re-suspended in sterilized water to OD_{600nm} 0.4. Cells were serially diluted (1:10) and spotted on SD medium supplemented as indicated. Plates were imaged after incubation for 5 days at 30 °C. WT denotes wild type.

3.1.3 Cytoplasmic Threonine Aldolase is the Main Source of *de novo* Synthesized Glycine Used for Heme Synthesis

In order to determine the source of *de novo* glycine for heme synthesis, I analyzed the contribution of glycine synthesis enzymes to the cellular pools of glycine and heme. The genes encoding the three enzymes (Figure 3.6) that catalyze the synthesis of glycine under dextrose-grown conditions in *S. cerevisiae* are *GLY1*, *SHM1*, and *SHM2*.

GLY1, *SHM1* and *SHM2* genes were deleted and the levels of glycine and heme were measured on dextrose-grown *gly1*Δ, *shm1*Δ and *shm2*Δ single mutant cells. Glycine levels decreased 50 % when the genes encoding the serine hydroxymethyltransferases Shm1 or Shm2 were deleted, whereas the levels of heme were not significantly diminished for either of these strains (Figure 3.7). In contrast, deletion of the *GLY1* gene, encoding cytoplasmic threonine aldolase, dramatically decreased the cellular level of heme by 75 % and glycine mass by 90 % .

As glycine deprivation upon Gly1 deficiency impacts on heme levels, I wanted to analyze how the absence of Hem25 would affect the phenotype of the cells lacking Gly1. Consistent with its partial glycine auxotrophy phenotype [59, 60, 82], *gly1*Δ cells grew as well as wild type and *hem25*Δ cells on glycine-supplemented SD medium, whereas in the absence of glycine supplementation, the growth of *gly1*Δ cells was impaired (Figure 3.8). The absence of *hem25*Δ did not affect the growth of *gly1*Δ in glycine-supplemented media but it exacerbated the growth defect on non-supplemented media. Whereas serine supplementation had a negligible effect on

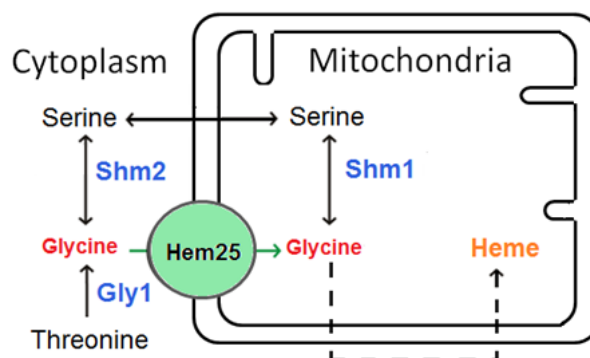


Figure 3.6: Glycine biosynthesis for fermenting yeast cells.

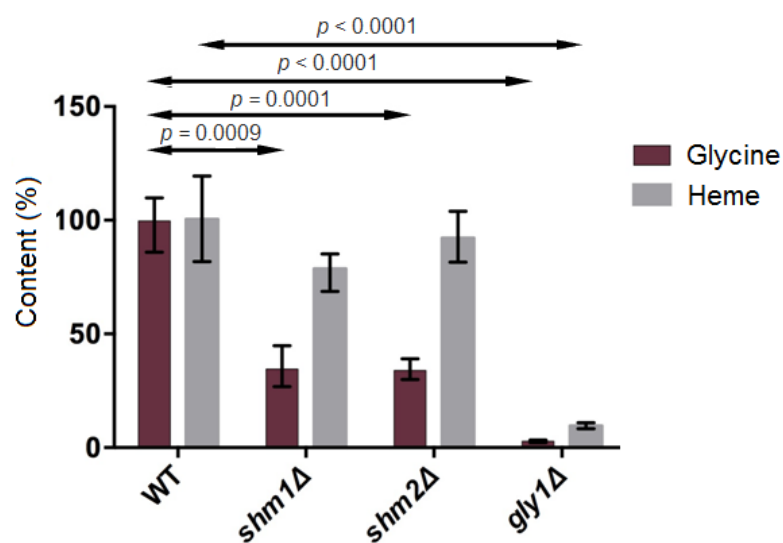


Figure 3.7: **Gly1 produces the main source of *de novo* synthesized glycine for heme synthesis.** Yeast cells of the indicated genotypes were grown at OD_{600nm} 0.6-1.0 in SD medium. Cells were processed for heme and glycine determination. Wild type glycine content was 6 nmol/ 10^8 cells and heme content 31 fmol/ μ g protein. The numbers represented in the graph are the percentages relative to wild type (WT). Values are the mean \pm SEM of three independent determinations.

*gly1*Δ *hem25*Δ growth, the addition of 5-Ala slightly improved the growth of the double mutant. The fact that 5-Ala supplementation did not restore the growth of *gly1*Δ *hem25*Δ to the level of *gly1*Δ suggests that other processes depending on mitochondrial glycine beyond heme synthesis are affected by Hem25 deficiency.

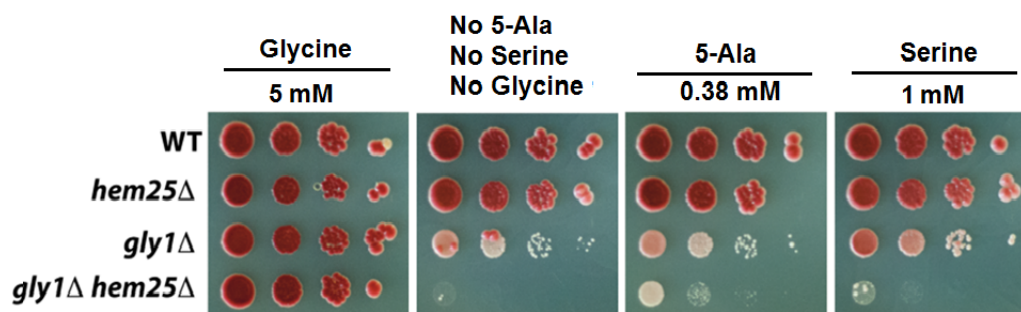


Figure 3.8: **The absence of Hem25 exacerbated the phenotype of *gly1*Δ cells.** Yeast cells of the indicated genotypes were grown into log phase at 30 °C in SC supplemented with 5 mM glycine and 0.38 mM 5-Ala to keep the double mutant cells growing without impairment. Cells were washed and re-suspended in sterilized water to OD_{600nm} 0.4. Cells were serially diluted (1:10) and spotted on SD solid medium containing glycine, serine, 5-Ala or non-supplemented. Plates were imaged after incubation for 5 days at 30 °C. WT denotes wild type.

I measured heme content in *hem25*Δ *gly1*Δ cells to determine whether the absence of Hem25 further decreases the heme levels of *gly1*Δ cells. The experiment showed that heme was not substantially diminished beyond what was observed in *gly1*Δ cells (Figure 3.9). This result is consistent with two proteins contributing to the same linear pathway leading to the downstream product.

3.1.4 Glycine and 5-Ala Rescue the Heme Biosynthetic Defect in the Yeast Model of CSA

Having identified a potential glycine defect in *hem25*Δ yeast, experiments aimed at finding a potential therapy for patients that suffer CSA were then undertaken, utilizing the *S. cerevisiae* model of CSA. I hypothesized three scenarios (Figure 3.10) to ameliorate the defect in heme synthesis in *hem25*Δ cells: (i) supplementation with high exogenous glycine to increase substrate availability for the first step in heme synthesis through a putative second glycine transporter, (ii) addition of excess serine to drive endogenous glycine synthesis in the mitochondria, or (iii) addition of the

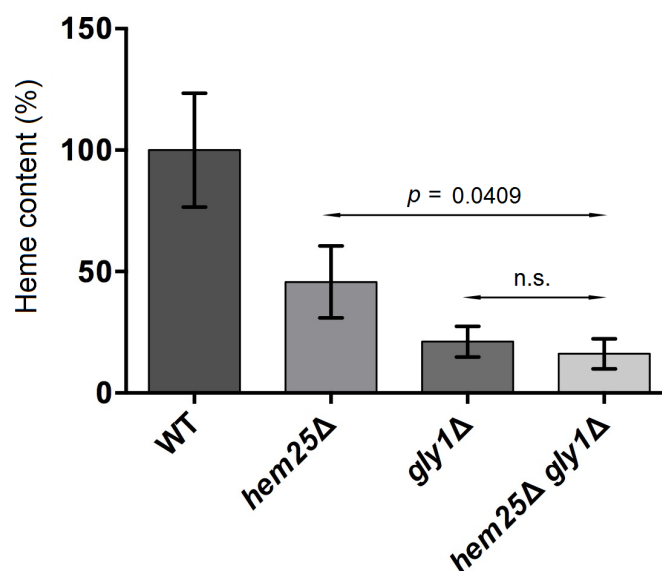


Figure 3.9: **Heme content in *hem25*Δ *gly1*Δ was not significantly decreased compared with *gly1*Δ.** Yeast cells of the indicated genotypes were grown to log phase at 30 °C in SC supplemented with 5 mM glycine and 0.38 mM 5-Ala to keep the double mutant cells growing without impairment. Cells were washed and reinoculated at OD_{600nm} 0.1 into SD medium. Cells were grown to mid-log phase and processed for heme determination. Heme values are the mean \pm SEM of three independent determinations. The numbers represented in the graph are the percentages relative to wild type (WT).

downstream metabolite, 5-Ala, within the heme biosynthesis pathway.

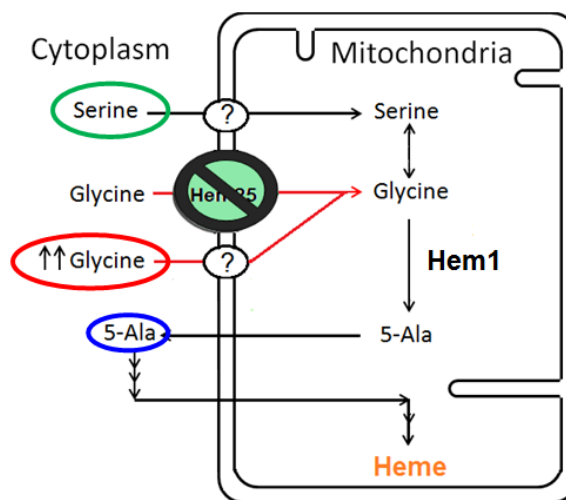


Figure 3.10: **Supplementation of CS yeast model.**

Each supplement was added to wild type, *hem25* Δ , and *hem1* Δ cells, and heme content was measured. As expected, the *hem1* Δ cells were dependent on supplementation with 5-Ala to synthesize heme, with glycine and serine providing no restoration of heme levels. Results showed that *hem25* Δ cells had a 50 % decrease in the level of heme, which was restored to wild type levels by the addition of 5-Ala or high concentration of exogenous glycine (Figure 3.11). Serine had no restorative effect on the *hem25* Δ cells. Supplementation to the wild type cells did not significantly change heme content.

Based on the decreased heme levels observed for the *gly1* Δ *hem25* Δ cells (Figure 3.9), I carried out an experiment where these double mutant cells were supplemented with serine, glycine and 5-Ala, and then measured heme content. The result of this experiment also showed that the addition of glycine or 5-Ala restored heme to normal levels in the *hem25* Δ *gly1* Δ cells, whereas serine did not restore heme content (Figure 3.12).

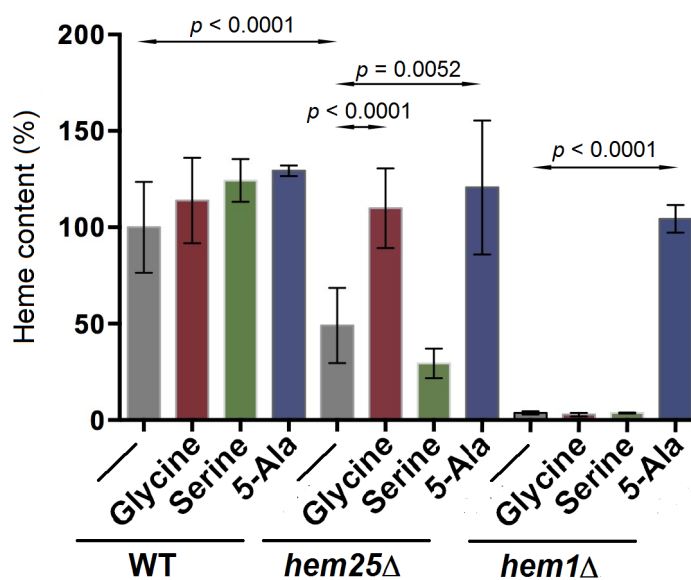


Figure 3.11: **Glycine and 5-Ala restored heme content in *hem25Δ* cells to wild type levels.** Yeast cells of the indicated genotypes were grown to mid-log phase in SD medium in the absence or presence of 5 mM glycine, 1 mM serine or 0.38 mM 5-Ala (the *hem1Δ* strain was grown in the presence 0.0038 mM 5-Ala as a basic growth condition). Cells were processed for heme determination. Heme values are the mean \pm SEM of at least six independent determinations. The values were normalized to wild type (WT).

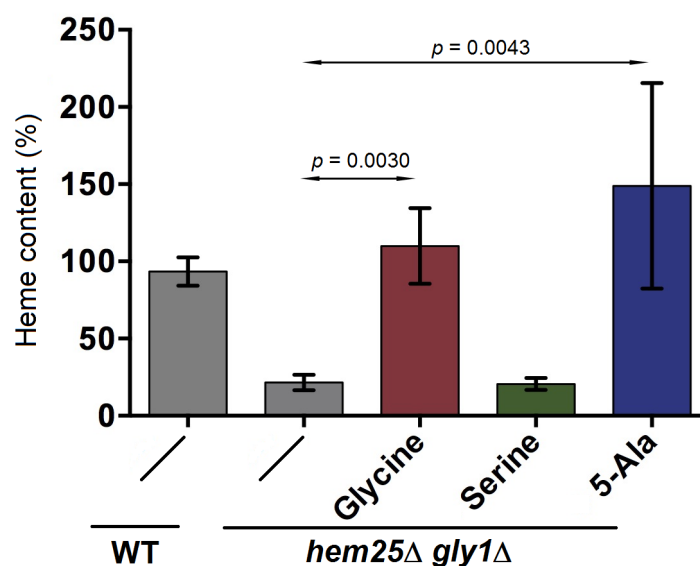


Figure 3.12: **Glycine and 5-Ala restored heme content in *hem25Δ gly1Δ* cells to wild type levels.** Yeast cells of the indicated genotypes were grown into log phase at 30 °C in SC supplemented with 5 mM glycine and 0.38 mM 5-Ala to keep the double mutant cells growing without impairment. Cells were washed and reinoculated at OD_{600nm} 0.1 into SD medium in the absence or presence of 5 mM glycine, 1 mM serine or 0.38 mM 5-Ala. Cells were grown to mid-log phase and processed for heme determination. Values were normalized to wild type (WT). Heme values are the mean \pm SEM of three independent determinations.

3.2 Discussion

3.2.1 Hem25 Participates in Heme Synthesis Facilitating Mitochondrial Glycine Import

The results presented here using the yeast *S. cerevisiae* as a model system of CSA support a role for Hem25 mediating the import of cytosolic glycine into the mitochondria. Consistent with the reduction of 5-Ala levels in *hem25* Δ cells observed by Guernsey *et al.* [37], here I report that loss of Hem25 function results in decreased cellular heme levels. Furthermore, the expression of human SLC25A38 in *hem25* Δ cells restored heme content to wild type levels. These observations support the use of yeast as a model to infer SLC25A38 function as a reduction of heme levels is a hallmark of CSA caused by mutation in *SLC25A38* gene.

To address the possible role of Hem25 as a mitochondrial glycine importer I investigated the dependence on Hem25 of two metabolic processes that require glycine import into the mitochondria. Yeast cells lacking the *SER1* gene (*ser1* Δ) are deprived of serine synthesized from 3-phospho-serine (glycolysis pathway), and rely on mitochondrial generation of one-carbon units through the GCV and serine synthesis by the reverse reaction catalyzed by mitochondrial Shm1 [59]. The absence of Hem25 aggravated the growth defect of *ser1* Δ cells, supporting the hypothesis that Hem25 is required for efficient import of glycine into the mitochondria. Consistent with this, glycine supplementation up to 1 mM slightly improved the growth of *ser1* Δ cells whereas *ser1* Δ *hem25* Δ cells were still significantly impaired. The utilization of glycine as a nitrogen source also depends on the activity of the GCV. Congruent with the proposed role of Hem25 facilitating glycine import, *hem25* Δ cells grew poorly on glycine as a nitrogen source. The result of these studies strongly imply that SLC25A38/Hem25 are required for efficient glycine import into the mitochondria.

The proposed role of Hem25 as a mitochondrial glycine importer implies that the cytosolic pool of glycine is the main source for heme synthesis. I measured cellular glycine content and heme levels in yeast cells deficient in each enzyme involved in glycine synthesis under dextrose-grown conditions. Consistent with the notion that Hem25 transports glycine into the mitochondria for heme synthesis, the absence of cytosolic Gly1, which is the main contributor to glycine biosynthesis [59, 60], led to

a 90 % decrease in glycine content and 75 % reduction in heme levels compared to wild type cells.

When the contribution of Shm1 and Shm2 to glycine and heme levels were studied, the results showed that glycine levels were decreased by 50 % by the inactivation of the *SHM1* or *SHM2* gene, whereas heme levels were not significantly reduced for either of these mutants. McNeil *et al.* [59] showed that cells lacking Shm1 had a wild type growth rate whereas *shm2* Δ cells had a slight decrease in growth rate. Interestingly, in their study the addition of glycine to *shm2* Δ cells did not improve growth rate whereas formate did, suggesting that the growth defect of cells lacking Shm2 was due to decreased generation of cytosolic one-carbon units and not because of reduced glycine levels. My results together with those of McNeil *et al.* indicate that glycine diminution upon *SHM1* or *SHM2* inactivation does not affect heme content or growth rate.

The absence of Gly1 leads to a 90 % reduction of cellular glycine that results in a partial glycine auxotrophy (Figure 3.7). Remarkably, the inactivation of *HEM25* exacerbated the phenotype of *gly1* Δ . Under glycine deprivation the role of Hem25 contributing to heme synthesis becomes evident as the severe growth defect of *gly1* Δ *hem25* Δ cells was partially alleviated by 5-Ala supplementation. This observation is consistent with Hem25 acting as a high affinity glycine transporter and being able to divert a fraction of the limited pool of cellular glycine resulting from loss of Gly1 to the mitochondria for functions other than just heme synthesis. Although the heme level is reduced in *gly1* Δ cells, its rate of synthesis does not limit growth as revealed by the fact that the growth of *gly1* Δ cells was not improved by 5-Ala supplementation. Instead, this suggests that other glycine dependent processes restrict *gly1* Δ proliferation. In the absence of Hem25 and Gly1, the rate of heme synthesis becomes one pathway that is contributing to the decrease in growth rate. The addition of 5-Ala would displace the heme metabolic block resulting in another mitochondrial glycine dependent process limiting cell growth. This other mitochondrial metabolic process that limits growth is yet to be determined. Why do heme levels for *gly1* Δ cells and *gly1* Δ *hem25* Δ cells not differ when their growth rates are different? The heme content for both strains might revolve around a minimum level per viable cell. They differ in the presence of Hem25 that, under glycine deprivation, allows a higher

rate of heme synthesis and consequent higher growth rate, although growth is still limited by some other glycine dependent process.

3.2.2 Hem25 Deficiency Elicits Partial Phenotypes

The analyses aimed to address the involvement of Hem25 on mitochondrial glycine metabolism support a role for Hem25 as a glycine importer. However, the phenotypes associated with Hem25 deficiency were partial and could be alleviated by high concentration of glycine supplementation.

3.2.3 Is Hem25 also a Serine Importer?

I have presented results that relate the activity of the GCV with the function of Hem25, revealing the involvement of Hem25 in mitochondrial glycine metabolism. However, the fact that certain phenotypes associated with Hem25 deficiency were alleviated by glycine but not by serine supplementation could also support the view that Hem25 transports serine, that the defects associated with *HEM25* inactivation arise from reduced mitochondrial serine import and that a low affinity glycine transporter mediates the beneficial effects of high glycine supplementation. I first consider the possibility that Hem25 transports serine exclusively. The reduction in 5-Ala [37] and heme levels (Figure 3.1) associated with Hem25 deficiency implies that serine transported by Hem25 is a major source of mitochondrial glycine, generated by Shm1 and consumed for 5-Ala synthesis by Hem1. However, the effect of *SHM1* inactivation on heme levels is very minor and not as profound as the reduction of heme levels observed for *hem25* Δ cells. This inconsistency implies that Hem25 is not a serine transporter or that Hem25 transports glycine in addition of serine.

It has been reported that *shm1* Δ cells grow with rates similar to wild type cells (doubling time: 2 hrs) whereas *shm2* Δ cells exhibit a growth impairment (doubling time 3.5 hrs) associated with shortage in one-carbon units that can be alleviated by formate supplementation [59]. Consistent with this observation, two studies have shown that *shm2* Δ *shm1* Δ cells are more impaired for growth than *shm2* Δ cells. McNeil *et al.* [59] reported the doubling times were 5 and 3.5 hrs for *shm2* Δ *shm1* Δ cells and *shm2* Δ , respectively. Kastanos *et al.* [44], working in another yeast genetic background, showed that the doubling time of *shm2* Δ *shm1* Δ was 21 hrs whereas

shm2 Δ cells duplicated every 6 hrs. Both studies showed that the addition of formate restored the growth rate to their respective wild type values. If Hem25 were a serine transporter, the growth phenotype of the *shm2* Δ *hem25* Δ cells should be worse than the single *shm2* Δ cells and close to the *shm2* Δ *shm1* Δ phenotype because the absence of Hem25 would prevent the entry of serine into the mitochondria and restrict the generation of one-carbon units by Shm1. However, my comparison of growth for *shm2* Δ versus *shm2* Δ *hem25* Δ cells did not reveal a significant difference (Figure 3.13). The absence of Hem25 does not aggravate the growth phenotype of *shm2* Δ cells suggesting that Hem25 is not a serine transporter.

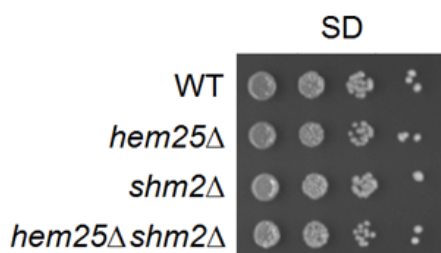


Figure 3.13: **Hem25 deficiency does not aggravate the growth phenotype of *shm2* Δ cells.** Yeast cells of the indicated genotypes were grown into log phase at 30 °C in SC, washed and re-suspended in sterilized water to OD_{600nm} 0.4. Then cells were serially diluted (1:10) and spotted on SD medium. Cells were grown for 5 days at 30 °C. WT denotes wild type

McNeil [59] reported that *shm2* Δ *gcv1* Δ cells had a slight growth defect, which was dramatically worsened by inactivation of *SHM1*. Both strains had their growth rates restored to wild type by formate addition. If Hem25 was involved in serine import, *shm2* Δ *gcv1* Δ *hem25* Δ cells should grow slower than *shm2* Δ *gcv1* Δ cells, as the absence of Hem25 would deprive Shm1 of its substrate serine, limiting the generation of one-carbon units. The growth comparison of five independent *shm2* Δ *gcv1* Δ isolates and six independent *shm2* Δ *gcv1* Δ *hem25* Δ isolates showed that the double and triple mutants strains grew with similar rate (data not shown), indicating that Hem25 does not contribute to substrate supply for Shm1.

3.2.4 Glycine and 5-Ala Rescue the Heme Biosynthetic Defect in the Yeast Model of CSA

Since the cure for CSA due to mutations in *SLC25A38* has not yet been found, I searched for potential treatments using yeast as a model. The rationale was to treat the *hem25* Δ cells with 5-Ala, glycine or serine, and then measure the heme content to see if it was restored to wild type values. The therapy with high concentrations of glycine was based on the evidence that there is likely a second mitochondrial glycine importer. Supplementation of the *hem25* Δ cells with high concentration of glycine restored heme content to wild type levels. The explanation is that this increases the mitochondrial glycine availability for the first step in heme synthesis. Supplementation of the *hem25* Δ cells with 5-Ala, a downstream metabolite within the heme pathway, also restored heme synthesis. Finally, the supplementation of the *hem25* Δ cells with serine to drive endogenous glycine synthesis by the mitochondria did not show any significant increase in heme content. The same treatment was applied to *hem25* Δ *gly1* Δ cells, which had a more severe defect in growth and had a similar defect in heme content. For double mutant cells grown with glycine or 5-Ala supplementation, heme content was restored to wild type levels. Interestingly, these two studies are congruent with results of the study by Guersney *et al.* [37], where they showed that cells lacking Hem25 were not able to reduce sodium nitroprusside (note that this reaction requires heme). However, when *hem25* Δ were supplemented with glycine or 5-Ala, cells were able to reduce sodium nitroprusside, implying restoration of heme levels by the addition of these two supplements. A potential biomedical application of this part of my work is that high concentrations of glycine and 5-Ala could be used as a potential therapy for CSA.

3.2.5 Fluctuation of the Steady State Concentration of Some Metabolites Does Not Alter the Growth Rate

I report here that *hem25* Δ cells grew as well as wild type cells on SD media despite the fact that heme content in *hem25* Δ cells was 50 % of wild type levels. These observations are consistent with data reported by Kardon *et al.* [43], showing that a *hem25* Δ strain in the W303 background exhibited a 75 % decrease in porphyrin levels compared to wild type cells, and the growth rate of *hem25* Δ cells was unaffected.

They characterized Mcx1 as a chaperone that contributes to heme synthesis by facilitating the binding of the cofactor PLP to Hem1. Even though the *mcx1* Δ strain, as well as a strain where Hem1 levels were reduced (using an allele *HEM1*-DAmP) strain exhibited a significant diminution of 5-Ala and porphyrin levels, their growth on dextrose-based media were also not impaired. These results suggest that a further diminution of heme level is required to hinder growth for fermenting yeast cells and are consistent with my observations of an absence of a growth defect phenotype for *hem25* Δ cells.

The main findings described in this chapter are that SLC25A38/Hem25 is likely a glycine transporter required for the synthesis of heme. The *de novo* source of glycine for heme synthesis is synthesized by cytoplasmic Gly1, consistent with the requirement for a glycine importer for the synthesis of heme. Glycine synthesized by cytoplasmic and mitochondrial Shm2 and Shm1, respectively, is only a minor contributor to heme synthesis under the conditions used here. Finally, the requirement for glycine needed for heme synthesis can limit cell growth, but it is not the only glycine metabolic pathway within the mitochondria that limits growth. Further research will be required to determine the presence of other mitochondrial processes that limit growth when glycine import is limiting. The other major outstanding question is the identity of the putative second mitochondrial transporter. I performed a genetic screen in yeast to determine which other *SLC25* family members affected growth of cells lacking Hem25 function to (i) gain insight into mitochondrial processes that require efficient glycine import and (ii) determine if any of the *SLC25* family members encode the second glycine importer.

Chapter 4

Growth Impairment by Simultaneous Deletion of *HEM25* and Members of the *SLC25* Family

The results presented so far strongly suggest a role for Hem25 in mitochondrial glycine import. These results also reveal that the phenotypes associated with Hem25 deficiency are only partial: inactivation of the *HEM25* gene impaired the ability of cells to grow when glycine was the sole nitrogen source, although not to the extent observed for inactivation of Lpd1 (Figure 3.3). In addition, the fact that the supplementation with 5 mM glycine in the growth media restored heme content of *hem25* Δ strain to wild type levels (Figure 3.11) and restored growth of the *ser1* Δ *hem25* Δ strain (Figure 3.5) suggests that another carrier imports glycine into the mitochondria when glycine is present at high concentrations. Based on these observations and the importance of such a putative second transporter might have for the treatment of CSA patients with high doses of glycine supplementation, my research work was aimed to find such a carrier.

From here on, all experiments were carried out using *S. cerevisiae* strains of the BY4741/BY4742 genetic background. The systematic yeast gene deletion collection was made in this background, and it was chosen here to facilitate genetic interaction analysis. The BY4741/BY4742 strain carries a Ty1 element inserted in the 3' region of the *HAP1* open reading frame, which makes this mutant allele act as a *HAP1* null allele [33]. The *HAP1* gene encodes a complex transcriptional regulator of many genes, and it is involved in regulation of gene expression in response to heme and oxygen levels. For this reason strains from the BY4741/BY4742 background used in this study were transformed with a plasmid carrying a wild type allele of *HAP1* (named NDY 601).

4.1 Results

4.1.1 Phenotypic Analysis Using Glycine as the Sole Nitrogen Source

I performed a genetic screen looking for a putative second glycine importer among the members of the SLC25 family. The screen was based on the ability of yeast to grow utilizing glycine as a nitrogen source. For yeast cells to grow using glycine as a sole nitrogen source, glycine is imported into the mitochondria where it becomes a substrate of the GCV for the generation of NH_3 (Figure 3.2). I hypothesized that a strain defective in both Hem25 function and a second glycine importer would exhibit a more severe growth impairment on glycine as a sole nitrogen source than *hem25* Δ cells. It has to be considered that genes involved in other processes could also be identified in this analysis. These other processes could be affected by the deletion of carriers participating in other mitochondrial functions that in combination with *HEM25* deficiency exacerbate the defect for glycine utilization.

Double gene deletion strains were constructed by standard genetic crosses of *hem25* Δ with the thirty one single gene deletion strains carrying mutations for each member of the yeast *SLC25* family, followed by sporulation and haploid cell selection. Each single or double mutant was grown overnight in liquid SD-Ura medium containing 1 g/L of ammonium sulfate. Cells were washed twice and resuspended in liquid SD-Ura medium containing 30 g/L glycine as the sole nitrogen source. An OD_{600nm} 0.1 was standardized for every culture at time 0. Cells were grown at 30 °C and OD_{600nm} was monitored for five days. The results presented in Figure 4.1 represent the growth of relevant strains reached after five days of cultivation.

In addition of wild type and *hem25* Δ cells, *lpd1* Δ cells were included in the analysis as controls. Lpd1 is the dihydrolipoamide dehydrogenase, which is an essential subunit of the glycine cleavage system. In the absence of Lpd1, NH_3 cannot be made from glycine.

From the thirty-one double mutant strains studied in this analysis, six grew slower than the respective single mutant strains (Figure 4.1). The genes that, when deleted, worsened the growth of *hem25* Δ cells were: *FLX1*, *PET8*, *MTM1*, *ORT1*, *SFC1* and *YMC1*. The function of these genes are shown in Table 4.1. The strain with deletion of both *AAC3* and *HEM25* showed an impairment to grow compared with *aac3* Δ

cells, but not with respect to *hem25* Δ cells. The reason *AAC3* was included in the list of genes that interact with *HEM25* is because it showed genetic interaction with *HEM25* by another phenotypic analysis that is shown later in this chapter.

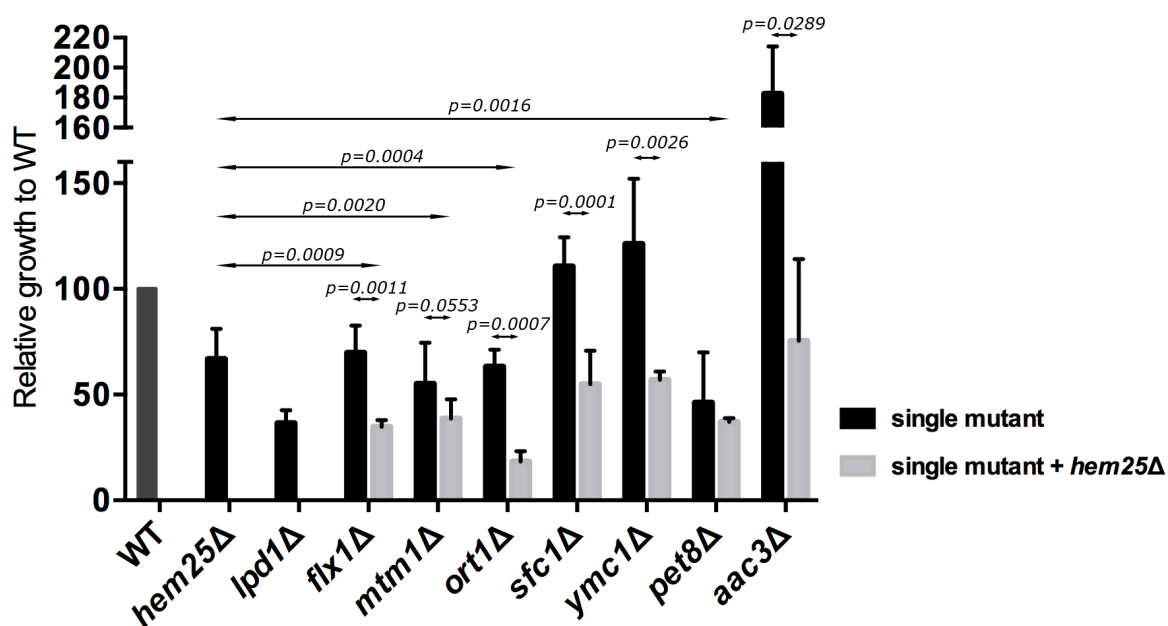


Figure 4.1: Growth analysis on glycine as sole nitrogen source for interactors of the *SLC25* family with *HEM25*. Cells of the indicated genotypes were grown in SD-Ura medium using 1 g/L of ammonium sulfate, washed and inoculated at OD_{600nm} 0.1 in SD-Ura medium with 30 g/L glycine as sole nitrogen source. OD was measured after 5 days of growth. Differences between single deletion strains and double deletion strains were determined using a fixed factor ANOVA test. At least three independent experiments were done to calculate the *p* values, mean and standard deviation. The numbers represented in the graph are the percentages relative to wild type (WT). The WT OD was 0.912.

Table 4.1: **Function of the candidate genes that showed genetic interaction with *HEM25***. SGD description from <http://www.yeastgenome.org/>

Standard Name	Systematic Name	SGD description
<i>FLX1</i>	<i>YIL134w</i>	Mitochondrial flavin adenine dinucleotide transporter; FAD is a synthesis product of riboflavin; human homolog <i>SLC25A32</i> is implicated in multiple acyl-CoA dehydrogenase deficiency (MADD) or glutaric aciduria type II (GAI), and can complement yeast null mutant
<i>MTM1</i>	<i>YGR257c</i>	Mitochondrial protein of the mitochondrial carrier family; high affinity pyridoxal 5'-phosphate (PLP) transporter, important for delivery of PLP cofactor to mitochondrial enzymes; involved in mitochondrial iron homeostasis and in activating mitochondrial Sod2p by facilitating insertion of an essential manganese cofactor
<i>ORT1</i>	<i>YOR130c</i>	Ornithine transporter of the mitochondrial inner membrane; exports ornithine from mitochondria as part of arginine biosynthesis; functionally complemented by human ortholog, <i>SLC25A15</i> , which is associated with hyperammonaemia-hyperornithinaemia-homocitrullinuria (HHH) syndrome, but HHH-associated variants fail to complement
<i>SFC1</i>	<i>YJR095w</i>	Mitochondrial succinate-fumarate transporter; transports succinate into and fumarate out of the mitochondrion; required for ethanol and acetate utilization
<i>YMC1</i>	<i>YPR058w</i>	Putative mitochondrial inner membrane transporter; proposed role in oleate metabolism and glutamate biosynthesis; member of the mitochondrial carrier (MCF) family; localizes to vacuole in response to H ₂ O ₂ ; <i>YMC1</i> has a paralog, <i>YMC2</i> , that arose from the whole genome duplication
<i>PET8</i>	<i>YNL003c</i>	S-adenosylmethionine transporter of the mitochondrial inner membrane; member of the mitochondrial carrier family; required for biotin biosynthesis and respiratory growth
<i>AAC3</i>	<i>YBR085w</i>	Mitochondrial inner membrane ADP/ATP translocator; exchanges cytosolic ADP for mitochondrially synthesized ATP; expressed under anaerobic conditions; similar to <i>Aac1p</i> ; has roles in maintenance of viability and in respiration; <i>AAC3</i> has a paralog, <i>PET9</i> , that arose from the whole genome duplication

Double mutant strains *flx1*Δ *hem25*Δ, *mtm1*Δ *hem25*Δ and *ort1*Δ *hem25*Δ showed a severe growth impairment, similar to the control strain *lpd1*Δ (Figure 4.1). The double mutants *sfc1*Δ *hem25*Δ and *ymc1*Δ *hem25*Δ had a significant growth defect compared with *sfc1*Δ and *ymc1*Δ cells respectively, but they did not have a significantly decreased growth rate compared with *hem25*Δ cells. The *pet8*Δ *hem25*Δ cells showed a significant decrease in growth rate compared with *hem25*Δ cells, but

not with *pet8* Δ cells. Although the last three double mutant strains only showed a significant decrease in growth rate with respect to one of its respective single mutants, they were included in the list of genes that interact with *HEM25*. This is because they exhibited genetic interaction with *HEM25* through other phenotypic analyses that are shown later in this chapter.

This phenotypic analysis based on the capacity of cells to grow with glycine as a sole nitrogen source revealed genes that when inactivated interacted with loss of *HEM25*. Based on this first approach to find a putative second glycine transporter, any of these members of the SLC25 family could be a potential transporter of glycine. However, to further study of these interactions is needed in order to determine if Flx1, Mtm1, Ort1, Pet8, Sfc1 or Ymc1 could be the putative glycine importer.

4.1.2 Phenotypic Analysis Showed Heme Content Was Decreased in a Subset of the Double Mutant Cells

From the screen based on the ability of yeast to grow utilizing glycine as a nitrogen source, I identified *HEM25* interacting genes that were already known to be involved in heme synthesis. *MTM1* codes for a PLP transporter [100]. PLP is required as a cofactor for GCV and Hem1 [97, 89]. Aac3 has been proposed as a heme precursor transporter in humans [5], which suggests that its deletion would impact heme levels. Sfc1 imports succinate into the mitochondria [68], which is a precursor for succinyl-CoA. Succinyl-CoA is a substrate for the first reaction in the heme synthesis pathway. Flx1 transports flavin adenine dinucleotide (FAD) into the mitochondria [95]. Hem14 is a mitochondrial enzyme that catalyzes the seventh reaction in the pathway of heme synthesis and it has a FAD-binding site [46]. The absence of FAD in the mitochondria could impact heme synthesis. Although the functions of Pet8, Ymc1 or Ort1 are not known to be involved in heme synthesis, they were included in this study to determine whether they also affect heme synthesis.

I was interested in determining how the absence of these other mitochondrial carriers impacted heme levels *per se*, and also in combination with deletion of *HEM25*. I found that there was a significant decrease in heme content in the double mutant strains of *HEM25* with *FLX1*, *ORT1*, *SFC1*, *YMC1* and *PET8*, compared with their respective single mutant cells (Figure 4.2). In contrast, the double mutant *aac3* Δ

hem25 Δ had a significant reduction in heme content compared with *aac3* Δ cells, but it did not decrease when compared to *hem25* Δ cells (Figure 4.2). When *MTM1* was deleted in *hem25* Δ cells, heme content did not significantly decrease compared to that in *mtm1* Δ cells.

Cells lacking *LPD1* were also included in the analysis. *LPD1* codes for Lpd1, which is also one of the subunits of the ketoglutarate dehydrogenase complex (KGD) complex. This complex makes succinyl-CoA, which is one of the substrates required for heme synthesis. The *lpd1* Δ cells showed a significant decrease in heme content compared with wild type cells.

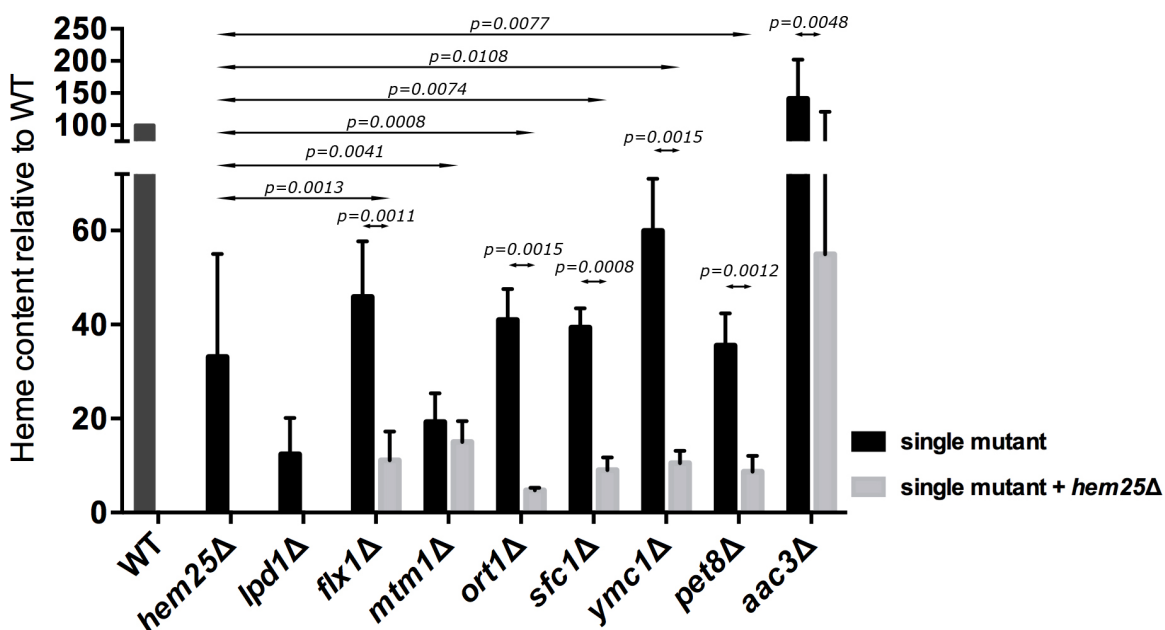


Figure 4.2: **Heme content for selected interactors of *HEM25*.** Cells of the indicated genotypes were grown at OD_{600nm} 0.6-1 in SD-Ura. Cells were harvested and processed for heme determination. Differences between single deletion strains and double deletion strains were determined using a fixed factor ANOVA test. At least three independent experiments were done to calculate the *p* values, mean and standard deviation. Values are normalized to wild type (WT).

The use of this analysis based on measuring heme content showed that the genetic interactions of *HEM25* with the *SLC25* family members *FLX1*, *ORT1*, *SFC1*, *YMC1* and *PET8* extended beyond the defect in the utilization of glycine as nitrogen source. Also, this phenotypic analysis could indicate which of these carriers could be the second mitochondrial glycine importer. The absence of Hem25 reduced by 50 % the heme content in cells. It can be speculated that if the second glycine importer is

deleted together with *HEM25*, the heme content would be lower than the heme level observed for *hem25* Δ cells.

4.1.3 Phenotypic Analysis Based on Growth in Fermentable and Non-Fermentable Medium

The analysis of the genetic interactions between *HEM25* and the seven members of the *SLC25* family was extended by scanning the ability to grow under fermentable and non-fermentable conditions. I also analyzed the effect of glycine or 5-Ala addition on cell growth under both regimens. The rationale behind this analysis was that if any of the interacting genes encoded a putative second glycine importer, a null mutant of this gene in combination with *hem25* Δ could result in a severe growth defect due to heme deficiency. Such heme deficiency would be bypassed by the addition of 5-Ala but not by glycine supplementation.

For this phenotypic analysis, single and double mutants were grown overnight in liquid SC-Ura medium supplemented with 5 mM glycine and 0.38 mM 5-Ala to keep the double mutants alive. Cells were washed twice, resuspended in sterile water at OD_{600nm} 0.4, serially diluted and spotted on SD-Ura media with dextrose or lactate. Each medium was also supplemented with 5 mM glycine or 0.38 mM 5-Ala. After three days of growth on dextrose and seven days of growth on lactate the growth phenotype was recorded. The *hem25* Δ cells showed a mild growth defect compared with wild type cells on the media with dextrose or lactate without supplements, but not when supplemented with glycine or 5-Ala.

Double mutant cells for null alleles of *AAC3*, *SFC1*, *FLX1* and *YMC1* with *hem25* Δ showed decreased growth on lactate media. The *aac3* Δ *hem25* Δ strain had a severe growth impairment compared with the single mutants on lactate medium. Neither glycine nor 5-Ala restored the growth defect of the double mutant cells.

The *sfc1* Δ cells had a severe impairment to grow on lactate, but the addition of glycine or 5-Ala restored growth (Figure 4.3). However, the double mutant *sfc1* Δ *hem25* Δ did not grow on lactate and only glycine slightly improved its growth. The *flx1* Δ *hem25* Δ cells did not grow in any of the conditions on lactate media, whereas the single mutant *flx1* Δ showed a growth impairment on non-supplemented media, which was restored by the addition of glycine or 5-Ala. The *ymc1* Δ *hem25* Δ cells

showed a small growth impairment compared with the respective single mutants on lactate medium. The supplementation of the double mutant cells with 5-Ala slightly restored growth defect, whereas glycine did not.

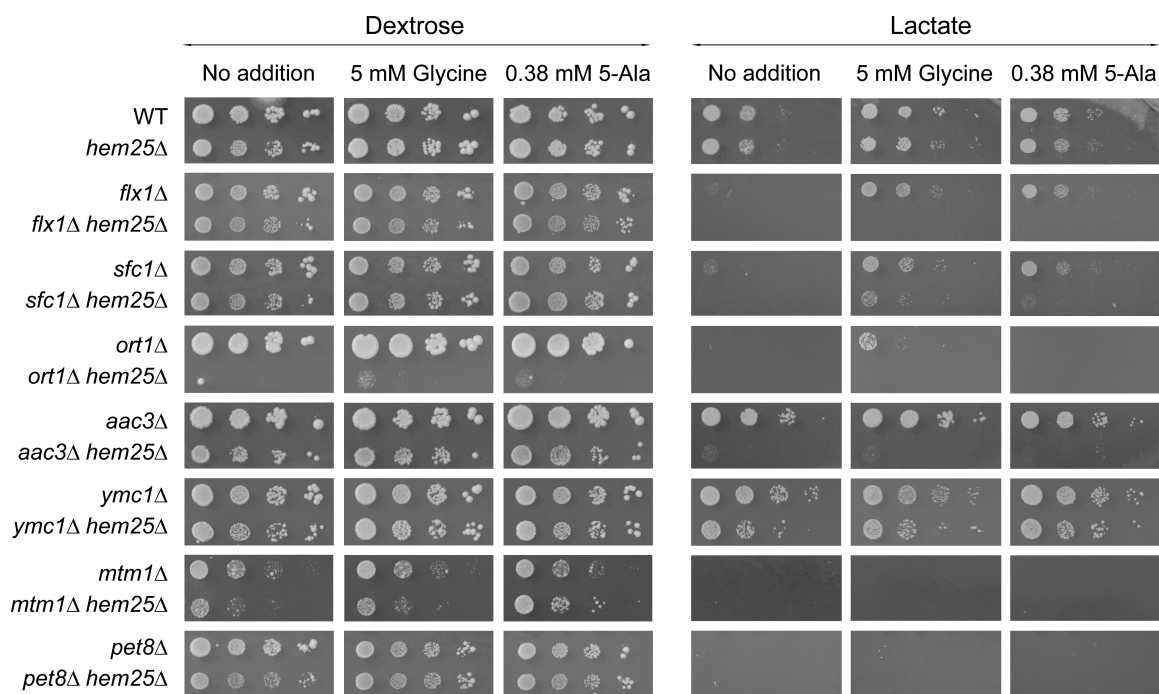


Figure 4.3: **Growth analysis on fermentable and non-fermentable media of selected interactors of the *SLC25* family with *HEM25*.** Yeast strains of the indicated genotypes were grown to early stationary phase in SC-Ura medium supplemented with 5 mM glycine and 0.38 mM 5-Ala to keep the double mutant cells growing without impediment. Cells were washed and re-suspended in sterilized water to OD_{600nm} 0.4, then serially diluted (1:10) and spotted on SD-Ura solid media containing dextrose or lactate. Plates were imaged after 3 and 7 days incubation at 30 °C for dextrose and lactate, respectively. WT denotes wild type.

In the condition where cells grew in dextrose, I found two genes that interacted with *HEM25*: *MTM1*, *ORT1* and *PET8*. The *ort1Δ hem25Δ* cells had a severe growth impairment compared with single mutants on dextrose. The double mutant cells showed only a slight growth improvement when supplemented with glycine or 5-Ala. The *hem25Δ mtm1Δ* cells showed a growth defect on non-supplemented medium, which was alleviated by the addition of 5-Ala. The *hem25Δ pet8Δ* cells showed a very slight growth impairment that was improved by the addition of 5-Ala or glycine.

Based on the rationale of this analysis, Ymc1 and Mtm1 would be candidates for

second glycine importers. This is because the growth defect of the double mutant cells was slightly bypassed by the addition of 5-Ala but not by glycine supplementation. However, Mtm1 is a PLP transporter, which is a cofactor of Hem1. Supplementation with 5-Ala likely restored the growth phenotype because it bypassed the loss of Hem1 function due to deficiency of the cofactor PLP. This would discard the possibility of Mtm1 being the putative glycine transporter.

4.1.4 Ymc1 as a Candidate for a Second Mitochondrial Glycine Importer

I further studied Ymc1 as a potential second glycine importer as the human homologue of *YMC1* (*SLC25A29*) was reported to encode an amino acid carrier [71] and under non-fermenting conditions 5-Ala slightly restored growth of the *ymc1* Δ *hem25* Δ mutant whereas glycine did not (Figure 4.3).

In order to analyze whether Ymc1 could be a second glycine carrier that contributed to heme synthesis, wild type, single mutants and *ymc1* Δ *hem25* Δ cells were grown in SD-Ura media in the absence and presence of either glycine or 5-Ala and heme levels were determined. The objective of this experiment was to determine if heme levels increased in the double mutant cells in the presence of 5-Ala, but not in the presence of glycine. The *ymc1* Δ *hem25* Δ cells had a significant reduction of heme levels compared with *hem25* Δ and *ymc1* Δ cells (Figure 4.4). Double mutant cells grown in media supplemented with 5 mM glycine did not show a significant increase in heme content. In contrast, the double mutant cells grown in media supplemented with 5-Ala showed heme content significantly increased compared with the double mutant cells grown in media without supplements. This experiment points to Ymc1 as a candidate for a second mitochondrial glycine importer. However, further studies are required to confirm this observation.

4.2 Discussion

The results presented in Chapter 3 support a role for Hem25 in mitochondrial glycine import. These results also suggest that there is at least another carrier that imports glycine into the mitochondria. Based on these observations, one objective of the

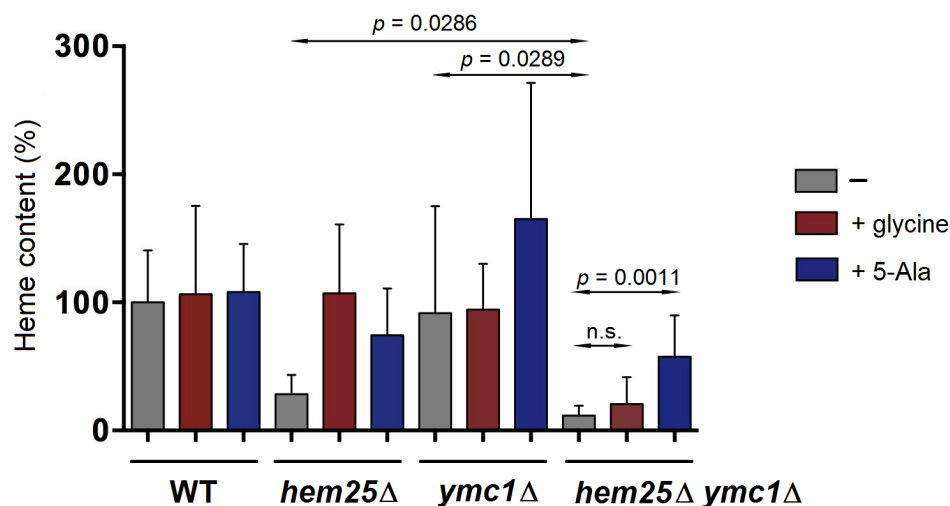


Figure 4.4: **Heme content of *ymc1*Δ *hem25*Δ cells was restored by the addition of 5-Ala, whereas glycine supplementation did not significantly increase heme levels.** Cells of the indicated genotypes were grown to OD_{600nm} 0.6-1 in SD-Ura medium with dextrose with or without 5 mM glycine or 0.38 mM 5-Ala. Cells were harvested and processed for heme determination. Differences between single deletion strains and double deletion strains were found using a fixed factor ANOVA test. Three independent experiments were done to calculate the p values, mean and standard deviation.

experiments shown in this chapter was to find a putative second mitochondrial glycine importer among the other members of the *SLC25* family.

Interestingly, *hem25*Δ cells showed a mild growth deficit compared to wild type when cells were grown in dextrose or lactate media. This growth phenotype was observed in the BY4741/BY4742 strains, whereas *hem25*Δ cells in W303 strain did not show a growth phenotype. Both results are consistent with two previously published results. On the one hand, a mild growth defect for *hem25*Δ cells in BY4741/BY4742 strains was also observed by Guernsey *et al.* [37]. On the other hand, the absence of phenotype in *hem25*Δ cells in W303 strain was also shown by Kardon *et al.* [43]. The differences in growth phenotype of *hem25*Δ cells observed between BY4741/BY4742 and W303 strains could be due to genetic differences between both backgrounds [79]. It is known that the BY4741/BY4742 strains carry a Ty1 element inserted in the 3' region of the *HAP1* open reading frame [33]. In the experiments carried out on BY4741/BY4742 strains in this study, *HAP1* was expressed from a plasmid. However, it is possible that the expression from the plasmid differs from expression from

the chromosome. Moreover, other genetic differences between these two strains could affect the mitochondrial physiology in combination with the absence of Hem25 function.

4.2.1 Ymc1 as a Candidate for a Second Mitochondrial Glycine Importer

I identified Ymc1 as a candidate for a putative second mitochondrial glycine importer among the members of the SLC25 family. When heme content was measured in *ymc1*Δ *hem25*Δ cells, the significant reduction in heme levels in the double mutant compared with its respective single mutants caught my attention. This would be consistent with the absence of two carriers that transport glycine, required for heme synthesis, into the mitochondria. Moreover, the human homologue of Ymc1 is the only one of the seven carriers analyzed in this chapter that was reported as an amino acid carrier in humans [71]. Porcelli *et al.* [71] reported that the human homologue of *YMC1*, i.e., *SLC25A29*, is a mitochondrial transporter for basic amino acids with a preference seen for arginine, lysine and histidine. The transport of glycine by *SLC25A29* was not studied.

For these reasons, I cultured *ymc1*Δ *hem25*Δ cells with glycine or 5-Ala, and measured heme content. A restoration of heme content with the addition of 5-Ala but not with the addition of glycine would be expected if Ymc1 were the second glycine transporter. Interestingly, the addition of glycine did not restore heme content in the cells, whereas a significant increase in heme levels was observed when the double mutant was supplemented with 5-Ala. Although the restoration in heme content did not reach wild type levels, these results suggest that Ymc1 could transport glycine. The absence of Hem25 together with Ymc1 would cause a greater depletion of glycine inside the mitochondria, which would severely decrease heme synthesis. Supplementation with 5-Ala would bypass the first reaction of heme synthesis, which led to a restoration of heme level in *ymc1*Δ *hem25*Δ cells. However, further analyses would be required to confirm the hypothesis of Ymc1 being a mitochondrial glycine importer.

4.2.2 New Genetic Interactors of *HEM25*

The use of several phenotypic analyses contributed to the identification of new genetic interactors of loss of *HEM25* function that are members of the *SLC25* family. The interaction of *HEM25* with *PET8* is the only one that has been reported in the *Saccharomyces* Genome Database (SGD). The genetic interactions between *HEM25* and *FLX1*, *ORT1*, *MTM1*, *SFC1* or *AAC3* have not been reported. These findings reported here contribute to the interactions now known between members of the *SLC25* family. *MTM1*, *FLX1* and *ORT1* genes were found to genetically interact with *HEM25* when the double mutant strains were grown with glycine as a sole nitrogen source. Under this growth condition *flx1* Δ *hem25* Δ , *mtm1* Δ *hem25* Δ and *ort1* Δ *hem25* Δ showed a severe growth impairment, which was similar to the *lpd1* Δ strain, which is defective for the GCV.

Interaction of *HEM25* with *MTM1*

MTM1 encodes a mitochondrial high affinity PLP transporter [100]. PLP is a cofactor required by dihydrolipoamide dehydrogenase (Lpd1) [15], which is one of the subunits of GCV. The growth defect of *mtm1* Δ *hem25* Δ cells when in glycine is the sole nitrogen source could be due to reduction of PLP levels in the mitochondria. This reduction could affect GCV function. At the same time Hem25 impairs the import of glycine, which is the substrate of the GCV. These two defects together would further decrease the capacity to use glycine as nitrogen source.

When heme was measured, a decrease in heme content was observed in *mtm1* Δ cells, but the double mutant cells had equal heme levels to the *mtm1* Δ cells. PLP is an essential cofactor of Hem1 [97], which is the first enzyme in the heme synthesis pathway [100]. The absence of Mtm1 would impact the function of Hem1 due to the decreased availability of the cofactor PLP. The double mutant cells probably did not have a heme level significantly lower than *mtm1* Δ cells because, under the situation where Hem1 function is impaired by decreased PLP levels, the availability of glycine would not determine the rate of heme synthesis as glycine lies upstream of Hem1 in the heme biosynthetic pathway.

In contrast, *mtm1* Δ *hem25* Δ cells also showed a growth defect relative to single mutants when cells grew in dextrose medium without supplementation. The growth

phenotype of the double mutant cells was improved by the addition of 5-Ala. 5-Ala is a downstream metabolite of Hem1, the first enzymatic reaction for heme synthesis and it would bypass the deficiency of PLP and glycine. A decreased ability to import PLP clearly affects growth of cells when mitochondrial glycine import is also decreased. This appears to be due to the fact that glycine is a substrate for the GCV and for heme synthesis, and both pathways contain PLP-dependent enzymes. Simultaneous restriction of both GCV and heme synthesis is the likely explanation for the decreased growth of cells lacking Hem25 and Mtm1 function.

Interaction of *HEM25* with *SFC1*

SFC1 encodes a mitochondrial succinate-fumarate transporter, which transports succinate into, and fumarate out of the mitochondria [69]. When heme content was measured in cells lacking Sfc1 and Hem25, a severe decrease in heme content was observed compared to *sfc1* Δ or *hem25* Δ cells. The import of succinate and export of fumarate would increase the availability of succinate in the mitochondria for synthesis of succinyl-CoA by the tricarboxylic acid (TCA) cycle. Succinyl-CoA and glycine are the substrates for the first reaction of heme synthesis. A deletion of *SFC1* and *HEM25* would deprive the mitochondria of the two substrates required for heme synthesis. However, the phenotypic analysis in lactate showed that the growth defect observed in *sfc1* Δ *hem25* Δ cells grown with supplements was not restored by the addition of 5-Ala. This result could indicate that succinyl-CoA and glycine are limiting other mitochondrial processes in addition to the synthesis of heme.

Interaction of *HEM25* with *ORT1*

ORT1 encodes an ornithine transporter of the mitochondrial inner membrane, which exports ornithine from mitochondria in exchange for protons [68]. *ORT1* had a strong genetic interaction with *HEM25* in the three phenotypic analyses that I carried out. When *ort1* Δ *hem25* Δ cells were grown in glycine as sole nitrogen source, cells had a severe growth impairment compared to the respective single mutants.

When heme content was measured, the double mutant *ort1* Δ *hem25* Δ cells had a severe decrease in heme levels compared to the single mutants. In the growth phenotypic analysis in dextrose, the double mutant cells showed a strong growth

impairment. *Ort1* interacts physically with cytoplasmic RNA-binding protein (*Sro9*) and the *ORT1* gene, genetically with *PET8*. The interaction of *ORT1* with *PET8* was observed in a high throughput analysis [91]. Interestingly, I observed an interaction between *HEM25* and *PET8* listed on SGD, and in the analyses shown in this chapter. *SRO9* encodes a component of the higher-order complex (HMC), which is involved in the regulation of gene expression in response to levels of heme and oxygen. It is difficult to explain why *ORT1* interacted with *HEM25* based on the little information known about these genes, but the connection could be through *PET8*. It is clear that there is a strong genetic interaction between *ORT1* and *HEM25*, which needs to be further studied.

Interaction of *HEM25* with *PET8*

The genetic interaction of *HEM25* with *PET8* found in the analyses carried out in this thesis may not be considered novel since it was being previously reported on SGD. Nevertheless, this study confirmed the interaction through new experiments. *PET8* encodes an S-adenosylmethionine (SAM) transporter of the mitochondrial inner membrane [56].

The *pet8* Δ *hem25* Δ cells did not show any additional growth deficit relative to the single mutants in the analyses done in fermentable media or when glycine was the sole nitrogen source. However, the *pet8* Δ *hem25* Δ cells showed a severe decrease in heme content compared with their respective single mutants. So far, it is not known how the absence of Pet8 can affect heme synthesis, but it is clear that both transporters impact heme synthesis. I looked at the genetic interactors of loss of function of *PET8* listed in SGD and I found several genes with relationship to components of the ETC. Some of the genes that interacted with *PET8* were *ALD5* (mitochondrial aldehyde dehydrogenase involved in regulation or biosynthesis of electron transport chain components), *CBP4* (mitochondrial protein required for assembly of cytochrome bc1 complex), *CYC1* (codes for cytochrome c), *QCR8* (codes for subunit 8 of ubiquinol cytochrome-c reductase of complex III), and *ATP14*, *ATP17* and *ATP20* (genes that encode subunits of ATP synthase). This information suggests that Pet8 is not only involved in the transport of SAM, but also is somehow related with ETC function. Indeed, cells lacking only Pet8 could not grow on non-fermentable medium, where

ATP must be supplied by the ETC (Figure 4.3), consistent with *Pet8* providing an essential function for the ETC. Processes within the ETC are heme-dependent, such as those that use cytochromes, and it is tempting to speculate that the decreased growth could be through an inability to properly synthesize heme and thus form an intact ETC in the absence of *HEM25* and *PET8*. The ETC does have roles beyond production of ATP, including regulation of apoptosis through cytochrome c release, aging, and maintaining proton gradients.

Interaction of *HEM25* with *AAC3*

The phenotypic analysis in non-fermentable media showed that *aac3Δ hem25Δ* cells had a severe growth defect in lactate media. *AAC3* encodes a mitochondrial inner membrane ADP/ATP translocator, which exchanges cytosolic ADP for mitochondrially-synthesized ATP [47]. It has a role in maintenance of viability in respiratory conditions. However, during exponential growth on dextrose under aerobic conditions, it acts in the opposite direction, importing ATP into mitochondria [94]. Moreover, competition experiments suggest that *Aac3* together with *Aac1* and *Aac2* bind heme and could transport heme [5]. Disruption of the *AAC* genes in yeast resulted in a reduction of heme biosynthesis by blocking the translocation of heme precursors into the matrix [5]. Although the reason why a reduction in growth is observed when *Hem25* and *Aac3* are absent in the cells is not known, a possible explanation for the growth defect is that heme synthesis could be impaired as proposed by Azuma *et al.* [5]. The reason why heme levels were not seen to be diminished in *aac3Δ* cells could be due to the three genes (*AAC1*, *AAC2* and *AAC3*) have to be deleted in order to decrease heme synthesis [5]. Furthermore, *AAC3* is downregulated by *HAP2* [13], which is part of the heme activation protein Hap2/3/4 complex.

The function of *Aac3* would have to be further studied to understand how its function is related with heme and why it has a genetic interaction with *HEM25*.

Interaction of *HEM25* with *FLX1*

FLX1 encodes a mitochondrial FAD transporter [95]. I found that the inactivation of *FLX1* exacerbated the growth defect of *hem25Δ* cells when they were grown in glycine as a sole nitrogen source. *Lpd1*, which is one of the subunits of the GCV, is

a flavoenzyme that contains a FAD-binding site and binds FAD as a cofactor [15]. The reduction of glycine utilization when Flx1 and Hem25 are absent could be due to reduced availability of FAD in the mitochondria, which is required for GCV, and a decrease of glycine inside the mitochondria, which in turn decreases substrate for GCV.

Furthermore, when heme content was measured in the single and double mutants, *flx1* Δ *hem25* Δ showed a severe decrease in heme content. The absence of Flx1 affects the import of FAD needed for functional flavoproteins. Protoporphyrinogen oxidase (Hem14) is a flavoprotein [36], which catalyzes the seventh reaction in the heme synthesis pathway. Another flavoprotein, Cyc2 plays a role in the insertion of heme into apocytochromes [36]. A defect in Flx1 and Hem25 together could impact the ETC and heme synthesis.

Another interesting piece of information was found listed on SGD, where Flx1 had a physical interaction with Sro9 [81]. Sro9 together with Hsp70-Ssa, Hsp90 and Ydj1 form the higher-order complex (HMC), which is involved in the regulation of gene expression in response to levels of heme and oxygen. In the absence of heme, Hap1 is bound by these four proteins and its activity is repressed [40]. The effect of loss of function of *FLX1* and *HEM25* is further described in Chapter 5.

Chapter 5

Simultaneous Defect of Hem25 and Flx1 Decreased the Abundance of Proteins of the Electron Transport Chain

5.1 Results

The genetic interaction of *FLX1* with *HEM25* was of interest in this study because absence of Flx1 and Hem25 could affect the activity of the electron transport chain (ETC) by different mechanisms. Flx1 transports FAD, which is a prosthetic group for flavoproteins, the majority of which are found in the mitochondria, where they participate in redox processes of the ETC [36], such as subunit 1 of succinate dehydrogenase (Sdh1) and NADH ubiquinone oxidoreductase (Ndi1). The flavoprotein protoporphyrinogen oxidase (Hem14) is involved in heme metabolism. Hem25 imports glycine into the mitochondria, which is required for heme synthesis. Heme molecules are required to form cytochromes in the ETC.

It is worth noting that the human homologue of *FLX1*, named *SCL25A32*, is a folate transporter. Spaan *et al.* [90] showed that the human homologue of *FLX1* was able to complement the function of the yeast homologue, which could indicate that Flx1 is a folate transporter as well. If Flx1 were a folate transporter, Flx1 could also be important for the formation of one-carbon units because this process requires folate to make THF [3].

Cells with deletions in both *HEM25* and *FLX1* showed decreased levels of heme. Heme and FAD are prosthetic groups required to form complexes in the ETC. To determine whether the absence of function of Hem25 and Flx1 affects the ETC, I performed western blot analyses to determine if the abundance of some subunits of the ETC was affected.

Western blot analyses were carried out with crude mitochondrial fractions from wild type, *hem25* Δ , *flx1* Δ and *flx1* Δ *hem25* Δ cells and antibodies against subunits that were part of complexes I-V. Mitochondrial porin (Por1) was used as a loading

control.

To determine whether complex I was affected in cells that contain the double deletion, the steady state level of NADH-ubiquinone oxidoreductase (Ndi1) was examined by western blotting using an antibody against Ndi1. A significant reduction of Ndi1 levels was observed in cells with the double deletion *flx1*Δ *hem25*Δ compared with the levels observed in the mitochondrial fractions of the single mutant cells (Figure 5.1).

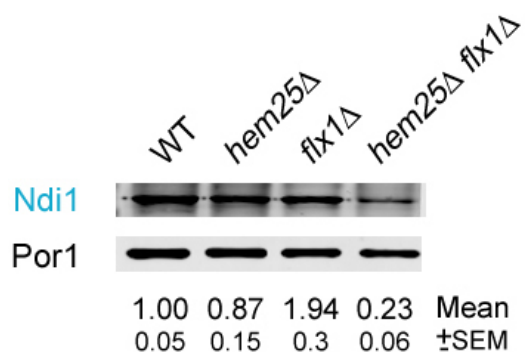


Figure 5.1: **Ndi1 level was significantly decreased in *flx1*Δ *hem25*Δ cells.** Cells of the indicated genotypes were grown to an OD of 1.0 in defined media with raffinose. Cells were then transferred and grown in lactate for 5 hours. Cells were harvested and crude mitochondrial fractions were prepared and analyzed by western blotting. Numbers under the lanes represent the mean of protein abundance normalized by the loading control and then to wild type (WT). The mean was calculated from three independent western blot analyses. Independent segregant strains of *flx1*Δ and *flx1*Δ *hem25*Δ were used. The figure shown is representative of three analyses. Pixel intensity was measured and calculated by using Odyssey Software. The deduced protein molecular weight (MW) of the bands revealed by anti-Ndi1 antibody was consistent with the MW of Ndi1 (57 kDa).

Sdh2 and the flavoprotein Sdh1 are subunits of succinate dehydrogenase and form part of succinate-ubiquinone oxidoreductase (complex II). Cells with the double deletion *flx1*Δ *hem25*Δ showed a decrease in Sdh1 levels compared with the single mutant cells (Figure 5.2). A significant decrease in Sdh2 level was also observed in the double mutant in comparison with the single mutants. Sdh1 and Sdh2 levels were also reduced in *hem25*Δ cells (Figure 5.2) but not to the extent of that observed in the double mutant cells.

Ubiquinol-cytochrome c oxidoreductase, which is complex III of the ETC, is composed of several subunits. I determined the abundance of subunit 2 (Cor2) and

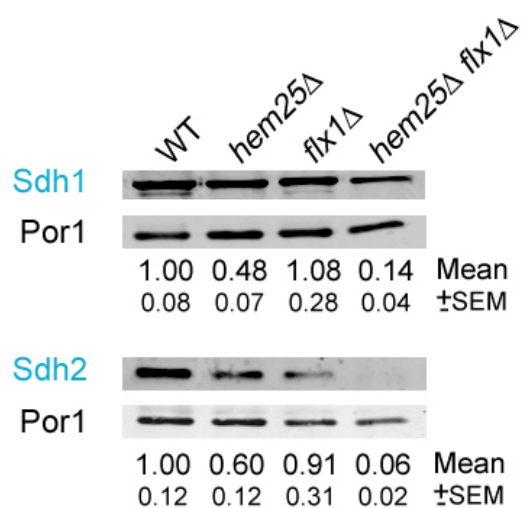


Figure 5.2: **Sdh1 and Sdh2 levels were significantly decreased in *flx1*Δ *hem25*Δ cells.** Cells of the indicated genotypes were grown to OD of 1.0 in defined media with raffinose. Cells were then transferred and grown in lactate for 5 hours. Cells were harvested and crude mitochondrial fractions were prepared for western blotting. Numbers under the lanes represent the mean of protein abundance normalized by the loading control and then to wild type (WT). The mean was calculated from three independent western blot analyses. Independent segregant strains of *flx1*Δ and *flx1*Δ *hem25*Δ were used. The figure shown is representative of three analyses. Pixel intensity was measured and calculated by using Odyssey Software. The deduced protein molecular weight (MW) of the bands revealed by anti-Sdh1 and anti-Sdh2 antisera were consistent with the MW of Sdh1 (69 kDa) and Sdh2 (30 kDa), respectively.

cytochrome c1 (Cyt1). Cor2 levels were significantly decreased in cells with the double deletion *flx1*Δ *hem25*Δ compared with the levels observed in the mitochondrial fractions of the single mutant cells (Figure 5.3). Cyt1 was also significantly reduced in the cells with the double mutation (Figure 5.3) in comparison with the single mutant cells. Both Cor2 and Cyt1 levels were also reduced in *hem25*Δ cells compared with wild type cells, but not to the extent observed in the double mutants.

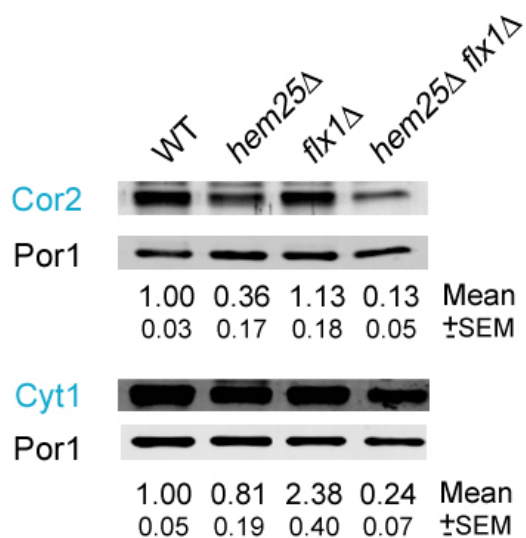


Figure 5.3: **Cor2 and Cyt1 levels were significantly decreased in *flx1*Δ *hem25*Δ cells.** Cells of the indicated genotypes were grown to an OD of 1.0 in defined media with raffinose. Cells were then transferred and grown in lactate for 5 hours. Cells were harvested and crude mitochondrial fractions were prepared for western blotting. Numbers under the lanes represent the mean of protein abundance normalized by the loading control and then to wild type (WT). The mean was calculated from three independent western blot analyses. Independent segregant strains of *flx1*Δ and *flx1*Δ *hem25*Δ were used. The figure shown is representative of three analyses. Pixel intensity was measured and calculated by using Odyssey Software. The deduced protein molecular weight (MW) of the bands revealed by anti-Cor2 and anti-Cyt1 antisera were consistent with the MW of Cor2 (40 kDa) and Cyt1 (34 kDa), respectively.

To determine whether ferrocycytochrome c oxygen oxidoreductase (complex IV) is affected, I studied subunits II (Cox2) and IV (Cox4) of cytochrome c oxidase. Cox2 and Cox4 were significantly reduced in the *hem25*Δ cells compared with wild type cells, but there was no reduction in protein levels in the double mutant *flx1*Δ *hem25*Δ compared with the *hem25*Δ cells (Figure 5.4).

Complex V, named ATP synthase, was also studied. There was no significant

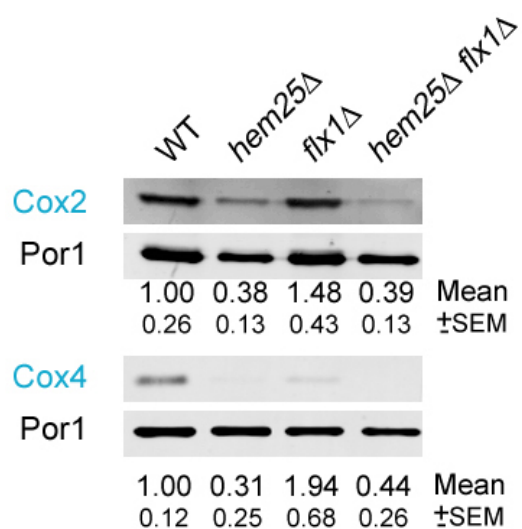


Figure 5.4: **Cox2 and Cox4 levels were not decreased in *flx1*Δ *hem25*Δ cells compared with *hem25*Δ cells.** Cells of the indicated genotypes were grown at OD of 1.0 in defined media with raffinose. Cells were then transferred and grown in lactate for 5 hours. Cells were harvested and crude mitochondrial fractions were prepared for western blotting. Numbers under the lanes represent the mean of protein abundance normalized by the loading control and then to wild type (WT). The mean was calculated from three independent western blot analyses. Independent segregant strains of *flx1*Δ and *flx1*Δ *hem25*Δ were used. The figure shown is representative of three analyses. Pixel intensity was measured and calculated by using Odyssey Software. The deduced protein molecular weight (MW) of the bands revealed by anti-Cox2 and anti-Cox4 antibodies were consistent with the MW of Cox2 (28 kDa) and Cox4 (17 kDa), respectively.

decrease in F1 α and F1 β protein levels in the double mutant cells in comparison with *flx1* Δ or *hem25* Δ cells (Figure 5.5).

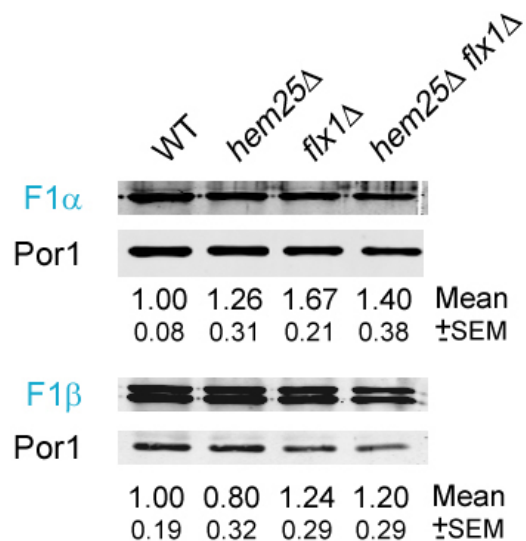


Figure 5.5: **F1 α and F1 β levels were not decreased in cells with the double mutation *flx1* Δ *hem25* Δ cells.** Cells of the indicated genotypes were grown to OD of 1.0 in defined media with raffinose. Cells were then transferred and grown in lactate for 5 hours. Cells were harvested and crude mitochondrial fractions were prepared for western blotting. Numbers under the lanes represent the mean of protein abundance normalized by the loading control and then to wild type (WT). The mean was calculated from three independent western blot analyses. Independent segregant strains of *flx1* Δ and *flx1* Δ *hem25* Δ were used. The figure shown is representative of three analyses. Pixel intensity was measured and calculated by using Odyssey Software. The deduced protein molecular weight (MW) of the band revealed by anti-F1 α and anti-F1 β antisera were consistent with the MW of F1 α (58 kDa) and F1 β (55 kDa), respectively.

5.2 Discussion

In this chapter, I presented evidence that the simultaneous loss of both *HEM25* with *FLX1*, perturbs the integrity of the ETC, revealed by the decreased levels of several of its components: Ndi1, Sdh1, Sdh2, Cor2 and Cyt1. I have already discussed in Chapter 4 the impact of the absence of Hem25 and Flx1 on heme levels. The abundance of Ndi1 subunit of complex I decreased in the *flx1* Δ *hem25* Δ cells compared with the levels in the single mutant cells. Complex I is the NADH-ubiquinone

oxidoreductase, which has a FAD prosthetic group [36]. The succinate-ubiquinone oxidoreductase is complex II, formed by four subunits, one of which is the flavoprotein Sdh1, which contains a FAD bound covalently [66, 19]. Sdh2 contains an Fe-S group. The four complex II subunits bind cytochrome b which contains a heme group. In the western blot analyses, Sdh1 and Sdh2 subunits were significantly decreased in the double mutant cells compared to single mutant cells. Kim *et al.* [45] showed that these subunits were also decreased in the single mutant *flx1* Δ cells. However, a significant reduction of Sdh1 or Sdh2 in the *flx1* Δ cells was not observed in this study. The discrepancies found between the two studies could be due to the use of different yeast backgrounds. The *flx1* Δ *hem25* Δ cells analyzed here also showed a decrease in the abundance of subunit 2 of ubiquinol cytochrome-c reductase (Cor2) and cytochrome c1 (Cyt1) proteins. Cor2 and Cyt1 form part of complex III, and both subunits contain heme molecules. Moreover, their transcription is regulated by Hap1, the Hap2/Hap3 complex and heme.

The data presented here suggest that Flx1 and Hem25 together are required for stability of specific ETC subunits. To my knowledge, this is the first time this has been reported. A possible reason for the reduced abundance of Ndi1, Sdh1, Sdh2, Cor2 and Cyt1 in *flx1* Δ *hem25* Δ cells could be related to a defect in the assembly of the subunits of the ETC. The respiratory chains of yeast take the form of supercomplexes [80]. The coordinated assembly of subunits is a poorly understood process, but it seems to be required for normal ETC activity. If there were a defect in one of the subunits, this could affect the assembly of the supercomplexes and therefore the stability of the subunits. If the absence of Hem25 affects heme synthesis and the absence of Flx1 affects FAD availability for heme and flavoproteins synthesis, the formation of complexes of the ETC that requires these cofactors could be impaired, and hence the stability of ETC subunits could be affected. A similar situation was observed in yeast cells with a mutation in subunit 4 of cytochrome c oxidase (Cox4) [63]. In the absence of Cox4, subunits Cox2 and Cox3 were not assembled into the higher order complex and were degraded more rapidly.

Interestingly, the western blot analysis of Cox2 and Cox4 proteins from complex IV here showed that the level of these proteins was decreased in *hem25* Δ cells but not further reduced in *flx1* Δ *hem25* Δ cells. This result showed that the absence

of Hem25 *per se* affects the stability of Cox4 and Cox2. Cox4 is essential for the assembly and function of the cytochrome c oxidase complex [52], and for assembly of complex I to form supercomplexes [18]. Moreover, Cox4 expression is regulated by the Hap2/Hap3/Hap4 complex, which is a heme regulatory factor. The decrease in heme levels observed in *hem25* Δ cells could affect the expression of Cox4. If Cox4 is decreased, the Cox2 subunit cannot assemble and is degraded [63]. Therefore, the level of Cox2 is reduced.

The subunits of the ATP synthase, F1 α and F1 β , were not diminished in abundance in *flx1* Δ *hem25* Δ cells compared to wild type cells. The ATP synthase does not contain a heme or FAD molecule in the complex, which might explain why the abundance of these two proteins was not affected.

The combined inability to synthesize heme at a wild type rate and import FAD into the mitochondria results in a decreased ability to assemble ETC complexes that require these cofactors, but does not affect the assembly of ETC complexes where these cofactors are absent. The decreased levels of the ETC proteins studied is likely due to a decrease in the capacity of ETC supercomplexes to properly assemble due to irregularities in the proper proportions of the proteins within these supercomplexes as I demonstrated by western blot analyses, although this will require further study of supercomplex assembly and stability to determine if this is indeed the case.

Chapter 6

Other Genes that Interacted with *HEM25*

6.1 Results

Glycine and succinyl-CoA are the substrates of the first enzyme of heme biosynthesis, i.e. Hem1. With this consideration, a genetic interaction analysis of *HEM25* with genes that are involved in succinyl-CoA metabolism, was undertaken as a complementary study to the role of Hem25 in providing glycine for heme synthesis.

I searched the SGD for genes functionally related with succinyl-CoA metabolism that had been identified as having genetic interactions with *HEM25* in a high-throughput screen [24] but that had not been confirmed by further studies using random sporulation or tetrad analysis. They were *KGD2* and *AIM22*. *KGD2* encodes one of the subunits of the ketoglutarate dehydrogenase complex (KGD). KGD catalyzes one of the reactions to synthesize succinyl-CoA in the TCA cycle. *AIM22* encodes a lipoate-protein ligase, which is required for lipoylation of Kgd2 [84]. Furthermore, another gene, *YHM2*, related with succinyl-CoA metabolism that was not listed in SGD was also selected for further study. This gene encodes a ketoglutarate mitochondrial transporter. Ketoglutarate is the substrate of KGD complex. The functions of these genes are shown in Table 6.1.

Table 6.1: **Candidate genes that showed negative genetic interaction with *HEM25*** (* indicates tested by random spore analysis). SGD description from <http://www.yeastgenome.org/>.

Standard Name	Systematic Name	SGD description
<i>AIM22</i>	<i>YJL046w</i>	Putative lipoate-protein ligase; required along with Lip2 and Lip5 for lipoylation of Lat1p and Kgd2p; similar to E. coli LplA; null mutant displays reduced frequency of mitochondrial genome loss
<i>KGD2</i>	<i>YDR148c</i>	Dihydrolipoyl transsuccinylase; component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes the oxidative decarboxylation of alpha-ketoglutarate to succinyl-CoA in the TCA cycle; phosphorylated

Table 6.1 – Continued from previous page

Standard Name	Systematic Name	SGD description
<i>YHM2*</i>	<i>YMR241w</i>	Citrate and oxoglutarate (ketoglutarate) carrier protein; exports citrate from and imports oxoglutarate into the mitochondrion, causing net export of NADPH reducing equivalents; also associates with mt nucleoids and has a role in replication and segregation of the mt genome

Double gene deletion strains were constructed by standard genetic crosses of *hem25* Δ with *aim22* Δ , *kgd2* Δ or *yhm2* Δ strains. The *HEM25* coding region was deleted and replaced with the *NatMX4* marker gene cassette while the *AIM22*, *KGD2* and *YHM2* genes were replaced with the *KanMX4* marker. Diploid cells were selected, transformed with NDY 601 plasmid and sporulated. Spores were isolated and plated, and haploid cells were selected as described in random spore analysis (RSA) in Section 2.3.3. The basic common medium for all the conditions was SD-Ura-His-Arg+canavanine. The medium did not contain uracil for selection of the plasmid carrying the *HAP1* gene. The presence of canavanine and the absence of histidine and arginine is for the counter selection of parental haploid cells and diploid cells. Four different solid media were used in this analysis: (i) SD-Ura-His-Arg with canavanine (no selection), which allowed the growth of the wild type, single mutant and the double mutant haploid cells; (ii) SD-Ura-His-Arg with canavanine and G418 (+G418), which allowed the growth of *aim22* Δ , *kgd2* Δ or *yhm2* Δ , and the respective double mutant haploid cells; (iii) SD-Ura-His-Arg with canavanine and ClonNAT (+Nat), which allowed the growth of the *hem25* Δ and the double mutant haploid cells; (iv) and the last condition, SD-Ura-His-Arg with canavanine, G418 and ClonNAT (+G418 +Nat), which allowed the growth only of the double mutant haploid cells. Colony sizes in each of the plates were scored after 4–5 days as a criteria of the genetic interaction between *HEM25* and the genes of interest.

For all the double deletions studied in these RSA analyses, it was observed that the colony size of the majority of the cells grown in the +Nat condition (where *hem25* Δ and double mutant cells grow) were not different to the colony size of the majority

of the cells grown in the “no selection” condition (where wild type, single and double mutant cells grow). Results from the RSA for the interaction *HEM25-KGD2* is shown as an example (Figure 6.1).

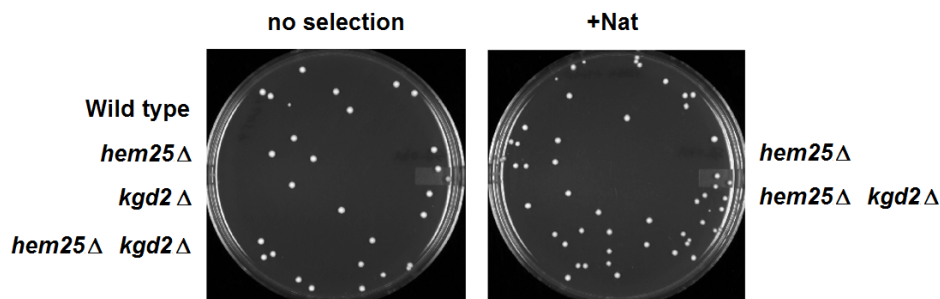


Figure 6.1: **Cells grown in “no selection” and +Nat conditions are shown as controls.** Spores were isolated and plated on the media to select the indicated genotypes: SD-Ura-His-Arg with canavanine (“no selection”) and SD-Ura-His-Arg with canavanine and ClonNAT (+Nat). Plates were imaged after 4–5 days incubation at 30 °C.

The RSA of the interaction of *HEM22* with *AIM22* showed that the colonies grown in the +G418 +Nat condition had a mild reduction in colony sizes compared with the colonies grown in +G418 condition (Figure 6.2(a)). The RSA of the interactions *HEM25-KGD2* and *HEM25-YHM2* showed that the colonies grown in the +G418 +Nat condition were significantly smaller than the colonies grown in +G418 condition (Figure 6.2(b) and (c)).

After the random sporulation analysis, I studied the growth of the double mutant cells. Single and double mutants of each gene analyzed by RSA were grown overnight in SC-Ura supplemented with glycine and 5-Ala. Cells were washed and serial dilutions were done in SD-Ura medium with dextrose, with and without supplementation of glycine or 5-Ala. Supplementation with glycine or 5-Ala was used to determine whether the decreased growth was due to the absence of the substrates of the first reaction in the pathway of heme synthesis. The *hem25Δ* cells showed a mild growth defect compared to wild type cells, which was restored by supplementation with glycine or 5-Ala. The *aim22Δ hem25Δ* cells showed decreased growth compared with the single mutant cells (Figure 6.3). The growth defect was improved by the addition of glycine or 5-Ala. The *kgd2Δ hem25Δ* and *yhm2Δ hem25Δ* cells showed a very slight growth defect compared to their respective single mutants (Figure 6.3).

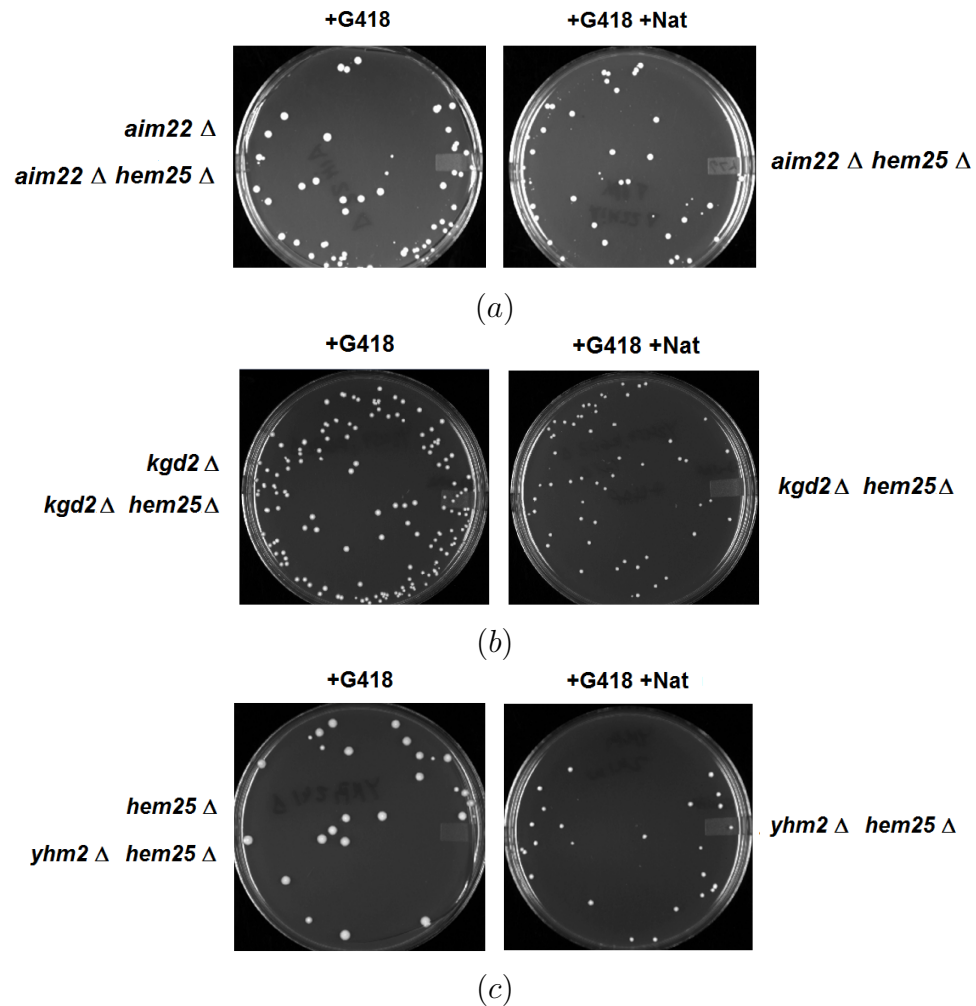


Figure 6.2: **Random spore analysis for interaction with *HEM25***. Spores were isolated and plated on SD-Ura-His-Arg with canavanine and G418 (+G418), and on SD-Ura-His-Arg with canavanine, G418 and ClonNAT (+G418 +Nat), to select for the indicated genotypes to address the interactions (a) *HEM25-AIM22*, (b) *HEM25-KGD2* and (c) *HEM25-YHM2*. Plates were imaged after for 4–5 days incubation at 30 °C.

Supplementation with glycine or 5-Ala restored the growth defect of double mutant cells.

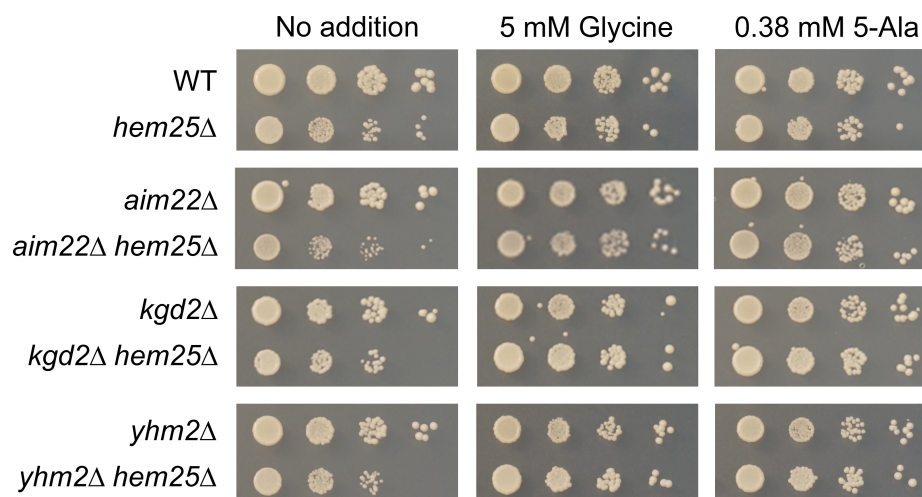


Figure 6.3: **Growth analysis.** Yeast strains were grown to early stationary phase OD_{600nm} 0.9-1 in SC-URA medium supplemented with glycine and 5-Ala. Cells were washed twice, suspended in sterilized water to OD_{600nm} 0.4, serially diluted (1:10) and spotted on SD-URA supplemented as indicated. Plates were imaged after 3 days incubation at 30 °C. WT denotes wild type.

6.1.1 Synthetic Genetic Array (SGA) Analysis to Identify Genes that Affect the Fitness of *hem25Δ* Cells

In this study, SGA analysis was performed with a *HEM25* deficient strain. The SGA analysis allowed the construction of cells with *HEM25* inactivated along with each non-essential yeast gene, which generate approximately 4700 double mutants. Growth of the double mutants cells in fermentable media was analyzed in order to identify those that resulted in decreased growth. This analysis was performed twice. A few genes whose loss of function resulted in significant decreased growth in the absence of Hem25 function are highlighted here (Table 6.2). There were genes with mitochondrial functions related with the KGD complex, such as *AIM22*, *KGD1* and *KGD4*. Also, there were genes related with cytochromes and the ETC, such as *CBP3*, *COR1*, *COX17* and *MSS2*. *PDX3* is a gene related with heme synthesis because it encodes a pyridoxine 5-phosphate oxidase, which catalyzes the last reaction of the PLP synthesis pathway (PLP is a cofactor of Hem1). Lastly, *PET8*, that encodes a

SAM transporter, also showed interaction with *HEM25* in the phenotypic analyses shown in Chapter 4. The rest of the genes found in both analyses are listed in the Appendix (Table A.1). These genetic interactions were not further studied, but they are mentioned as a reference for future work on the function of Hem25.

Table 6.2: **Candidate genes that showed negative genetic interaction with *HEM25* by SGA** (* indicates tested by random spore analysis). SGD description from <http://www.yeastgenome.org/>.

Standard Name	Systematic Name	SGD description
Genes related with alpha-KetoGlutarate Dehydrogenase complex		
<i>KGD1</i>	<i>YIL125w</i>	Subunit of the mitochondrial alpha-ketoglutarate dehydrogenase complex; catalyzes a key step in the TCA cycle, the oxidative decarboxylation of alpha-ketoglutarate to form succinyl-CoA
<i>KGD4</i>	<i>YFR049w</i>	Subunit of the mitochondrial alpha-ketoglutarate dehydrogenase; recruits E3 subunit (Lpd1p) to the E1-E2 (Kgd1p, Kgd2p) core; has similarity to human mitochondrial ribosomal protein MRP-S36
<i>AIM22*</i>	<i>YJL046w</i>	Putative lipoate-protein ligase; required along with Lip2 and Lip5 for lipoylation of Lat1p and Kgd2p; similar to <i>E. coli</i> LplA; null mutant displays reduced frequency of mitochondrial genome loss
Genes related with cytochromes and the ETC		
<i>CBP3</i>	<i>YPL215w</i>	Mitochondrial protein required for assembly of cytochrome bc1 complex; forms a complex with Cbp6p that binds to mt ribosomes near the polypeptide tunnel exit and promotes efficient translation of the <i>COB</i> mRNA; Cbp3p-Cbp6p complex also interacts with newly synthesized cytochrome b (Cobp) and Cbp4p to promote assembly of Cobp into the cytochrome bc1 complex; Cbp3p-Cbp6p complex is sequestered if assembly of Complex III is blocked, downregulating <i>COB</i> mRNA translation
<i>COR1</i>	<i>YBL045c</i>	Core subunit of the ubiquinol-cytochrome c reductase complex; the ubiquinol-cytochrome c reductase complex (bc1 complex) is a component of the mitochondrial inner membrane electron transport chain
<i>COX17</i>	<i>YLL009c</i>	Copper metallochaperone that transfers copper to Sco1p and Cox11p; eventual delivery to cytochrome c oxidase; contains twin cysteine-x9-cysteine motifs
<i>MSS2</i>	<i>YDL107w</i>	Peripherally bound inner membrane protein of the mitochondrial matrix; involved in membrane insertion of C-terminus of Cox2p, interacts genetically and physically with Cox18p
Other genes		
<i>PDX3</i>	<i>YBR035c</i>	Pyridoxine (pyridoxamine) phosphate oxidase; has homologs in <i>E. coli</i> and <i>Myxococcus xanthus</i> ; transcription is under the general control of nitrogen metabolism
<i>PET8*</i>	<i>YNL003c</i>	S-adenosylmethionine transporter of the mitochondrial inner membrane; member of the mitochondrial carrier family; required for biotin biosynthesis and respiratory growth 1

6.2 Discussion

A recent published work [43] has identified a chaperone, Mcx1, that contributes to heme synthesis. Mcx1 facilitates the binding of cofactor PLP into Hem1. They also showed that *MCX1* genetically interacts with *HEM1* and *HEM25*. The double mutant *mcx1* Δ *hem25* Δ cells had a severe growth defect compared to the single mutant cells when glycerol was the carbon source. This defect was restored by supplementation with 5-Ala. This result is in alignment with the results of this thesis where the involvement of Hem25 in heme synthesis has been shown. Moreover, when I looked at the interactome of *HEM25*, *HEM1* and *MCX1* on SGD, I found genes that are common interacting partners of these three genes. These were *KGD2*, *AIM22*, *CEM1* and *HTD2*. These genes all have functions related with succinyl-CoA synthesis. *CEM1* and *HTD2* code for enzymes involved in mitochondrial fatty acid synthesis required for octanoic acid production, which is a precursor for lipoic acid synthesis. I also searched for functional correlations between these genes in the HaploInsufficiency and HOmozygous Profiling (HipHop)chemogenomics database. This database records the sensitivity of the yeast deletion collection to an array of around 3500 compounds [51]. The absence of Hem25 produces a similar drug sensitivity profile to that of cells lacking Hem1, Pdx3 and Mcx1. These observations taken together support a role of Hem25 with Hem1, Mcx1 and Pdx3 in the early steps of heme synthesis.

The interactions of *KGD2* and *AIM22* genes with *HEM25* have been reported in SGD and these interactions were confirmed by the RSA shown in this chapter. The KGD complex promotes the oxidative decarboxylation of α -ketoglutarate to succinyl-CoA in the citric acid cycle [73]. This complex is composed of three components: dihydrolipoyl succinyltransferase (Kgd2), dihydrolipoyl dehydrogenase (Lpd1) and the alpha-ketoglutarate dehydrogenase (Kgd1). Kgd2 requires lipoic acid as a cofactor. *AIM22* encodes a lipoate-protein ligase homologue, which promotes the lipoylation of Kgd2. The enzymatic activity of Aim22 is essential for Kgd2 function [84].

The RSA revealed that the *aim22* Δ *hem25* Δ colonies had a mild reduction in size compared with the colonies grown in the +G418 growth condition (*aim22* Δ *hem25* Δ

cells and *aim22* Δ cells). A significant colony size reduction was observed in the RSA carried out to study the interaction of *KGD2* with *HEM25*. The serial dilution experiment showed that the haploid double mutant *aim22* Δ *hem25* Δ cells had a mild phenotype compared with its single mutant cells, and the double mutant *kgd2* Δ *hem25* Δ cells had a very slight growth defect compared to its single mutant cells. Glycine and 5-Ala restored the growth defect of the single mutant *hem25* Δ cells. Therefore, the growth defect of the double mutant cells was also restored by the supplementation with glycine or 5-Ala. Furthermore, the SGA analysis also revealed genetic interaction of *HEM25* with *AIM22*. The results from SGD, RSA and SGA analyses strongly suggest that simultaneous loss of Aim22 and Hem25 function results in sicker cells compared with loss of function of either protein alone. The RSA also showed that simultaneous loss of Kgd2 and Hem25 function results in sicker cells compared with loss of function of either protein alone. Kgd2 and Aim22 affect the synthesis of succinyl-CoA. When Aim22 or Kgd2 function is absent together with loss of Hem25 function, mitochondria succinyl-CoA and glycine levels may be reduced. The decrease of the two substances required for heme synthesis could impact cell growth.

YMH2 was another gene that showed a genetic interaction with *HEM25* by RSA. The RSA showed that the colonies of the *yhm2* Δ *hem25* Δ cells were smaller compared with the colonies grown in the +G418 growth condition (*yhm2* Δ and *yhm2* Δ *hem25* Δ cells). Yhm2 is a citrate and ketoglutarate carrier protein. It exports citrate from the mitochondria and it imports ketoglutarate into the mitochondria. Ketoglutarate is a substrate of Kgd1, which is a subunit of the KGD complex [72]. The absence of the ketoglutarate carrier could affect succinyl-CoA synthesis. Decreased levels of succinyl-CoA and glycine in *yhm2* Δ *hem25* Δ cells could produce a defect in heme synthesis, which could be the reason for the decreased colony size observed for these double mutants.

The interaction I determined between *HEM25* and *AIM22* or *KGD2* found by SGA is in alignment with the interactome of *HEM25* documented in the SGD. The interactome shows that some of the *HEM25* interactors are genes involved in succinyl-CoA synthesis such as: *AIM22*, *KGD2*, *CEM1* and *HTD2*. My SGA analyses revealed that there are negative genetic interactions between *HEM25* and genes related with

the KGD complex. The new-found interactor genes of *HEM25* were *KGD1* and *KGD4*. *KGD1* encodes the Kgd1 subunit, and *KGD4* encodes Kgd4, which is also a novel component identified in the KGD complex [39]. The interaction between *HEM25* and the genes that code for proteins that form the KGD complex was also observed when I carried out the RSA to confirm the interaction between *HEM25* and *AIM22* or *KGD2*.

Other interacting genes of *HEM25* encode proteins with functions related to cytochromes: *CBP3*, *COR1*, *COX17* and *MSS2*. *CBP3* encodes a protein required for the assembly of the cytochrome bc1 complex. *COR1* encodes a subunit of the cytochrome bc1 complex. *MSS2* encodes a protein involved in membrane insertion of subunit 2 of cytochrome c oxidase. *COX17* codes for a metallochaperone that transfers copper to Cox11 (protein required for delivery of copper to subunit 1 of cytochrome c oxidase). The absence of Hem25 function affects heme synthesis. The interaction of *HEM25* with these genes could be due to a reduced stability or a defect in assembly of the cytochrome subunits.

Also, the *pdx3* Δ cells genetically interact with *hem25* Δ cells in the SGA analysis. *PDX3* codes for the pyridoxine phosphate oxidase. This enzyme catalyzes the last step of the PLP synthesis, with PLP being a cofactor of Hem1 (the first enzyme in the heme synthesis pathway). This result is congruent with the results found in the HaploInsufficiency and HOmozygous Profiling (HipHop) chemogenomics database. The strains *pdx3* Δ and *hem25* Δ have similar drug sensitivity profiles.

The last interactor of *HEM25* was *PET8*. This interaction is in alignment with the results found in the phenotypic analyses shown in Chapter 4, and the data reported in SGD. The molecular explanation of this interaction will require further study.

Chapter 7

Conclusions

Mutations in *SLC25A38* have been associated with CSA, and the function of SLC25A38 had not been determined. The main focus of my thesis was to characterize SLC25A38 function. Experimental work presented in this thesis has provided support for the yeast homologue of *SLC25A38*, *HEM25*, whose absence causes a defect in heme biosynthesis, encodes a mitochondrial glycine importer. The defect in heme synthesis results in iron precipitation in the mitochondria of erythrocyte precursors in humans with CSA. Conservation of function between Hem25 and SLC25A38 was observed when the human protein was expressed in *S. cerevisiae*. Therefore, the function of Hem25 could be extrapolated to the human protein SLC25A38.

There are four other main contributions of this thesis to the knowledge of Hem25 relevance in mitochondrial biosynthetic pathways. First, I showed that in *S. cerevisiae*, maybe there is at least one other transporter involved in glycine import into the mitochondria. Separate lines of experimentation showed that Ymc1 is a possible candidate for the putative second glycine importer into the mitochondria among the members of the SLC25 family.

Second, interactions between *HEM25* with other members of the *SLC25* family were not previously known. Phenotypic analyses revealed that *FLX1*, *MTM1*, *ORT1*, *AAC3*, *SFC1* and *PET8* have genetic interaction with *HEM25*. Each of these interactions would require further study to understand their molecular basis, although their known functions led to hypotheses being proposed based on the requirement for specific cofactors that are substrates for these transporters being required for heme synthesis.

Third, this thesis also provides new evidence that simultaneous loss of function of Flx1 and Hem25 has dramatic effects on ETC stability. This interaction has not been reported in previous studies. Western blotting analyses showed that the disruption of *FLX1* together with *HEM25* causes a dramatic decrease of Ndi1, Sdh1, Sdh2, Cor2

and Cyt1 subunits. Components of ETC complexes that contained FAD and/or heme as cofactors were found to be decreased, whereas those that did not maintained there normal level. Simultaneous loss of the ETC cofactors heme and FAD results in much more dramatic loss in complex stability compared to loss of either just heme or FAD.

Finally, experiments in this thesis confirmed some of the genetic interactions found by high throughput analysis reported in the SGD. Using random sporulation, *HEM25* showed interaction with *AIM22*, *KGD2* and *YHM2*.

7.1 Future work

The work presented in this thesis has furthered our understanding of Hem25 function. However, there are still many unanswered questions. Experiments demonstrated that Hem25 is not the only glycine importer into the mitochondria. Ymc1 was identified as a potential candidate for a second glycine importer. *In vivo* or *in vitro* transport assay should be performed to confirm Hem25 and Ymc1 functions. In the future it would be necessary to work with mammalian cells, when possible with erythroid precursors from patients with CSA, in order to confirm the role of SLC25A38 as a mitochondrial glycine transporter. One could also work with pure SLC25A38 protein, however, as it is an integral membrane protein this would likely prove to be a challenge.

Cells lacking Hem25 and Gly1 function had a severe growth defect, which was partially restored by supplementation with 5-Ala. This would indicate that there are other mitochondrial processes beyond heme synthesis that are affected by low glycine mitochondrial content. The identification of the mitochondrial processes affected by decreased glycine levels would enhance our understanding of the molecular pathophysiology associated with mutations in the *SLC25A38* gene.

Moreover, future work should focus on further dissecting the mechanism by which loss of Hem25 function results in decreased cell fitness due to the absence of *FLX1*, *MTM1*, *ORT1*, *AAC3*, *PET8* or *SFC1*. It would be worth studying the mechanism by which the combined absence of Hem25 and Flx1 function impacts the abundance of Ndi1, Sdh1, Sdh2, Cor2 and Cyt1 subunits of the ETC. Lastly, the massive gene list from my SGA analysis has provided new insights in the knowledge of new interactors of *HEM25*. Further characterization of the processes identified by my analysis that compromise the growth in the absence of Hem25 function will be required.

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Appendix A

Supplementary Data

Table A.1: **Candidate genes that showed negative genetic interaction with *HEM25* in the SGA analysis** (not tested by random spore or tetrad analysis). SGD description from <http://www.yeastgenome.org/>.

Standard Name	Systematic Name	SGD description
Mitochondrial genes		
<i>MRPL10</i>	<i>YNL284c</i>	Mitochondrial ribosomal protein of the large subunit; appears as two protein spots (YmL10 and YmL18) on two-dimensional SDS gels.
<i>PRX1</i>	<i>YBL064c</i>	Mitochondrial peroxiredoxin with thioredoxin peroxidase activity; has a role in reduction of hydroperoxides; reactivation requires Trr2p and glutathione; induced during respiratory growth and oxidative stress; phosphorylated; protein abundance increases in response to DNA replication stress.
<i>MRX10</i>	<i>YDR282c</i>	Mitochondrial inner membrane protein of unknown function; associates with mitochondrial ribosome; localizes to the inner membrane with the C terminus facing the intermembrane space; ortholog of human <i>RMND1</i> , mutation in which is implicated in infantile encephaloneuromyopathy and defective mitochondrial translation.
<i>MSD1</i>	<i>YPL104w</i>	Mitochondrial aspartyl-tRNA synthetase; required for acylation of aspartyl-tRNA; yeast and bacterial aspartyl-, asparaginyl-, and lysyl-tRNA synthetases contain regions with high sequence similarity, suggesting a common ancestral gene.
<i>PET122</i>	<i>YER153c</i>	Mitochondrial translational activator specific for the <i>COX3</i> mRNA; acts together with Pet54p and Pet494p; located in the mitochondrial inner membrane.
<i>YIL060w</i>	<i>YIL060w</i>	Mitochondrial protein of unknown function; required for respiratory growth; mutant accumulates less glycogen than does wild type; null mutation results in a decrease in plasma membrane electron transport; <i>YIL060w</i> is not an essential gene
Nuclear/DNA/RNA related genes		
<i>RAD5</i>	<i>YLR032w</i>	DNA helicase/Ubiquitin ligase; involved in error-free branch of DNA damage tolerance (DDT) pathway; proposed to promote replication fork regression during postreplication repair by template switching; stimulates synthesis of free and PCNA-bound polyubiquitin chains by Ubc13p-Mms2p; required for error-prone translesion synthesis; forms nuclear foci upon DNA replication stress; associates with native telomeres, cooperates with homologous recombination in senescent cells.

Table A.1 – Continued from previous page

Standard Name	Systematic Name	SGD description
<i>CCR4</i>	<i>YAL021c</i>	Component of the Ccr4-Not transcriptional complex; Ccr4-Not is involved in regulation of gene expression; component of the major cytoplasmic deadenylase, which is involved in mRNA poly(A) tail shortening.
<i>NSI1</i>	<i>YDR026c</i>	RNA polymerase I termination factor; binds to rDNA terminator element, required for efficient Pol I termination; required for rDNA silencing at <i>NTS1</i> ; facilitates association of Sir2p with <i>NTS1</i> , contributes to rDNA stability and cell longevity; interacts physically with Fob1p and RENT subunits, Sir2p and Net1p; may interact with ribosomes, based on co-purification experiments; Myb-like DNA-binding protein; <i>NSI1</i> has a paralog, <i>REB1</i> , that arose from the whole genome duplication.
<i>SXM1</i>	<i>YDR395w</i>	Nuclear transport factor (karyopherin); involved in protein transport between the cytoplasm and nucleoplasm; similar to Nmd5p, Cse1p, Lph2p, and the human cellular apoptosis susceptibility protein, Cas1.
<i>MCM21</i>	<i>YDR318w</i>	Component of the kinetochore sub-complex COMA; COMA (Ctf19p, Okp1p, Mcm21p, Ame1p) bridges kinetochore subunits in contact with centromeric DNA with subunits bound to microtubules during kinetochore assembly; involved in minichromosome maintenance; modified by sumoylation; orthologous to human centromere constitutive-associated network (CCAN) subunit CENP-O and fission yeast mal2.
<i>ESC4</i>	<i>YHR154w</i>	Protein implicated in Mms22-dependent DNA repair during S phase; involved in recruiting the Smc5/6 complex to double-strand breaks; DNA damage induces phosphorylation by Mec1p at one or more SQ/TQ motifs; interacts with Mms22p and Slx4p; has four BRCT domains; has a role in regulation of Ty1 transposition; relative distribution to nuclear foci increases upon DNA replication stress.
<i>APQ12</i>	<i>YIL040w</i>	Protein required for nuclear envelope morphology; nuclear pore complex localization, mRNA export from the nucleus; exhibits synthetic lethal genetic interactions with genes involved in lipid metabolism.
<i>WSS1</i>	<i>YHR134w</i>	Metalloprotease involved in DNA repair, removes DNA-protein crosslinks at stalled replication forks during replication of damaged DNA; sumoylated protein localizing to the nuclear periphery of mother cells; localizes to a single spot on the nuclear periphery of mother cells but not daughters; interacts genetically with <i>SMT3</i> ; activated by DNA binding.
<i>MEF1</i>	<i>YLR069c</i>	Mitochondrial elongation factor involved in translational elongation.
<i>BUD22</i>	<i>YMR014w</i>	Protein required for rRNA maturation and ribosomal subunit biogenesis; required for 18S rRNA maturation; also required for small ribosomal subunit biogenesis; cosediments with pre-ribosomal particles; mutation decreases efficiency of +1 Ty1 frameshifting and transposition, and affects budding pattern.

Table A.1 – Continued from previous page

Standard Name	Systematic Name	SGD description
<i>RTT106</i>	<i>YNL206c</i>	Histone chaperone; involved in regulation of chromatin structure in both transcribed and silenced chromosomal regions; affects transcriptional elongation; has a role in regulation of Ty1 transposition; interacts physically and functionally with Chromatin Assembly Factor-1 (CAF-1).
<i>DUN1</i>	<i>YDL101c</i>	Cell-cycle checkpoint serine-threonine kinase; required for DNA damage-induced transcription of certain target genes, phosphorylation of Rad55p and Sml1p, and transient G2/M arrest after DNA damage; Mec1p and Dun1p function in same pathway to regulate both dNTP pools and telomere length; also regulates postreplicative DNA repair.
Ribosomal genes		
<i>RPL41A</i>	<i>YDL184c</i>	Ribosomal 60S subunit protein L41A; comprises only 25 amino acids; <i>rpl41a rpl41b</i> double null mutant is viable; homologous to mammalian ribosomal protein L41, no bacterial homolog; <i>RPL41A</i> has a paralog, <i>RPL41B</i> , that arose from the whole genome duplication.
<i>RPL1B</i>	<i>YGL135w</i>	Ribosomal 60S subunit protein L1B; N-terminally acetylated; homologous to mammalian ribosomal protein L10A and bacterial L1; <i>RPL1B</i> has a paralog, <i>RPL1A</i> , that arose from the whole genome duplication; <i>rpl1a rpl1b</i> double null mutation is lethal.
<i>RPL43A</i>	<i>YPR043w</i>	Ribosomal 60S subunit protein L43A; null mutation confers a dominant lethal phenotype; homologous to mammalian ribosomal protein L37A, no bacterial homolog; <i>RPL43A</i> has a paralog, <i>RPL43B</i> , that arose from the whole genome duplication.
Other genes		
<i>SPO7</i>	<i>YAL009w</i>	Putative regulatory subunit of Nem1p-Spo7p phosphatase holoenzyme; regulates nuclear growth by controlling phospholipid biosynthesis, required for normal nuclear envelope morphology, premeiotic replication, and sporulation.
<i>CPA1</i>	<i>YOR303w</i>	Small subunit of carbamoyl phosphate synthetase; carbamoyl phosphate synthetase catalyzes a step in the synthesis of citrulline, an arginine precursor; translationally regulated by an attenuator peptide encoded by <i>YOR302w</i> within the <i>CPA1</i> mRNA 5'-leader.
<i>YOR309c</i>	<i>YOR309c</i>	Dubious open reading frame; unlikely to encode a functional protein, based on available experimental and comparative sequence data; partially overlaps the verified gene <i>NOP58</i> .
<i>THI3</i>	<i>YDL080c</i>	Regulatory protein that binds Pdc2p and Thi2p transcription factors; activates thiamine biosynthesis transcription factors Pdc2p and Thi2p by binding to them, but releases and de-activates them upon binding to thiamine pyrophosphate (TPP), the end product of the pathway; has similarity to decarboxylases but enzymatic activity is not detected.

Table A.1 – Continued from previous page

Standard Name	Systematic Name	SGD description
<i>LYS14</i>	<i>YDR034c</i>	Transcriptional activator involved in regulating lysine biosynthesis; involved in the regulation of genes of the lysine biosynthesis pathway; requires 2-aminoadipate semialdehyde as co-inducer.
<i>VAB31</i>	<i>YEL005c</i>	Subunit of the BLOC-1 complex involved in endosomal maturation; interacts with Vps21p-GFP; has potential role in vacuolar function, as suggested by its ability to bind Vac8p; likely member of; Vab2p-GFP-fusion localizes to cytoplasm in punctate pattern.
<i>PEX10</i>	<i>YDR265w</i>	Peroxisomal membrane E3 ubiquitin ligase; required for for Ubc4p-dependent Pex5p ubiquitination and peroxisomal matrix protein import; contains zinc-binding RING domain; mutations in human homolog cause various peroxisomal disorders.
<i>ATP17</i>	<i>YDR377w</i>	Subunit f of the F0 sector of mitochondrial F1F0 ATP synthase; F1F0 ATP synthase is a large, evolutionarily conserved enzyme complex required for ATP synthesis.
<i>HNT2</i>	<i>YDR305c</i>	Dinucleoside triphosphate hydrolase; has similarity to the tumor suppressor FHIT and belongs to the histidine triad (HIT) superfamily of nucleotide-binding proteins.
<i>YDR517w</i>	<i>YDR517w</i>	Acetylated <i>cis</i> -Golgi protein, involved in ER to Golgi transport; homolog of human <i>GRASP65</i> ; forms a complex with the coiled-coil protein Bug1p; mutants are compromised for the fusion of ER-derived vesicles with Golgi membranes; protein abundance increases in response to DNA replication stress.
<i>DUT1</i>	<i>YBR252w</i>	deoxyuridine triphosphate diphosphatase (dUTPase); catalyzes hydrolysis of dUTP to dUMP and PPi, thereby preventing incorporation of uracil into DNA during replication; critical for the maintenance of genetic stability; also has diphosphatase activity on deoxyinosine triphosphate.
<i>FSH1</i>	<i>YHR049w</i>	Putative serine hydrolase; localizes to both the nucleus and cytoplasm; sequence is similar to <i>S. cerevisiae</i> Fsh2p and Fsh3p and the human candidate tumor suppressor OVCA2.
<i>YHR138c</i>	<i>YHR138c</i>	Protein of unknown function; similar to Pbi2p; double null mutant lacking Pbi2p and Yhr138cp exhibits highly fragmented vacuoles; protein abundance increases in response to DNA replication stress.
<i>YIL028w</i>	<i>YIL028w</i>	Dubious open reading frame; unlikely to encode a functional protein, based on available experimental and comparative sequence data.
<i>XBP1</i>	<i>YIL101c</i>	Transcriptional repressor; binds promoter sequences of cyclin genes, <i>CYS3</i> , and <i>SMF2</i> ; not expressed during log phase of growth, but induced by stress or starvation during mitosis, and late in meiosis; represses 15% of all yeast genes as cells transition to quiescence; important for maintaining G1 arrest and for longevity of quiescent cells; member of Swi4p/Mbp1p family; phosphorylated by Cdc28p; relative distribution to nucleus increases upon DNA replication stress.

Table A.1 – Continued from previous page

Standard Name	Systematic Name	SGD description
<i>ZAP1</i>	<i>YJL056c</i>	Zinc-regulated transcription factor; binds to zinc-responsive promoters to induce transcription of certain genes in presence of zinc, represses other genes in low zinc; regulates its own transcription; contains seven zinc-finger domains.
<i>YJL055w</i>	<i>YJL055w</i>	Putative protein of unknown function; functions together with Ham1p to mediate resistance to 5-FU; specifically reduces the incorporation of 5-FU into RNA, without affecting uptake or incorporation of uracil into RNA; proposed to be involved in the metabolism of purine and pyrimidine base analogues; deletion mutants are sensitive to HAP and AHA.
<i>PEP8</i>	<i>YJL053w</i>	Vacuolar protein component of the retromer; forms part of the multimeric membrane-associated retromer complex involved in vacuolar protein sorting along with Vps35p, Vps29p, Vps17p, and Vps5p; essential for endosome-to-Golgi retrograde protein transport; interacts with Ypt7p; protein abundance increases in response to DNA replication stress.
<i>YJL049w</i>	<i>YJL049w</i>	Putative protein of unknown function; <i>YJL049w</i> is a non-essential gene.
<i>IXR1</i>	<i>YKL032c</i>	Transcriptional repressor that regulates hypoxic genes during normoxia; involved in the aerobic repression of genes such as <i>COX5b</i> , <i>TIR1</i> , and <i>HEM13</i> ; binds DNA intrastrand cross-links formed by cisplatin; HMG (high mobility group box) domain containing protein which binds and bends cisplatin-modified DNA, blocking excision repair; <i>IXR1</i> has a paralog, <i>ABF2</i> , that arose from the whole genome duplication.
<i>FPS1</i>	<i>YLL043w</i>	Aquaglyceroporin, plasma membrane channel; involved in efflux of glycerol and xylitol, and in uptake of acetic acid and the trivalent metalloids arsenite and antimonite; role in mediating passive diffusion of glycerol is key factor in maintenance of redox balance; member of major intrinsic protein (MIP) family; phosphorylated by Hog1p MAPK under acetate stress; deletion improves xylose fermentation.
<i>YLR235c</i>	<i>YLR235c</i>	Dubious open reading frame; unlikely to encode a functional protein, based on available experimental and comparative sequence data; overlaps ORFs <i>TOP3/YLR234w</i> and <i>YLR236c</i> .
<i>JIP3</i>	<i>YLR331c</i>	Dubious open reading frame; unlikely to encode a functional protein, based on available experimental and comparative sequence data; not conserved in closely related <i>Saccharomyces</i> species; 98% of ORF overlaps the verified gene <i>MID2</i> .
<i>YMR141c</i>	<i>YMR141c</i>	Dubious open reading frame; unlikely to encode a functional protein, based on available experimental and comparative sequence data.
<i>RCH1</i>	<i>YMR034c</i>	Putative transporter; member of the SLC10 carrier family; identified in a transposon mutagenesis screen as a gene involved in azole resistance; <i>YMR034c</i> is not an essential gene.

Table A.1 – Continued from previous page

Standard Name	Systematic Name	SGD description
<i>SCJ1</i>	<i>YMR214w</i>	One of several homologs of bacterial chaperone DnaJ; located in the ER lumen where it cooperates with Kar2p to mediate maturation of proteins.
<i>YML037c</i>	<i>YML037c</i>	Putative protein of unknown function; has some characteristics of a transcriptional activator; may be a target of Dbf2p-Mob1p kinase; GFP-fusion protein co-localizes with clathrin-coated vesicles; <i>YML037c</i> is not an essential gene.
<i>YML033w</i>	<i>YML033w</i>	Merged open reading frame; does not encode a discrete protein; <i>YML033w</i> was originally annotated as an independent ORF, but was later demonstrated to be an exon of an adjacent ORF, <i>YML034W</i> .
<i>TSA1</i>	<i>YML028w</i>	Thioredoxin peroxidase; acts as both ribosome-associated and free cytoplasmic antioxidant; self-associates to form high-molecular weight chaperone complex under oxidative stress; chaperone activity essential for growth in zinc deficiency; required for telomere length maintenance; protein abundance increases, forms cytoplasmic foci during DNA replication stress; <i>TSA1</i> has a paralog, <i>TSA2</i> , that arose from the whole genome duplication.
<i>HOR7</i>	<i>YMR251w-A</i>	Protein of unknown function; overexpression suppresses Ca ²⁺ sensitivity of mutants lacking inositol phosphorylceramide mannosyltransferases Csg1p and Csh1p; transcription is induced under hyperosmotic stress and repressed by alpha factor; <i>HOR7</i> has a paralog, <i>DDR2</i> , that arose from the whole genome duplication.
<i>TDA7</i>	<i>YNL176c</i>	Cell cycle-regulated gene of unknown function; promoter bound by Fkh2p; null mutant is sensitive to expression of the top1-T722A allele; <i>TDA7</i> has a paralog, <i>YDL211c</i> , that arose from the whole genome duplication.
<i>BUD17</i>	<i>YNR027w</i>	Putative pyridoxal kinase; a key enzyme in vitamin B6 metabolism; involved in bud-site selection; diploid mutants display a random rather than a bipolar budding pattern; similarity to yeast <i>BUD16</i> and human pyridoxal kinase (<i>PDXK</i>).
<i>YNR029c</i>	<i>YNR029c</i>	Putative protein of unknown function; deletion confers reduced fitness in saline.
<i>URE2</i>	<i>YNL229c</i>	Nitrogen catabolite repression transcriptional regulator; inhibits <i>GLN3</i> transcription in good nitrogen source; role in sequestering Gln3p and Gat1p to the cytoplasm; has glutathione peroxidase activity and can mutate to acquire GST activity; altered form creates [<i>URE3</i>] prion.
<i>SWT1</i>	<i>YOR166c</i>	RNA endoribonuclease involved in perinuclear mRNP quality control; involved in perinuclear mRNP quality control via the turnover of aberrant, unprocessed pre-mRNAs; interacts with subunits of THO/TREX, TREX-2, and RNA polymerase II; contains a PIN (PilT N terminus) domain.

Table A.1 – Continued from previous page

Standard Name	Systematic Name	SGD description
<i>YNL089c</i>	<i>YNL089c</i>	Dubious open reading frame unlikely to encode a functional protein; almost completely overlaps <i>YNL090w/RHO2</i> which encodes a small GTPase of the Rho/Rac subfamily of Ras-like proteins.
<i>ALG12</i>	<i>YNR030w</i>	Alpha-1,6-mannosyltransferase localized to the ER; responsible for addition of alpha-1,6 mannose to dolichol-linked Man7GlcNAc2; acts in the dolichol pathway for N-glycosylation; human homolog <i>ALG12</i> complements yeast null mutant.
<i>SOL1</i>	<i>YNR034w</i>	Protein with a possible role in tRNA export; shows similarity to 6-phosphogluconolactonase non-catalytic domains but does not exhibit this enzymatic activity; homologous to Sol3p and Sol4p; SOL1 has a paralog, <i>SOL2</i> , that arose from the whole genome duplication; protein abundance increases in response to DNA replication stress.
<i>YOR296w</i>	<i>YOR296w</i>	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; expressed during copper starvation; <i>YOR296w</i> is not an essential gene.
<i>YOR022c</i>	<i>YOR022c</i>	Putative carboxylic ester hydrolase; similar to bovine phospholipase A1; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies.
Lipid related genes		
<i>SUR2</i>	<i>YDR297w</i>	Sphinganine C4-hydroxylase; catalyses the conversion of sphinganine to phytosphingosine in sphingolipid biosynthesis.
<i>NSG1</i>	<i>YHR133c</i>	Protein involved in regulation of sterol biosynthesis; specifically stabilizes Hmg2p, one of two HMG-CoA isoenzymes that catalyze the rate-limiting step in sterol biosynthesis; forms foci at the nuclear periphery upon DNA replication stress; relocates to the cytosol in response to hypoxia; homolog of mammalian INSIG proteins; <i>NSG1</i> has a paralog, <i>NSG2</i> , that arose from the whole genome duplication.
<i>FAA1</i>	<i>YOR317w</i>	Long chain fatty acyl-CoA synthetase; activates imported fatty acids with a preference for C12:0-C16:0 chain lengths; functions in long chain fatty acid import; accounts for most acyl-CoA synthetase activity; localized to lipid particles; involved in sphingolipid-to-glycerolipid metabolism; forms ER foci upon DNA replication stress; <i>FAA1</i> has a paralog, <i>FAA4</i> , that arose from the whole genome duplication.
<i>FIG4</i>	<i>YNL325c</i>	Phosphatidylinositol 3,5-bisphosphate (PtdIns[3,5]P) phosphatase; required for efficient mating and response to osmotic shock; physically associates with and regulated by Vac14p; contains a Sac1-like domain; homologous to human <i>FIG4</i> , which is associated with CMT4J, a form of Charcot-Marie-Tooth disorder.
<i>NCR1</i>	<i>YPL006w</i>	Vacuolar membrane protein; transits through the biosynthetic vacuolar protein sorting pathway, involved in sphingolipid metabolism; cells lacking Ncr1p exhibit high levels of long chain bases (LCB), similar to the accumulation of high amounts of lipids observed in patients with Neimann-Pick C, a disease caused by loss-of-function mutations in <i>NPC1</i> , the functional ortholog of <i>NCR1p</i> .

Table A.1 – *Continued from previous page*

Standard Name	Systematic Name	SGD description
<i>HNF1</i>	<i>YDL125c</i>	Adenosine 5'-monophosphoramidase; interacts physically and genetically with Kin28p, a CDK and TFIIK subunit, and genetically with <i>CAK1</i> ; member of the histidine triad (HIT) superfamily of nucleotide-binding proteins and similar to Hint; protein abundance increases in response to DNA replication stress.

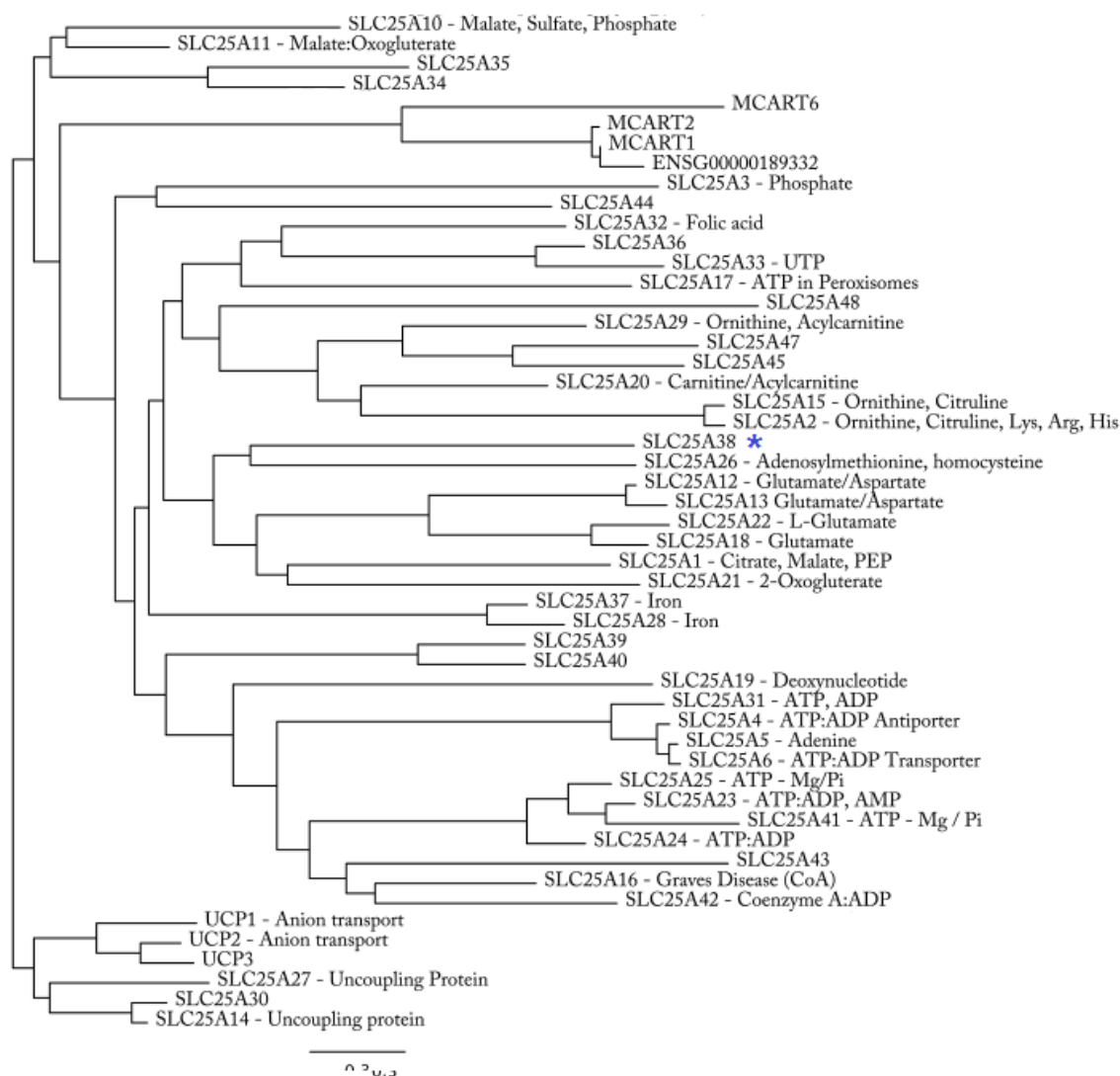


Figure A.1: **Maximum-likelihood phylogenetic tree of human sequences in the SLC25 protein family of mitochondrial transporters.** SLC25A38 is marked with an asterisk and sequences are annotated according to their transport roles. SLC25-family members were selected from Refseq based on previous analyses and aligned using HMMER and automatically edited using the AliMask-CS (Alignment Masking with Confidence Scores) algorithm. A maximum-likelihood phylogenetic tree was constructed using FastTree2 with the WAG substitution matrix and the resulting tree visualized with Figtree. This analysis was done by Daniel Gaston (Department of Pathology, Dalhousie University).