DIPLOMARBEIT

“Screening for multicopy suppressors of a yeast *mrs3/4*Δ mutant: an attempt to discover novel mitochondrial iron transporters“

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„Magister der Naturwissenschaften“

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Zusammenfassung

Eisen ist ein essentielles Spurenelement für alle Organismen. Seit 1962 das erste eisenhältige Protein – Ferredoxin – entdeckt wurde, wuchs auch das wissenschaftliche Interesse an der Beteiligung von Eisen an molekularbiologischen Prozessen. Obwohl heute viele dieser Prozesse aufgeklärt sind, gibt es noch Unklarheiten. Insbesondere wirft der Transport von Eisen durch die innere Mitochondrienmembran viele Fragen auf. Zwei Proteine der „Mitochondrial Carrier Family", Mrs3p und Mrs4p, weisen Eisen - Transportaktivität auf, spielen aber wohl nur eine Nebenrolle, da diese Aktivität nur auf Eisen-limitierenden Medien nachweisbar ist. Das Ziel der vorliegenden Arbeit war es daher, weitere mitochondriale Eisentransporter zu finden. In einem genetischen Screen wurden multi-copy Suppressoren gesucht, welche den Wachstumsphänotyp einer *mrs3/4*Δ Mutante supprimieren können.

Im ersten Teil der Arbeit wurde dieser Screen mit einer üblichen Genbank durchgeführt. Dabei wurde neben *MRS3* nur *MRS12* gefunden. Rim2p/Mrs12p, welches ursprünglich als Transporter für Pyrimidinbasen charakterisiert wurde, konnte den Phänotyp des *mrs3/4*Δ Stammes sehr gut supprimieren.

Um zu verhindern, dass immer wieder dieselben Gene gefunden werden, wurde der Screen mit einer Genbank wiederholt, in der die Gene *MRS12, MRS3* und *MRS4* fehlten. Auf diese Weise wurden weitere Suppressoren gefunden, welche jedoch den Phänotyp nur sehr schwach supprimieren konnten. Die annotierte Funktion der entsprechenden Gene lässt vermuten, dass die Suppressoren nur eine untergeordnete Rolle im Eisenhaushalt spielen.

In einem weiteren Experiment wurde ermittelt, ob eine Überexpression von *MRS3/4* die Resistenz gegenüber Cadmium erhöhen kann. Eine Komplementation des Wachstumsdefekts, welcher durch eine erhöhte Sensibilität des *mrs3/4*Δ Stammes gegenüber Schwermetallen zustande kommt, konnte bestätigt werden, nicht jedoch eine Erhöhung der Resistenz an sich.

Abstract

Iron is an essential trace element for all organisms. Since the first iron-containing protein – ferredoxin – was discovered in 1962, the scientific interest in the involvement of iron in molecular processes has been growing. Although many of these processes are well understood these days, some obscurities remain. In particular the transport of iron across the inner mitochondrial membrane raises many questions. Two proteins of the mitochondrial carrier family, Mrs3p and Mrs4p, show iron transport activity. However, both proteins appear to play a minor role, because transport activity becomes evident under iron limiting conditions only.

The goal of the present work was therefore to discover novel mitochondrial iron transporters.

In a genetic screen I searched for multi-copy suppressors of the growth phenotype of an *mrs3/4*Δ mutant.

In the first part of this study I performed the screen with a common library. In addition to *MRS3*, I did only discover *MRS12*. Rim2p/Mrs12p, which had initially been characterized as pyrimidine transporter, was able to suppress the growth phenotype of the *mrs3/4*Δ mutant very well.

To detect weaker suppressors, the screen was repeated with a library lacking *MRS12, MRS3* and *MRS4* under less stringent conditions. In this manner, I was able to discover more suppressors, which were able to suppress of the phenotype to a low degree only, however. The annotated functions of the corresponding genes indicate that the suppressors may only play a minor role for iron homeostasis.

In an additional experiment I investigated whether over-expression of *MRS3/4* is able to increase the resistance of yeast to cadmium. I was able to demonstrate that over-expression of both genes can complement the growth defect emerging from the hypersensitivity of the *mrs3/4*Δ strain to heavy metals, but an increase in heavy metal resistance was not observed.

1. Introduction

1.1 Chemical characteristics and biological role of iron

Because of its abundance and ubiquity in the biosphere as well as its flexible chemistry, iron has been an important player in the origin of life (Wächtershäuser, 2007). Still, after the evolution of higher organisms, iron is part of many complex biochemical processes, due to properties such as the easily occuring transition between its oxidation states (II) and (III), the tendency of hydroxy-complexes to condensate to oligomers, the flexibility of iron towards the nature of its ligands, the various co-ordination numbers and the co-ordination geometry.

Iron can appear in various oxidation states, but only iron(II) and iron(III) are exceptionally notable for biological processes. Free iron is toxic to the cell because it causes the production of free radicals. Furthermore, free iron will adhere unspecifically to cell membranes. Hence, iron is perpetually bound to proteins and its distribution within the cell is highly regulated. In organisms, iron is an essential cofactor for heme proteins including cytochromes, hemoglobin or myoglobin. Thus, iron participates in many redox reactions within the cell and is indispensable for oxygen transport in the cardiovascular system. In addition, iron is found in iron-sulphur-cluster proteins like hydrogenase and nitrogenase and in non-heme iron proteins like ribonucleotide-reductase and bacterial methane-monooxigenase.

Beyond its general function in biomolecules, iron was already linked to particular topics like aging and immunology. After Denham Harman proposed the free radical theory of aging (Harman D., 1956), a theory suggesting the accumulation of oxidatively modified cellular components during an organism's lifespan, leading to progressive decline of cellular functions, scientists tried to reveal the main catalyst for these modifications. According to a recent publication, it seems that "labile iron" (iron in its redox reactive form) is the main catalyst that mediates extensive oxidative modifications (Galaris et al, 2008). Furthermore, iron is sequestered in infected mammals (Marx, 2002), whereas modified iron levels in patients with iron related disorders cause immunodeficiencies in the innate and adaptive immune system (Subramaniam, 2007). Finally it has to be mentioned that iron is involved in many diseases. Some of these iron-related diseases will be highlighted below.

1.2 Iron related disorders and their connection to yeast studies

The recent advances made in yeast metal transport research, in particular the investigation of iron and copper regulation, contributed to our understanding of many iron related disorders in man. In addition, it was shown that mitochondria with defective regulation of iron homeostasis are involved in some of these disorders.

The best example, how basic research in yeast strengthened the connection of a gene involved in human disease to mitochondrial function, is *Friedreich's Ataxia*. Clinical symptoms include progressive neurodegeneration and hypertrophic cardiomyopathy. In most cases, the genetic background of this disease is a homozygous mutation in the gene *FRDA1*. The mutation is a $GAA_{(n)}.TTC_{(n)}$ hyper-expansion which results in decreased expression levels of the corresponding protein frataxin. Patients with *Friedreich's Ataxia* accumulate iron in mitochondria but strikingly, despite the vast excess of iron, show symptoms of iron deficiency. At the time of its identification, the amino acid sequence of frataxin revealed no clue to its function, except for its mitochondrial localization sequence. Later however, *YFH1*, the yeast homologue to frataxin, was identified as a high copy number suppressor of a mutant defective in intracellular iron usage (Babcock et al., 1997).

Recent studies demonstrate that the GAA expansion inhibits frataxin expression through a heterochromatin-mediated repression mechanism and that novel therapeutic compounds can revert the pathological processes of the disease (Rai et al, 2008). In addition, it was suggested that the secondary structure of the hyper-expansion may impair transcription and that UUC runs may lead to a rapid degradation of transcripts (Krasilnikova et al, 2007).

Frataxin constitutes a very important iron chelator in mitochondria, where it binds to ferrochelatase, the enzyme involved in heme formation, and Isu, the scaffold protein required for Fe-S protein assembly (Cook et al., 2006). Low levels of frataxin result in mitochondrial iron overload, and downstream regulatory effects lead to decreased cytosolic iron levels and additional downregulation of frataxin expression (Li et al, 2008). FA patients show symptoms of iron deficiency because they accumulate iron in mitochondria but can not use it for iron utilizing processes.

In addition to the characteristics discussed above, it is likely that oxidative stress plays an important role in FA. Accumulating iron may lead to the production of reactive oxygen species via the Fenton reaction. However, the involvement of oxidative stress in FA is controversial. Increased antioxidant defense by overexpressing a MnSOD mimetic in mice did not show positive effects on the cardiac pathophysiology, and moreover, complete frataxin-deficiency did not induce oxidative stress in neuronal tissues (Seznec et al., 2005).

Although *Friedreichs's Ataxia* might thus be a neurodegenerative disease not associated with oxidative damage, neurodegenerative diseases of the brain are most certainly accompanied with increased oxidative stress - and iron levels seem to influence many of these diseases. Most striking is the impact of iron overload on Alzheimers Disease, Parkinsons Disease and Multiple Sclerosis. It was demonstrated that iron accumulates in the brain and leads to motoric and cognitive dysfunctions in the elderly and that iron overload leads to free radical production, lipid peroxidation and neuronal damage (Stankiewicz et al., 2007). Iron deposits were found in the brain gray matter of AD patients, in the substantia nigra of PD patients and in the thalamus of patients suffering from MS.

There is more evidence that dysregulation of brain iron homeostasis can be a primary cause of neurondegeneration. The discovery of Aceruloplasminemia and Neuroferritinopathy, two iron disorders which also occur in younger patients, revealed that mutations in certain genes can result in special disorders of the brain. Loss-of-function mutations in the ceruloplasmin gene and mutations in the light chain of ferritin cause iron overload in the brain (Madsen and Gitlin, 2007). The symptoms of affected patients generally resemble those seen in the elderly (Wills et al., 2002).

Although these diseases only concern higher eukrayotes, yeast may be relevant for future studies. The protein ceruloplasmin has analogous function to the yeast iron transporter Fet3p, although both proteins only share low sequence similarity. While Fet3p imports iron into yeast, ceruloplasmin is important for the transferrin-mediated iron export of the mammalian cell. Both proteins meet their function via their muticopper oxidase activity (Xu et al., 2004). Yeast may therefore be instrumental in the analysis of many current diseases, as it already was for Friedreich's Ataxia.

1.3 Regulation of iron homeostasis in yeast

Much of what we know about iron uptake, intracellular compartimentalisation, iron prosthetic group biosynthesis and regulation derives from studies in *Saccharomyces cerevisiae*. In *S. cerevisiae* there are three distinct mechanisms to achieve iron homeostasis. Baker's yeast can react very quickly to changes in iron availability by (i) regulation of iron-uptake systems, (ii) storage of iron or mobilisation of intracellular stored iron and (iii) metabolic adaptation (Philpott and Protchenko, 2008).

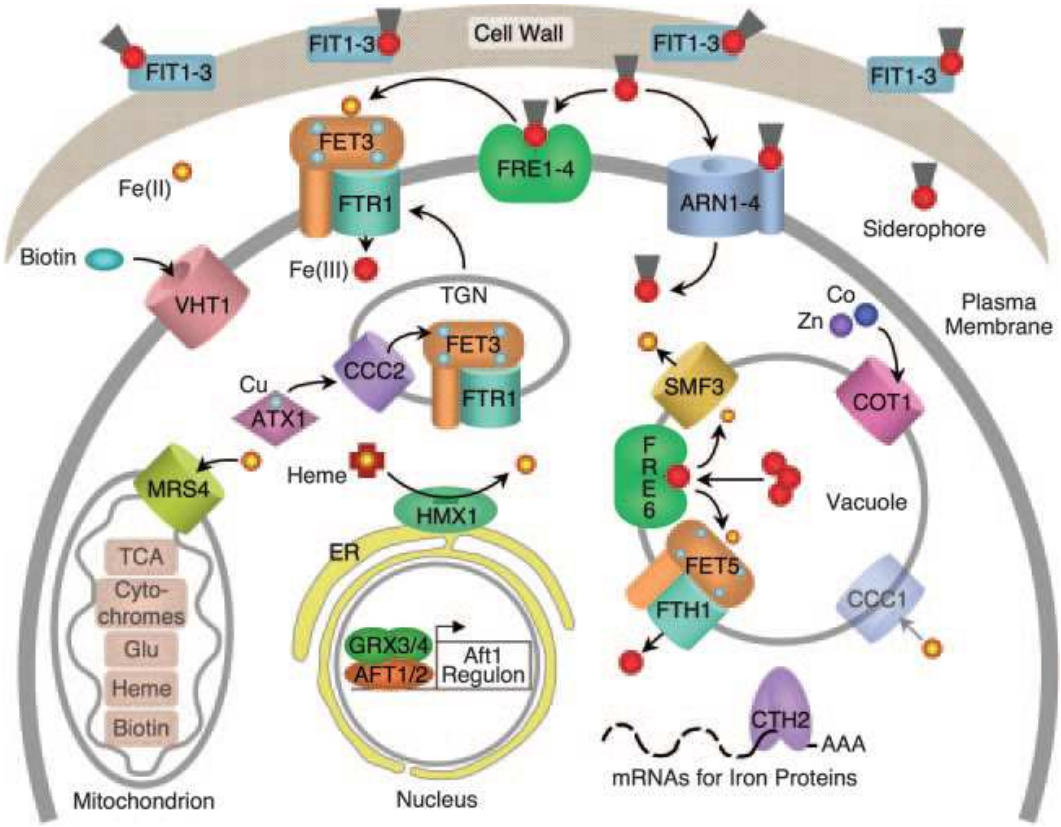


Figure 1: Regulation of iron transport in yeast.
Philpott and Protchenko, "Response to Iron Depriviation in Saccharomyces cerevisiae", 2008.

Cellular iron uptake is mediated by one of three mechanisms (Kosman, 2003). Under most laboratory conditions, iron is imported by the ferroxidase-permease dependent transport system. In the ferroxidase reaction, the multicopper oxidase Fet3p catalyzes the oxidation of Fe^{2+} to Fe^{3+}. The latter is then imported by the permease Ftr1p and reduced to Fe^{2+} in the cytoplasm.

The second mechanism of iron uptake, the direct import of ferrous iron, only plays a minor role in *S. cerevisiae*. Ferric iron (Fe^{3+}) is reduced to its ferrous form (Fe^{2+}) by the PM-localized reductase complex Fre1(2,3)p and subsequently imported by Fet4p.

Yeast is also a perfect scavenger of siderophore iron. Although it does not produce siderophores of its own, it can use multiple classes of external siderophores. Yeast therefore expresses a number of different plasma membrane permeases, or alternatively releases ferrous iron from siderophores at the Fre1(2,3)p complex, which can then enter the cell via the ferrous iron uptake mechanism or the ferroxidase-permease dependent transport mechanism. Fit(1-3)p are proteins associated with the cell wall that bind siderophores and faciliate iron uptake via this alternative pathway (Protchenko et al, 2001).

In yeast, iron is stored mainly in the vacuole. Newly arrived cellular iron does accumulate in the vacuole, and vacuolar iron is used to support essential metabolic activities like respiration (Kwok and Kosman, 2005). The main exporter of vacuolar iron is the Fet5p/Fth1p ferroxidase/permease complex, whereas *CCC1* appears to support iron uptake into the vacuole (Li et al., 2001).

Many genes that participate in iron homeostasis rely on the iron response-regulators Aft1p and Aft2p. Binding of Aft1p/Aft2p to their responsive elements (*ARE*) is not directly influenced by iron levels, but regulation is achieved by localization of these proteins (Rutherford and Bird, 2004). Unless iron levels are low, Aft1p and Aft2p remain in the cytosol. At low iron levels, Aft1p and Aft2p localize to the nucleus and activate the transcription of genes belonging to the "iron regulon". How Aft1p/Aft2p sense iron levels is not fully understood. Also, the expression of the major siderophore transporters (encoded by *ARN1-4)*, was shown to be under the control of Aft1p (Yun et al., 2000).

Metabolic adaptation is accomplished by Cth2p (Puig et al., 2005). The expression of *CTH2* is also up-regulated by Aft1p. Cth2p binds to mRNAs involved in iron utilization and leads to their degradation. When iron is limited, this mechanism allows the cell to use iron for essential pathways instead of wasting it to less important processes.

Besides the cell membrane, the vacuole and the nucleus, a very important player in iron homeostasis is the mitochondrion. Being the primary "end-user" of cellular iron, homeostasis depends primarily on the equilibrium beween iron availability and the mitochondrial demand for iron. Thus, all above mentioned mechanisms are only one side of this interplay.

1.4 Iron transport in yeast mitochondria

Iron has an essential role in mitochondria. Components of the electron transport chain like cytochrome C and Fe/S proteins require iron as co-factor. Heme formation and assembly of Fe/S clusters are the two major processes that consume iron in mitochondria.

Ferrous iron from the matrix is inserted into protoporphyrin IX to form heme. This final step of heme biosynthesis is catalyzed by ferrochelatase, an enzyme which is associated with the matrix side of the inner mitochondrial membrane. The insertion is probably tied to transport across the inner mitochondrial membrane (Lange, Kispal, Lill, 1999). At the initial step of Fe/S cluster formation, Nfs1p removes sulphur from cysteine and ligates it to iron that is probably supplied by the chelator Yfh1p. Fe/S clusters are formed on the scaffold proteins Isu1/2p and subsequently transferred to apo-Fe/S proteins. Yfh1p seems to play a central role in iron handling and might be a regulatory point between heme synthesis and Fe/S cluster assembly. A simple model of iron consuming processes in mitochondria is depicted below.

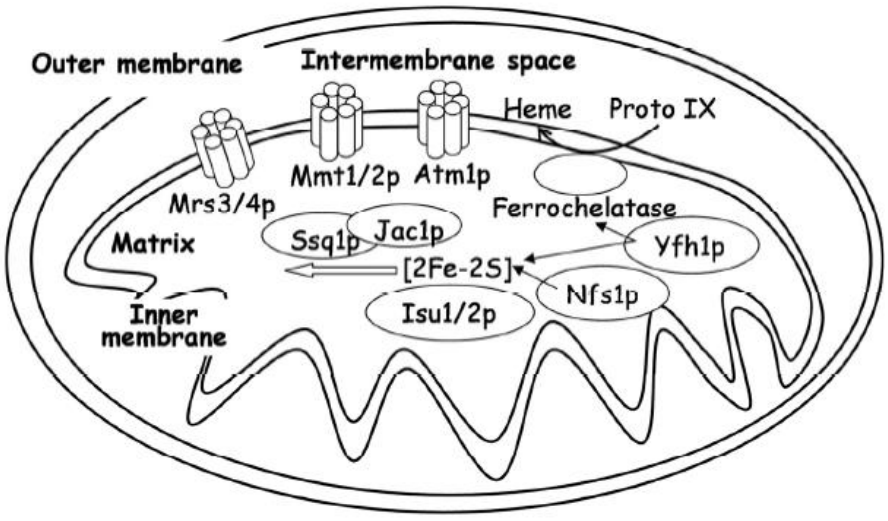


Figure 2: Proteins involved in iron trafficking and utilization in *S. cerevisiae* mitochondria
Kwok and Kosman, "Iron in Yeast: Mechanisms involved in homeostasis", 2005.

The demand for iron by mitochondria is high, but it was not yet discussed how this metal ion gets into the organelle. To date, it is not entirely clear how iron passes the mitochondrial membranes. Studies to investigate iron uptake into mammalian mitochondria began in the 1960s (Strickland and Davis, 1965). It was demonstrated that bovine heart mitochondria bind Fe^{2+} and Fe^{3+} in an energy independent reaction and that iron is bound to different sites than copper and calcium (Cederbaum and Wainio, 1972). An important study showed that the transport of iron across the inner mitochondrial membrane requires reducing equivalents supported by the respiratory chain, which indicates that iron passes the inner mitochondrial membrane only in the ferrous form (Flatmark and Romslo, 1975). In the same study evidence was presented that iron accumulates in mitochondria via an unidirectional flux of Fe(II) to the matrix where it is tightly bound to ligands. It was furthermore observed that this accumulation is energy dependent, although it was not clear whether the energy is supplied by coupled respiration, ATP hydrolysis or a cation gradient.

Experiments addressing the molecular basis of mitochondrial iron uptake demonstrated that import of ferrous iron was dependent on a membrane potential (Lange, Kispal and Lill, 1999). By measuring the incorporation of ^{55}Fe into protoporphyrin in presence or absence of the uncoupling agent CCCP, it was shown that only 25% of heme was formed in presence of CCCP. In addition, iron import turned out to be independent of ATP. Neither internal nor extramitochondrial ATP had a stimulatory effect on heme synthesis, and depletion of ATP by apyrase did not reduce heme formation.

Two genes discovered shortly before this publication, *MMT1* and *MMT2*, were discussed in terms of iron transport across the mitochondrial membranes. However, quantitative analysis of heme synthesis in mitochondria isolated from Δ*mmt1*Δ*mmt2* cells showed that these proteins are not required for the transport of iron to ferrochelatase. Also, experimental data showed that both genes might not provide iron for the biogenesis of Fe/S proteins. These results indicate that *MMT1* and *MMT2* are not essential for iron import into mitochondria. Another mitochondrial transporter, *ATM1*, was demonstrated to be important for export of Fe/S clusters only (Kispal, Csere et al., 1999). The transport activity found in the study of Lange et al. could not be explained by transporters of the inner mitochondrial membrane which were known so far.

Later two other genes were discovered and considered to encode proteins participating in mitochondrial iron transport. *MRS3* and *MRS4*, which belong to the *mitochondrial carrier family*, will be discussed in detail because they played a vital role for the present work. *MRS3* and *MRS4* had originally been found in a multicopy suppressor screen of an mtRNA splicing defect in yeast (Wiesenberger et al., 1991). *MRS3* and *MRS4* can suppress the phenotypes of a yeast strain lacking *MRS2* (Waldherr et al, 1993), a gene that was demonstrated to be important for Mg^{2+} homeostasis (Kolisek et al., 2003). It was therefore assumed that *MRS3/4* might be involved in metal ion transport as well.

Additional information about the role of *MRS4* was obtained from gene expression analysis. Genes of the *iron regulon* were induced by up-regulation of the transcription factors Aft1p or Aft2p (Rutherford et al., 2001). In one of the mutants (*AFT2-1*^{up}), it was shown that *MRS4* was induced. These data established a connection between *MRS4* and iron. Experiments in an *yfh*Δ mutant confirmed the role of *MRS3/4* in iron transport. *MRS3/4* deletion can suppress the phenotype in a yeast strain lacking *YFH1* (Foury and Roganti, 2002). Like already mentioned above, *frataxin* is an essential mitochondrial iron chelator. *Yfh1*Δ strains accumulate much more free iron in mitochondria than wild type cells and loose their mitochondrial genome. In a strain that lacks both *YFH* and *MRS3/4*, this accumulation is alleviated and the iron content is restored to almost wild type levels. The data suggest that toxic iron overload in mitochondria of an *yfh*Δ strain is relieved when iron import is additionally reduced via *MRS3/4* deletion.

A comprehensive study about *MRS3* and *MRS4* was carried out by Mühlenhoff et al., 2003. Expression profiling revealed that both overexpression and deletion of *MRS3/4* lead to up-regulation of several genes belonging to the *iron regulon*. It was therefore possible that Mrs3p and Mrs4p either have a regulatory function or directly participate in the transport of iron. As different expression levels of *MRS3/4* did not alter the membrane potential, it was very likely that these carriers are directly involved in mitochondrial iron uptake. Uptake of ^{55}Fe into mitochondria of *mrs3/4*Δ and *MRS3/4n* cells was tested *in vivo* and *in organello*. Iron uptake was determined by measuring *de-novo* formation of heme and Fe/S proteins. A decline in the formation of heme and the Fe/S protein Bio2p was observed in *mrs3/4*Δ cells, while both processes were reproducably increased in *MRS3/4n* cells. These data indicate that Mrs3/4p constitutes a transport system allowing iron influx.

However, the authors observed that iron transport became evident under iron-limiting conditions only. On lactate media containing standard concentrations of iron, different *MRS3/4* expression levels had no influence on the formation of Fe/S proteins. It is thus obvious that there must be another mitochondrial transporter with affinity to iron, which is active when iron is sufficiently available.

1.5 The aim of this work

Mrs3p and Mrs4p might not account for the transport activity detected by Lange et al. Mühlenhoff et al. provide strong evidence for the existence of another transport mechanism. The goal of the present work was therefore the finding of novel genes encoding mitochondrial transporters with iron transport activity.

New transporters might be found with various techniques, like isolating a protein in a biochemical approach or using a genetic screen. A genetic screen – a traditional forward genetic technique to identify novel genes – could be set up in different ways. We could screen for synthetically lethal random mutations in an *mrs3/4*Δ strain, or screen for multi-copy suppressors of a phenotype associated with the *MRS3/4* double deletion. We went for the latter method, because it would yield results more swiftly.

An *mrs3/4*Δ strain shows a growth defect on iron-limiting media. Furthermore, it was shown that the induction of the iron regulon in *mrs3/4*Δ cells is associated with increased sensitivity to cadmium. Both phenotypes were utilized for the multi-copy suppressor screen.

2. Materials and Methods

2.1 Strains and media

Table 1 gives an overview on all strains that were used in this work. For detailed information on the media please refer to table 2.

Name	**Background**	**Genotype**	**Notes**
DH10B		*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139Δ(ara, leu)7697 galU galK λ- rpsL nupG*	Invitrogen ™
NEB5α	DH5α	*fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs™
W303		*MATa, ura3–1, ade2–1, trp1–1, his3–11,15, leu2–3,112*	ATCC no. 208353
DBY747		*MATa, his3-Δ1, leu2-3, leu2-112, ura3-52, trp1-289a*	ATCC no. 204659
GW400	W303-1A	*MATa, ura3–1, ade2–1, trp1–1, his3–11,15, leu2–3,112; mrs3Δ::loxP; mrs4Δ::loxP*	strains created by Dr. Gerlinde Wiesenberger
GW403	DBY747	*MATa, his3-Δ1, leu2-3, leu2-112, ura3-52, trp1-289a; mrs3Δ::loxP; mrs4Δ::loxP*	
OS1-3c	W303	*MATa, ura3–1, ade2–1, trp1–1, his3–11,15, leu2–3,112; mrs12::kanMX4*	
OS1-2d	W303	*MATa, ura3–1, ade2–1, trp1–1, his3–11,15, leu2–3,112; mrs3Δ::LEU2; mrs4Δ::URA3; mrs12::kanMX4*	

Table 1: Bacterial and yeast strains

Name	Constituents	Supported organism	Optional supplements
LB (Luria Bertani)	1% tryptone, 0.5% yeast extract, 1% NaCl, 0.1% dextrose	*E. coli*	100 mg/L ampicilin
SOC	2% tryptone, 0,5% yeast extract, 10mM NaCl, 2,5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM Dextrose	*E. coli*	
YPD	1% yeast extract, 2% peptone, 2% dextrose	*S. cerevisiae*	80 -100µM BPS and 0 - 2µM $FeCl_2$ or 20 - 40µM $CdCl_2$
2xYPAD	2% yeast extract, 4% peptone, 4% dextrose, 0.004% adenine	*S. cerevisiae*	
YPG	1% yeast extract, 2% peptone, 3% glycerol	*S. cerevisiae*	
SD	0,67% yeast nitrogen base, 2% dextrose, supplemented with required amino acids	*S. cerevisiae*	80 µM BPS and 5 - 15µM $FeCl_2$ or 20 - 40µM $CdCl_2$

Table 2: Media used in this work. Plates contained additional 2% agar

To establish media allowing selection for suppressors, the iron chelator BPS or $CdCl_2$ were added to standard media. Plates containing BPS were supplemented with additional iron ($FeCl_2$), if required.

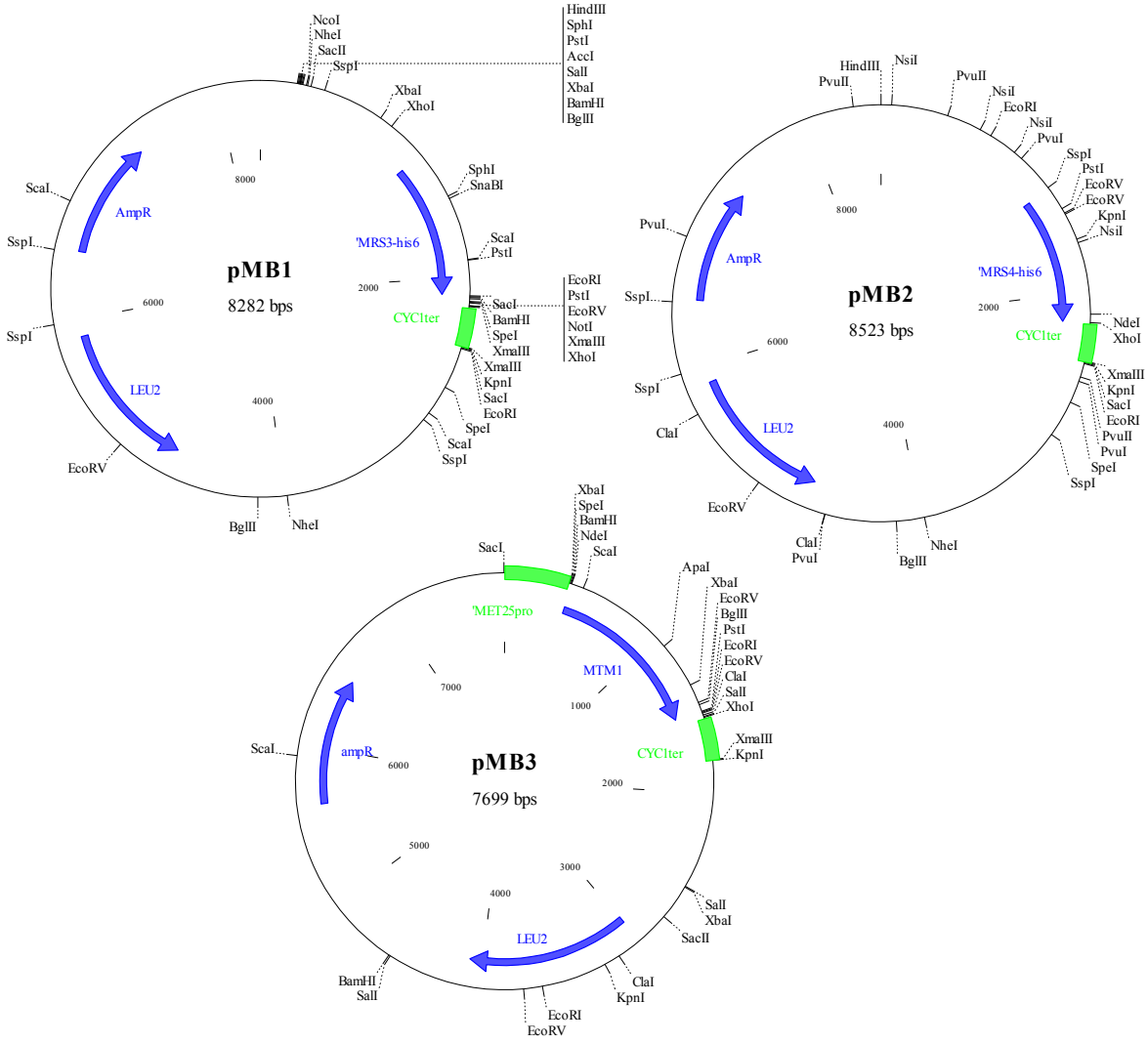
Bacteria were incubated overnight at 37°C. Yeast strains were incubated one to several days at 28°C or 37°C.

2.2 Plasmid constructs

Plasmids that were created during this work are listed in table 3. For information about further plasmids please refer to 5.1 (Appendix).

name	Background	marker	gene	promoter	Notes
pMB1	YCplac111 (CEN4-ARS1)	*LEU2*	*MRS3*	MET25	6x his tag, CYC1-ter
pMB2	YCplac111 (CEN4-ARS1)	*LEU2*	*MRS4*	MET25	6x his tag, CYC1-ter
pMB3	Yeplac181 (yeast 2 micron)	*LEU2*	*MTM1*	MET25	CYC1-ter

Table 3: plasmids constructed during this work



2.3 Transformation of $CaCl_2$ competent *E. coli*

E. coli strain DH10B was inoculated in 5 ml of LB liquid medium and grown for approximately 14 hours. This overnight culture was added to 1 litre of pre-warmed LB medium. The culture was incubated at 37°C until it reached an optical density of 0.6. The cells were harvested and resuspended in 500 ml of ice-cold $CaCl_2$ (0.1 M), followed by an incubation time of 15 minutes. Centrifugation, resuspension and incubation of cells were repeated with 250 ml and 100 ml of fresh $CaCl_2$ respectively. After the last incubation step, cells were centrifuged and resuspended in 10 ml of 0.1 M $CaCl_2$, containing 10% glycerin. Cells were split in 650 µl aliquots and used immediately or frozen at -80°C. For transformation, frozen cells were thawed on ice. 100 µl aliquots of the cell suspension were mixed with 2 µl of plasmid DNA. The reaction tubes were incubated at 4 °C for 30 minutes and thereafter at 42 °C for 2 minutes (heat shock). Cells were plated on LB_{amp} selective medium.

2.4 Transformation using electro-competent *E. coli*

Ligation reactions were diluted ten-fold and incubated at 65°C for 20 min to inactivate the ligase. 50 µl NEB5α electro-competent cells were thawed on ice. 2 µl of the diluted ligation reaction were mixed with the cells and transferred into electroporation cuvettes with 1 mm gap (BioRad #165-2089). Air bubbles were removed by tapping the cuvettes. Electroporation was performed with the BioRad gene pulser electroporator at 1,7 kV, 200 Ohm and 25 µF. The usual time constant was 4.1. 1 ml of pre-warmed (37°C) SOC was added to the cells. The suspension was transferred into 50 ml falcon tubes (T2318, Greiner®) and incubated at 37°C for one hour. 200 µl aliquots of the cell suspension were streaked out onto 146 mm LB plates containing 100 mg/L ampicillin and incubated overnight.

2.5 High-efficiency yeast transformation

Yeast cells were grown in 2xYPAD overnight and diluted in fresh medium to an OD_{600} of 0.5. The cells were incubated for at least two cell divisions (OD_{600} of 2.0), harvested by centrifugation at 4500 rpm for 4 min and washed with 10 ml of sterile water. The pellet was resuspended in 10 ml of 0.1 M LiAc and incubated at 30°C for 10 min. 1-ml aliquots (~10^8 cells) of this suspension were used for each transformation reaction.
The cells were spun down in micro-centrifuge tubes. After removal of the supernatant, the pellets were resuspended in 358 µl of transformation mix, consisting of 240 µl of 50% PEG, 36 µl of 1 M LiAc, 52 µl of carrier DNA (2 mg/ml) and 30 µl of sterile water. To each aliquot, 2 µl of plasmid DNA was added. The mix was incubated at 30°C for 30 min, followed by a heat shock at 42°C for 20 min. The cells were spun down, resuspended in sterile water and plated on selective media.

2.6 Plasmid preparation from bacteria

2 ml of LB_{amp} were inoculated with bacteria and grown overnight. 1.5 ml of the cell suspension were centrifuged and the supernatant was discarded. The cell pellet was resuspended in 100 µl of ice cold "lysis buffer" (50 mM dextrose, 25 mM Tris.Cl pH 8.0, 10 mM EDTA pH 8.0). For the following steps the reaction tubes were kept on ice. 200 µl of "alkaline SDS solution" (0.2 N NaOH, 1% SDS) was added and thoroughly mixed with the cell suspension by inverting the tube 4 times. Cell lysis was stopped by immediately adding 150 µl of ice cold high salt solution (3 M potassium / 5 M acetate) and vortexing the tube. The preparation was incubated on ice for 5 minutes and subsequently centrifuged for 5 minutes at 16 000 rpm. 400 µl of the supernatant was carefully transferred into a new microcentrifuge tube and mixed with 1 ml of 96% ethanol to precipitate plasmid DNA. After incubation at room temperature for another 5 minutes, the mixture was centrifuged at high speed (12 000 rpm, 10 minutes) and the supernatant was carefully discarded. The DNA pellet was washed with 1 ml of 70% ethanol. Ethanol was completely removed, and the DNA pellet was air-dried for 5-10 minutes. DNA was dissolved in 50 – 100 µl of TE buffer (10 mM Tris.Cl pH 8, 1 mM EDTA pH 8).

2.7 Plasmid preparation from yeast

Yeast cells were grown in selective media overnight. Cells were harvested by centrifugation and resuspended in 100 µl of STET buffer (8% (w/v) sucrose, 50 mM Tris.Cl pH 8.0, 50 mM EDTA pH 8.0, 5% (v/v) Triton X-100). 0.2 g of 0.45 mm glass beads were added. The tube was vortexed five times for 1 minute with a short cooling period after every minute. Another 100 µl of STET were added and the mixture was vortexed briefly and boiled for 3 minutes. The preparation was cooled on ice and centrifuged at 15 000 rpm for 10 minutes. 100 µl of the supernatant were added to a fresh microcentrifuge tube containing 50 µl of 7.5 M ammonium acetate. The tube was shaken and incubated at -20 °C for one hour, followed by a centrifugation step at 16 000 rpm for 10 minutes. 100 µl of the supernatant were transformed into a new tube and mixed with 200 µl of ice-cold ethanol. The DNA was precipitated at 4 °C at 13 000 rpm for 10 minutes and air-dried for 5 minutes. The supernatant was carefully removed and the DNA pellet was washed with 70% ethanol. The pellet was then dissolved in 20 µl of TE pH 8.0 buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA pH 8.0). Usually, 2 µl of this preparation were transformed into *E. coli* and yielded a convenient number of transformants on selective plates.

2.8 Isolation of genomic DNA

Isolation of genomic DNA was performed using the Blood and Cell Culture DNA Maxi Kit (Qiagen™). Because of divergences to the standard protocol of the kit, all steps are described below. For detailed information on the buffers and solutions used, please refer to the manual. Before starting with the protocol, 30 µl of RNase A (100 mg/ml) was added to 15 ml of buffer G2. Also, 12 µl of β-mercaptoethanol was added to 12 ml of buffer Y1. Strain *OS1-2d* (*mrs3/4/12*Δ) was inoculated in 100 ml of YPD and grown to a late logarithmic state (OD_{600} ~ 1.0 – 2.0). Cells were harvested by centrifugation for 4 minutes at 4500 rpm and washed with 12 ml of TE buffer (10 mM Tris.Cl / 1 mM EDTA, pH 8.0). The pellet was then resuspended in 12 ml of buffer Y1 containing β-mercaptoethanol as described. 1 ml of Zymolyase (1000 U/ml) was added to the cell suspension and the suspension was shaken at 120 rpm and 28 °C for 30 minutes.

Spheroblasting was tracked by microscopy and photometric measurement of the cells bursting in water. The spheroblasts were collected and lysed in 15 ml of buffer G2 containing RNase A. 400 µl of Proteinase K (20 mg/ml) was added and the mix was incubated at 50 °C for 30 minutes. Subsequent centrifugation steps were performed in autoclaved glass tubes (30 ml Corex® aluminosilicate glass tubes). The mixture, consisting of several cell components, was centrifuged at 5000 x *g* and 4 °C for 10 minutes. The supernatant was carefully removed from the cell debris. A "Genomic Tip" was equilibrated with 10 ml of buffer QBT. The supernatant was vortexed and put onto the top of the Genomic Tip. After the DNA was bound to the column, it was washed two times with 15 ml of buffer QC. DNA was eluted with 15 ml of buffer QF. 10.5 ml of isopropanol was added to the eluate and the DNA was spun down at >5000 x *g* for 10 minutes at 4°C (we used Sorvall centrifuge, rotor SS34 at 9500 rpm). The pellet was washed with 4 ml of ice-cold 70% ethanol and again centrifuged. The ethanol was removed completely by pipeting and the pellet was air-dried for 10 minutes. The genomic DNA was dissolved in 300 µl of TE buffer pH 8.0 at 50°C.

2.9 Partial digestion of genomic DNA

The genomic DNA was partially digested with the restriction enzyme Sau3A. To find the optimal enzyme concentration, pilot experiments were carried out in 10 µl reactions. 2 µl of isolated DNA (~100 ng/µl) were incubated with 2 µl (8 units) of Sau3A or a 10-, 100-, 1000- or 10 000 – fold dilution of this enzyme concentration, respectively, for exactly 30 minutes. Before dilutions of enzyme were added, the reaction mix was pre-warmed to 37 °. The digestion was stopped by adding 2 µl of a loading dye containing 100 mM EDTA. Further digestions with intermediate steps of enzyme dilutions were necessary. The optimal reaction, yielding mainly fragments between 3 and 10 kb, was scaled up 25-fold and 5 µg of genomic DNA were digested.

2.10 Isolation of DNA fragments

To isolate DNA fragments between 3 and 10 kilobases in size, the digested DNA was separated on 1% TAE agarose for at least 45 minutes. The fragments were either isolated using a gel extraction kit or using TEAE (tri-ethyl-aminoethyl-) cellulose. The gel was cut at 3 and 10 kb, the part of the gel below 3 kb was discarded and TEAE cellulose was put into the incision at 10 000 base pairs. Reversing the polarity caused the genomic DNA fragments to migrate towards the TEAE paper, where it was bound. To elute the DNA from the paper, a microcentrifuge tube was punctured with a needle and put into another open microcentrifuge tube. The TEAE cellulose was inserted into the punctured tube on top of a layer of silanized glass wool. The TEAE paper was washed twice with 200 µl of washing buffer (150 mM NaCl, 50 mM Tris.HCl pH 7.5 and 1 mM EDTA). After this washing step the DNA was eluted with 300 µl of pre-warmed (50°C) high salt elution buffer (1 M NaCl, 50 mM Tris.HCl and 1 mM EDTA). DNA was subsequently precipitated with ethanol, washed with 1 ml of 70% EtOH and dissolved in 100 µl of TE buffer (pH 8).

2.11 Creation of a multi-copy genomic library

The plasmid YEplac181 was cleaved with BamHI and dephosphorylated using antarctic phosphatase. To find the optimal amount of Antarctic phosphatase, several reactions with different phosphatase concentrations were performed. For optimal results, 2 units of phosphatase were used per µg DNA. The processed plasmid was purified by gel electrophoresis and gel extraction. The best ratio of plasmid and insert was determined for the ligation reaction using the integrated α-complementation system of the vector (plating of bacterial cells on IPTG/X-gal) to estimate positive cloning events. It turned out that a 5-fold concentration of insert compared to the vector was favorable. 50 ng of plasmid and 250 ng of genomic DNA were ligated using 2 units of T4 DNA ligase (Roche). The ligation reaction was carried out overnight at 4°C. Electroporation of bacteria was performed as described above. 10 aliquots of electrocompetent cells were used for the creation of the genomic library. The colonies were washed from the plates with 300 ml of liquid LB_{amp}, using a cell culture scraper and glass pipettes. Cells were

diluted to an OD_{600} of 1.0 and incubated for one division. Ten 1-ml aliquots were diverted from the suspension and cells were harvested by centrifugation. The pellet was resuspended in fresh LB_{amp} containing 10% glycerol and frozen. From the residual cells, library DNA was isolated using a Plasmid Maxi-Prep Kit (Quiagen™).The DNA was dissolved in 1ml of TE buffer pH8 and aliquoted into 5 portions à 200µl.

2.12 Serial dilution "complementation assays"

Yeast strains were diluted to an optical density (600 nm) of 1.0 and transferred into the first well of a 96-well plate. The cell suspensions were diluted 10-, 100- and 1000-fold from the left to the right. The cells were spotted on selective media and incubated at 28°C until a growth difference was visible. Additional plates were incubated at 35.5 / 37 °C to study the temperature sensitive growth of the transformed strains.

2.13 Finding the optimal screening conditions

1 µl of pLH4 (*MRS4* in YEplac111) was mixed with 49µl of empty YEplac111 to create a "mock library". 0.1, 0.2, 0.5, 1 and 2 µl of a yeast wild type library (Nasmyth) or the "mock library" DNA were transformed into GW400. Transformants were replica plated on YPD plates containing 50, 100 or 150 µM of $CdCl_2$ or 80 µM BPS supplemented with 0-5 µM $FeCl_2$, respectively. Plates were inspected after 2-3 days to find the optimal conditions for the screen. For iron deprived media, a second replica plating step was necessary. YPD plates containing BPS were therefore replica plated on SD -leu selective plates, containing 80µM BPS and 0-5 µM $FeCl_2$.

2.14 “Whole-cell” - PCR

In the suppressor screen, PCR was used to identify recurring clones before sequencing. PCR was performed on whole cells for easy handling. A few cells were scraped off with a toothpick and applied to the inner wall of a PCR tube. The cells in the PCR tube were microwaved for one minute to open up the cell wall and expose the DNA. 2,5 µl of 10x PCR buffer, 2,5 µl of Mg_2Cl (25 µM), 2,5 µl of dNTPs (2 µM), 2,5 µl of both primers (10 µM), 1 µl of *Taq-polymerase* (1 U/µl) and 11,5 µl of water were added to the cells. DNA was amplified in a thermocycler using the conditions listed below.

PCR conditions (apply to all primers):

94 °C	10 min			
	denaturation	94 °C	30 sec	} 30 circles
	annealing	49°C	30 sec	
	elongation	72°C	60 sec	
73°C	10 min			
4°C	∞			

Primers were designed to bind to the coding region of the genes. The oligonucleotides used in this study are shown in table 4.

MRS3-5fw	5′- GTA CCA TCA ACT TAT AGC G -3′
MRS3-6rev	5′- ACT TGA TAT ATG GCA CTG G -3′
MRS4-3fw	5′- TGC TCC GCT ACA TTC CC -3′
MRS4-4rev	5′- CAA ATG GGA TAT TCA TGG C -3′
MRS12-1fw	5′- AAT GGG AAG AGG ATG CC -3′
MRS12-2rev	5′- ACT GTT CTC ATC AAA TGC G -3′
ORT1-1fw	5′- GCA TCC AAC GTG TTC CC -3′
ORT1-2rev	5′- CCA AAT ATC TTC TTC ACC G -3′
SSB1-1fw	5′- TTT CCA AGG TGC TAT CGG -3′
SSB1-2rev	5′- TTT AGA AGA CAA GAC TGG G-3′

Table 4: PCR primers used in this study

3. Results

Motivation

It is still not fully understood how iron passes the inner mitochondrial membrane. In 1999, Lange et al. published experimental data about the molecular mechanism of iron import.

To date, the respective transporter is still unidentified. Two transporters, *MRS3* and *MRS4*, show iron transport activity, yet only under iron limiting conditions. When iron is not restricted, another transporter has to accomplish this function. The present work is aimed at identifying novel iron transporters of the inner mitochondrial membrane.

We decided that a genetic screen would be the best approach to find novel candidates. A multi-copy suppressor screen of an *mrs3/4*Δ mutant had several advantages over a screen for synthetic lethal mutations. I was familiar with three growth phenotypes of an *mrs3/4*Δ mutant and possessed a genomic library of a yeast strain (obtained from *Kim Nasmyth*). Furthermore, a multi-copy suppressor screen would yield results faster than a screen for synthetic lethal mutations.

However, this method has a major weakness. Although the chance to reveal novel interesting proteins is high, potential transporters might remain undiscovered, because their affinity to iron might be too low or their over-expression might inhibit cell growth.

3.1 Screening for new putative iron transporters

First of all, I had to figure out the right conditions for the screen. On this account, many pilot tests had to be carried out. The pilot experiments are discussed below. Subsequently the screen is explained in detail, and the results are shown.

3.1.1 Pilot experiments

Experiments were carried out regarding the following questions: Which strain would be proper for the multi-copy suppressor screen? Which vector would be the most suitable backbone to carry the inserts of the genomic library? Which phenotype(s) could be utilized to search for suppressors? Because I used a growth phenotype on special media in this work, another question had to be answered: How much of chemicals have to be added to the media and how much of DNA has to be transformed to achieve an optimal number of transformants?

In earlier studies investigating *MRS3* and *MRS4*, the *Saccharomyces cerevisiae* strain DBY747 was used. The genomic library (*Kim Nasmyth*) had been created in the vector system YEplac181, which carries a leucine marker. However, it was observed that *mrs3/4*Δ strains show a growth defect on leucine - deficient synthetic media, even when transformed with a plasmid expressing *LEU2*, which complements *leu2*Δ of the yeast strains used. Very likely, deletion of *MRS3/4* is associated with functional impairment of *LEU1*, which is a cytosolic Fe/S protein. As mentioned in the introduction, *MRS3/4* deletion has a strong influence on the formation of Fe/S clusters. My main concern was that reduced growth of *mrs3/4*Δ strains on synthetic media lacking leucine could lead to a bias towards *MRS3* and *MRS4* in the screen, because both genes not only complement reduced growth on iron limiting media, but also reduced growth on leucine selective plates. Detection of suppressors would then be hampered because their growth might be reduced on leucine selective media, compared to *MRS3* and *MRS4*. It was observed earlier that the this growth difference between wild-type and isogenic *mrs3/4*Δ strain on –leu is less pronounced in other strains than DBY747, like W303. Thus, it might be beneficial to work with W303 instead of DBY747, or to use another vector system with a different selective marker, which of course would require to create a new genomic library. To decide whether a switch to an alternative strain or to another vector system was necessary, I carried out a simple growth test. DBY747 and W303 wild type and *mrs3/4*Δ strains were transformed with the empty plasmids YEplac181 or YEp352, allowing either growth on lecuine- or uracil- selective media. *mrs3/4*Δ strains were additionally transformed with the single copy vectors pLH3 (*MRS3* in YCplac111) and pLH4 (*MRS4* in YCplac111).

Complementation assays were carried out on leucine- and uracil- selective media and on relevant "screening media". Growth behaviour was observed after 3 days.

The results are presented in figure 3.

GW403 (DBY747 *mrs3/4*Δ) showed reduced growth not only on leucine - selective media, but also on uracil - selective media. W303 wild-type and GW400 (W303 *mrs3/4*Δ) strains exhibited a more similar growth on selective media. In addition, growth difference was clearly visible between wild-type and mutant strain on BPS- and cadmium- containing media, which was essential for the following screen. For these reasons, W303 was used with the original library from Kim Nasmyth (*LEU* marker).

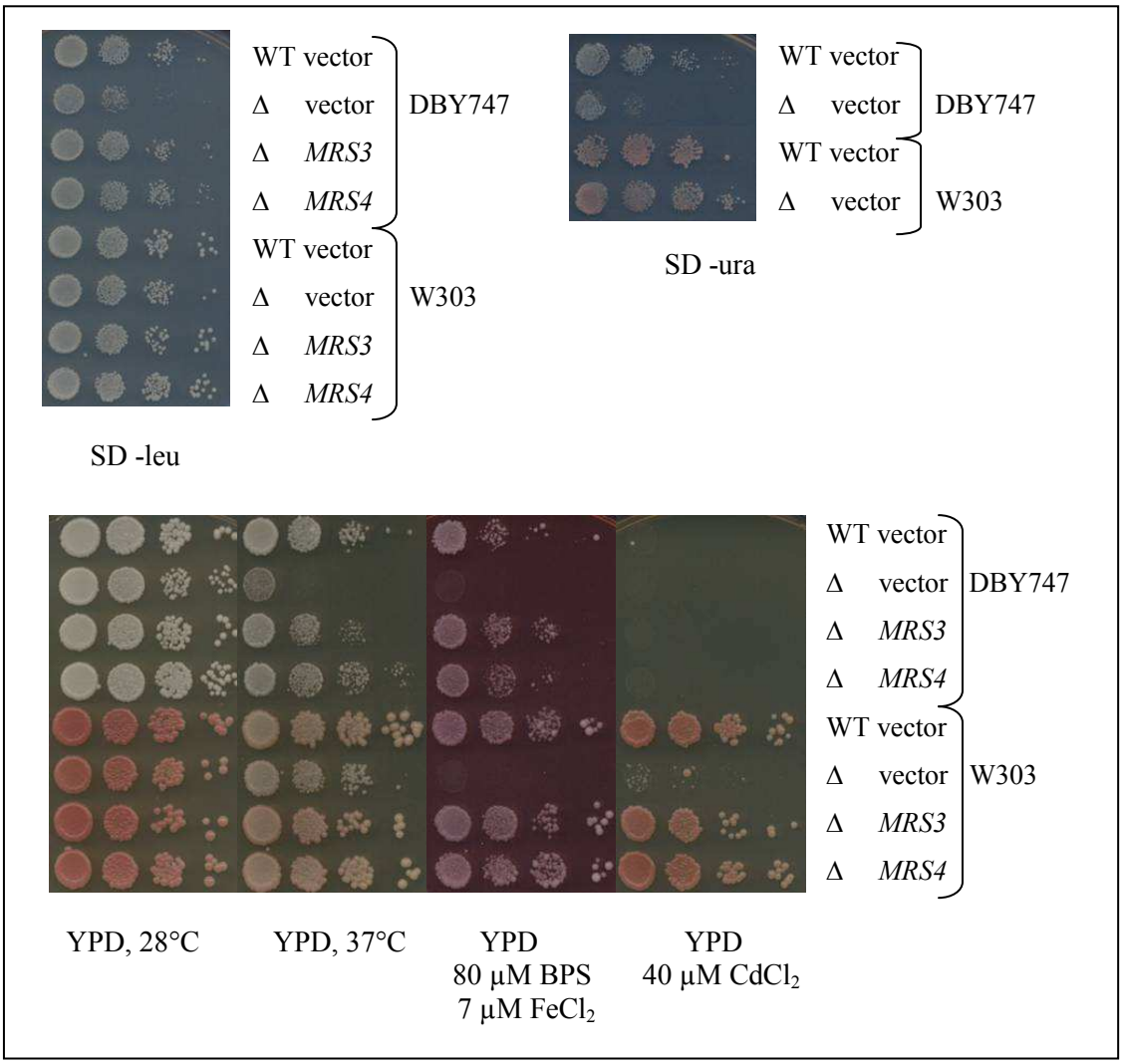
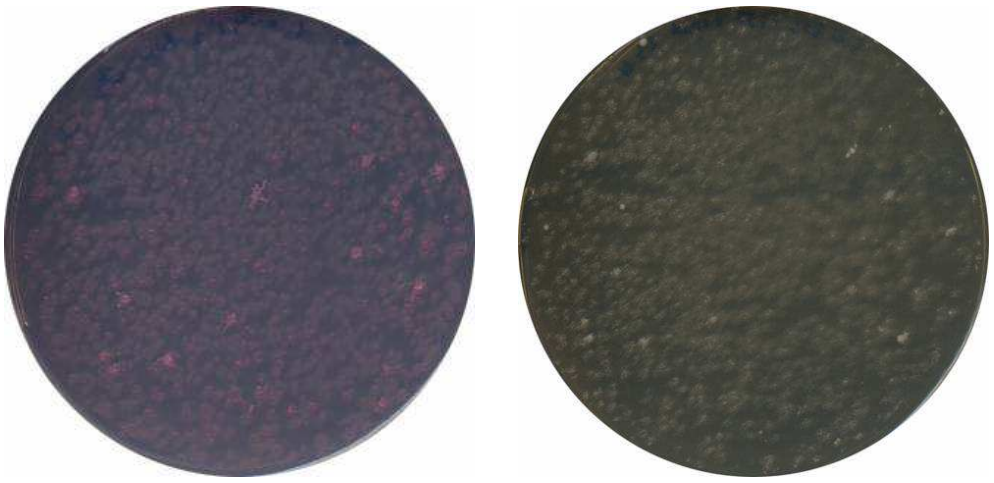


Figure 3: W303 is a more convenient strain for the screen than DBY747.
DBY747 and W303 wild-type and *mrs3/4*Δ strains were transformed with the "empty" plasmids YEplac181 (*LEU2*) or YEp352 (*URA3*), or with YEplac181 over-expressing either *MRS3* or *MRS4*.
"W303 Δ": GW403 (DBY747 *mrs3/4*Δ) "DBY747 Δ": GW400 (W303 *mrs3/4*Δ);
"vector": YEplac181

In an earlier suppressor screen, the growth defect of an *mrs3/4*Δ strain at 37°C had been utilized to search for suppressors. In the present work I used the growth defect of an *mrs3/4*Δ strain on iron limiting media and on media containing cadmium. To create iron limiting conditions, I added the iron chelator bathophenanthroline-disulfonate (BPS) to complex (YPD) or synthetic (SD) media, supplemented with $FeCl_2$, if necessary. The optimal amount of BPS and iron supplements, or cadmium, respectively, that had to be added to the media were figured out. In the same test it was possible to determine the optimal amount of DNA for the transformation reaction.

Different volumes of library and "mock library" DNA were transformed into yeast and the transformants were replica-plated on various selective plates, as described in materials and methods (2.13). The results of the experiment are shown in figure 4. The strongest growth difference between suppressors and background cells was seen on media containing 50 µM cadmium or 80 µM BPS + 5 µM $FeCl_2$, respectively.



SD -leu 80 µM BPS/ 5 µM $FeCl_2$ YPD 50 µM $CdCl_2$

Figure 4: The pilot experiment demonstrates that suppressors may be found in media containing 80 µM BPS / 5 µM $FeCl_2$ or 50 µM $CdCl_2$, respectively.

3.1.2 Multi-copy suppressor screen using the "wild-type" genomic library (Kim Nasmyth)

GW400 was transformed with 1.5 µl of the yeast genomic library. 16 transformation reactions were performed and each cell suspension was plated onto a 146 mm Petri dish. Transformation of the library yielded about 37 000 colonies per plate. Transformants were replica-plated on iron - deficient media. Approximately 100 promising colonies were streaked out on iron - deficient media to obtain single colonies. Plasmids were isolated from yeast cells and amplified in *E. coli*.
Primers were designed to identify clones containing *MRS3* or *MRS4*. PCR was carried out on whole cells as explained in materials and methods (2.14), and clones expressing *MRS3* or *MRS4* were discarded. The remaining clones were digested with EcoRI and HindIII, each enzyme cutting at one side of the insert and maybe fragmenting the insert. The restriction patterns of the inserts were compared.
From clones sharing similar restriction patterns, only one clone was sequenced. All clones were retransformed before sequencing to confirm that the suppression was due to the plasmid and not due to a random mutation or reversion in the yeast strain.

A gene which appeared very often in the screen was identified as *MRS12 (RIM2),* a transporter of pyrimidine nucleotides in yeast mitochondria. Additional primers were designed which allowed exclusion of clones containing *MRS12*. All further suppressors isolated were identified as *MRS12* or *MRS3* in the subsequent PCRs, so the screen was stopped. A summary of the results is shown in figure 5.

Most candidates that were sequenced turned out to be *MRS12* or *MRS3*. Only one of the isolated inserts contained another DNA fragment than *MRS12* or *MRS3* and showed the ability to suppress the phenotype. This fragment was matching a region downstream of the 21S rRNA (mitochondrial DNA), and was not further investigated.

MRS4 was never found in the screen. It is unlikely that the library did not contain *MRS4*. I rather assume that *MRS4* did not show up, because over-expression doesn't lead to a growth advantage under the conditions used in the screen. In a former screen (performed by *Liz Heaver*), only *MRS4* and not *MRS3* was found. It is very likely that only one of both genes can be found in a screen. Even though both genes can complement the phenotype of the double mutant alone, their expression levels in vivo might differ. If one gene is expressed adequately in a screen, probably the other gene is expressed too weak to show complementation, or too strong, leading to reduced growth.

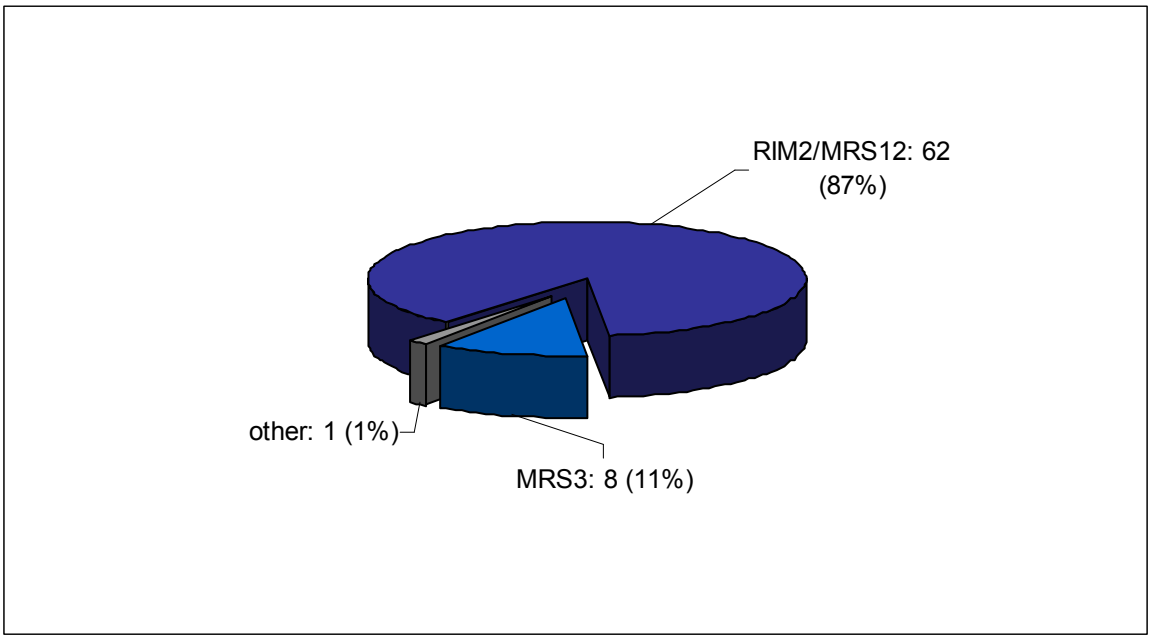


Figure 5: Most candidates found in the screen with the Nasmyth library account for *MRS12*. "other" refers to the fragment of mitochondrial DNA, which was found.

In a complementation assay (described in materials and methods; 2.12), some of the clones were compared to cells over-expressing *MRS3*, *MRS4* or *MRS12* on media containing BPS or cadmium (figure 6). Clones containing *RIM/MRS12* can suppress the growth phenotypes of the *mrs3/4*Δ strain very well, as is demonstrated in figure 6.
I assume that the screen was too stringent to detect weaker suppressors. For this reason, I decided to perform another screen under less stringent conditions.
In the pilot tests, where I tried to figure out the optimal concentration of chemicals, I stopped as soon as I located cells with a growth advantage over the "cell background".
To find conditions favouring growth of weaker suppressors and to avoid isolation of *MRS3*, *MRS4* and *MRS12*, I created a genomic library that contained none of these genes.

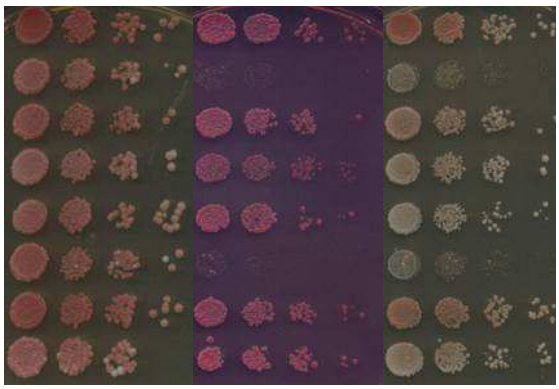


Figure 6: Isolated clones expressing *MRS12* or *MRS3* can suppress the phenotype.
A clone containing *MRS3*, a clone containing *MRS12* and a clone that did not suppress after retransformation were transformed into the *mrs3/4*Δ strain. Strains over-expressing *MRS3*, *MRS4* or *MRS12* in YEplac181 were used as controls.
"WT": W303; "Δ": GW400 (W303 *mrs3/4*Δ); "vector": YEplac181

3.1.3 Growth of the *mrs3/4*Δ and *mrs3/4/12*Δ strains and the synthetic phenotype of the *mrs3/4/12*Δ strain.

The isolation of genomic DNA from an *mrs3/4/12*Δ strain for the purpose of creating a genomic library was a challenge, because the yield of genomic DNA was generally low. To increase DNA yield, it was required that cells were harvested in a logarithmic phase, that's why I plotted a growth chart for the *mrs3/4/12*Δ strain. Because this modified yeast strain shows a quite interesting nature (including a synthetic phenotype), the growth chart is demonstrated below. Yeast strains lacking *MRS12* loose their mitochondrial genome (Van Dyck et al, 1995).

In the growth chart, strains lacking *MRS12* were compared to several rho^+ and rho^0 strains. Yeast strains W303, W303 *mrs3/4Δ*, W303 ρ^0, W303 *mrs3/4Δ* ρ^0, W303 *mrs12Δ* and W303 *mrs3/4/12Δ* were incubated overnight in YPD or YPG liquid media and diluted to an optical density (600nm) of 0.150. Cells were incubated under vigorous shaking at 28°C. Growth behaviour was determined by measuring the optical density every 2 hours for at least 16 hours.

The results of this growth test are shown in figures 7 and 8. The wild-type strain showed a characteristic growth, including the diauxic shift. The W303 *mrs3/4*Δ strain showed a long lag phase at the beginning, compared to the wild-type strain. Rho^0 strains showed a long lag - phase and no diauxic shift. This was expected because the mitochondrial genome contains proteins that are important for respiration.

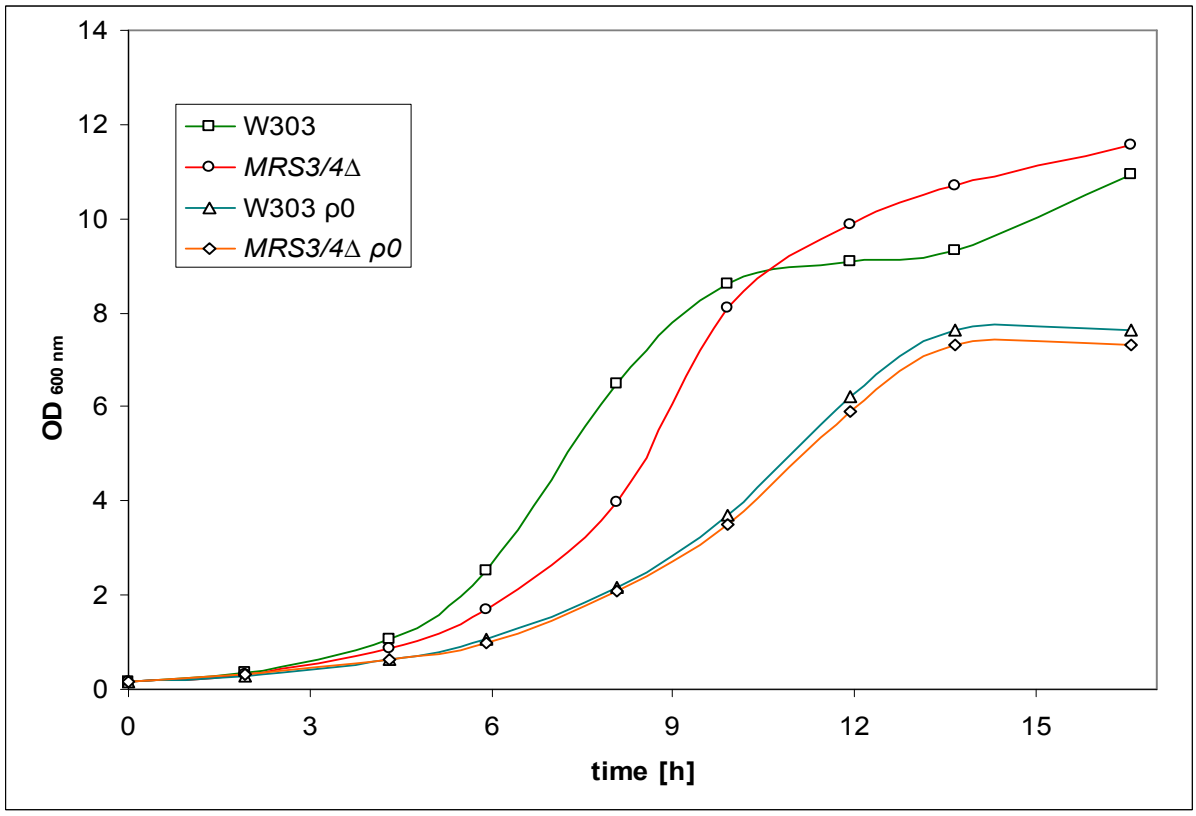


Figure 7: Growth chart of W303 wt, W303 *mrs3/4*Δ, W303 ρ^0 and W303 *mrs3/4*Δ ρ^0 strains in fermentable substrate (YPD).

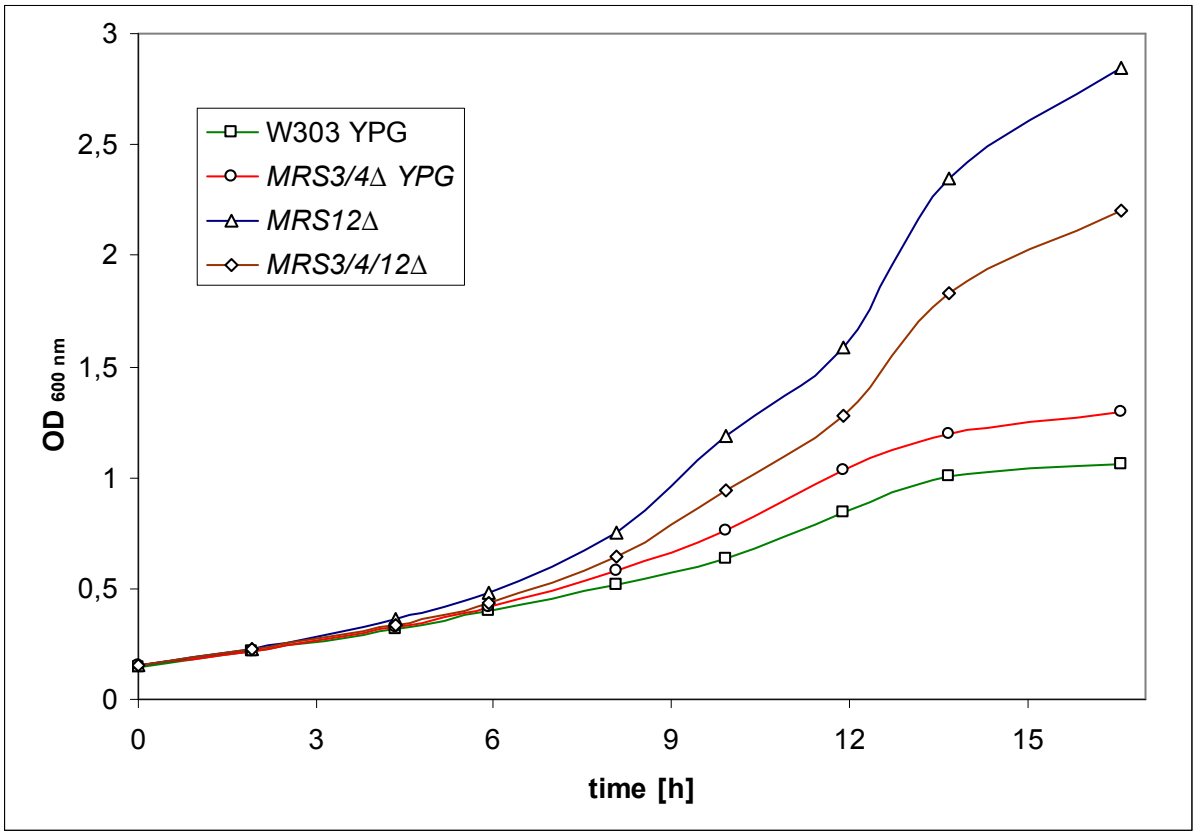


Figure 8: Growth chart of W303 *mrs12*Δ and W303 *mrs3/4/12*Δ in YPD and of W303 wt and W303 *mrs3/4*Δ strains in non-fermentable substrate (YPG).

As it was already mentioned, strains lacking *MRS12* loose their mitochondrial genome. In the growth chart, the *mrs12Δ* strain exhibited a very slow growth and only reached an optical density (600nm) of 2.0. The *mrs3/4/12Δ* strain showed similar, yet even worse growth. I therefore assume that there is a synthetic phenotype when *MRS3* and *MRS4* are deleted and *MRS12* is additionally deleted. The term "synthetic phenotype" is used whenever mutations in two (or more) genes lead to a significant phenotype which does not appear when the genes are mutated alone. The synthetic phenotype can be observed in the growth chart, but is even more obvious on plates, as is demonstrated in figure 9.

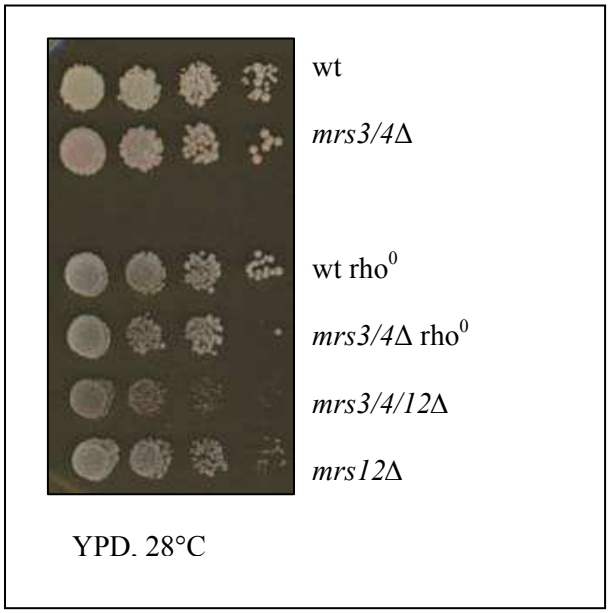


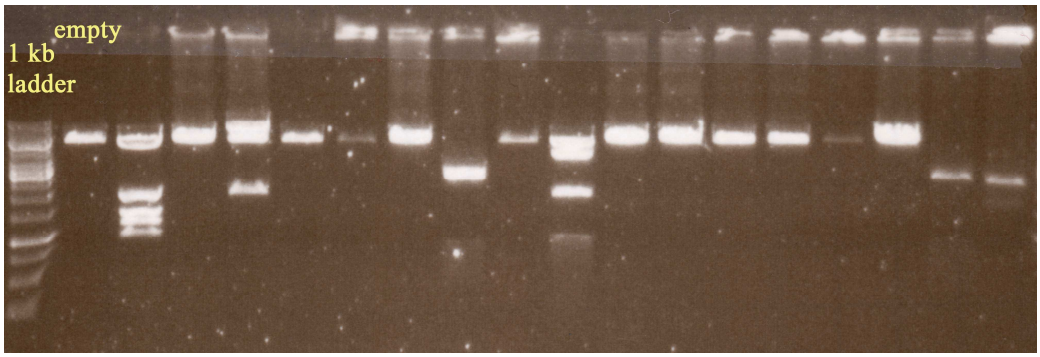
Figure 9: An *mrs3/4/12*Δ strain shows a synthetic phenotype. Several dilutions of W303 wild-type, *mrs3/4*Δ rho^0, *mrs3/4/12*Δ and *mrs12*Δ strains were plated on YPG media.

3.1.4 Multi-copy suppressor screen with a genomic library isolated from an *mrs3/4/12*Δ strain

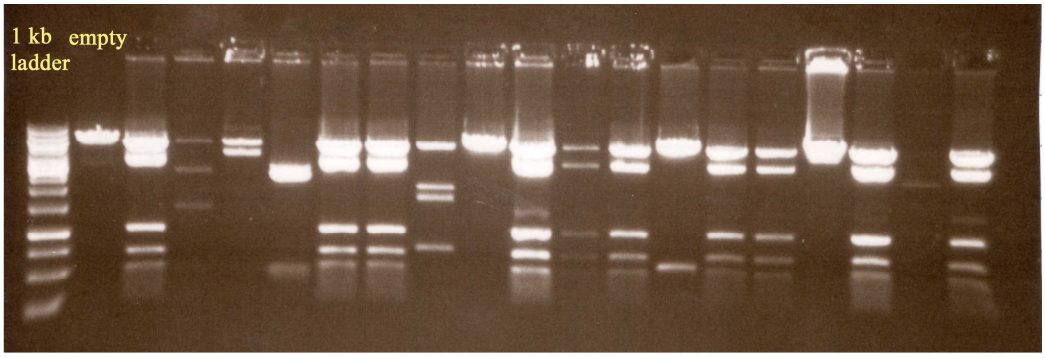
Creation of the genomic library:

When I created the new genomic library, I had to get over many obstacles. First, I encountered some difficulties I had not expected previously, like insufficient yield of genomic DNA from the *mrs3/4/12*Δ strain, exonuclease digestion / breaking of DNA fragments when eluted from the TEAE cellulose, and low transformation efficiency of chemically competent cells (before electrocompetent cells were used). Second, many steps required pilot experiments which also consumed much DNA, like determining the best amount of Sau3A for partial digestion, the best concentration of phosphatase for vector dephosphorylation and the optimal ratio of vector and insert for the ligation reaction. Third, the quality of the finished library often showed was not sufficient.

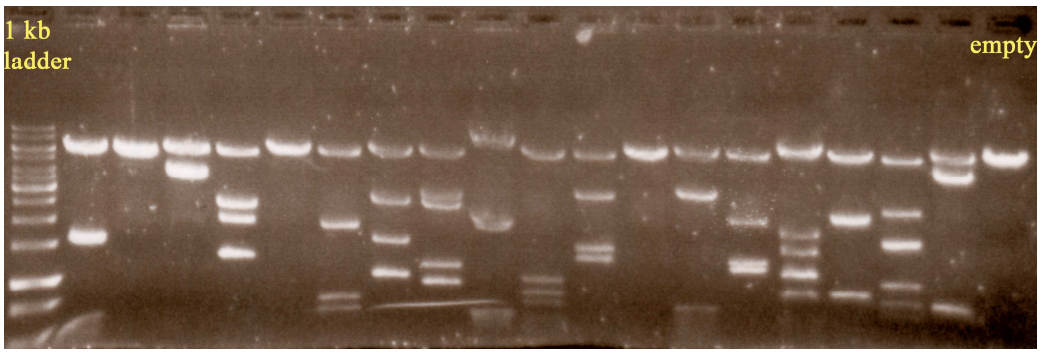
To prove the reliability and significance of this screen, a quality assays for each created library was indispensable. Because of the encountered difficulties, three libraries had to be created. A section of each quality assay is depicted below (Figure 10).
In the first library created, the transformation yielded about 200 000 colonies, but only a few clones contained inserts. From 24 clones analyzed, only 4 (16,6%) contained heterogeneous inserts. All remaining clones did not contain any insert (religated vectors) or were too short (assumed contamination with another plasmid). In the second library, transformation yielded about 80 000 colonies. Most of the clones analyzed carried one and the same insert. Only 11 of 50 clones (22% of clones) showed a different restriction pattern. In addition, I found out that several clones contained *MRS4* or *MRS12* – the library was obviously contaminated with these genes. It was required that I created a third genomic library. Fortunately I achieved a better result this time. Again about 80 000 colonies were washed from the plates. Almost all clones contained a different insert. 48 of 57 clones analyzed showed a different restriction pattern (84%).



The first *mrs3/4/12*Δ library shows a high proportion of vector religation.



One clone is over-represented in the second *mrs3/4/12*Δ library which is further contaminated with *MRS3* and *MRS4*.



The third *mrs3/4/12*Δ library contains many different clones.

Figure 10: Section of the control assays of all created genomic libraries.
Each newly created genomic library was transformed into *E. coli*. At least 24 colonies were incubated overnight in LB_{amp} at 37 °C. Plasmids were isolated and 2 µl of DNA were cleaved with EcoRI and HindIII, thereby releasing the insert from the vector. DNA was run on 0.7% agarose. Library clones were compared concerning frequency and diversity of inserts.
YEplac181 was similarly cut and used as control (designated ”empty”).

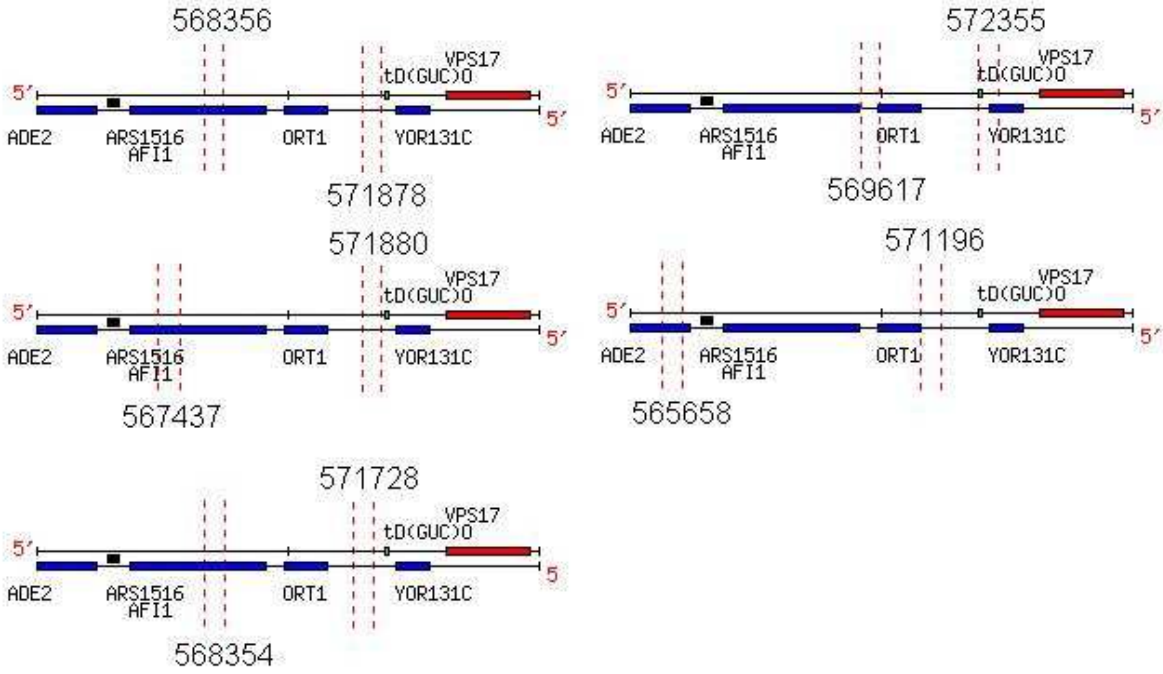
Results of the screen:

A screen was carried out with the first library as well as with the third library. Like in the prior screen, conditions for replica-plating had to be determined in a pilot experiment. This time, it was appropriate to define less stringent conditions to allow growth of weaker suppressors. Again, two steps of replica-plating were necessary for selection on iron-limiting media. Curiously, in the pilot experiment it turned out that cells had to be replica plated on iron deficient SD media first and on iron deficient YPD media afterwards. No colony growth was observed when cells were plated from complex media onto selective media (which was the order in the screen with the "*Nasmyth* library"). Because cells from the second replica plating step were taken for single-colony streak-outs (also on YPD/BPS), many clones lost their plasmids and I was only able to analyze very few.

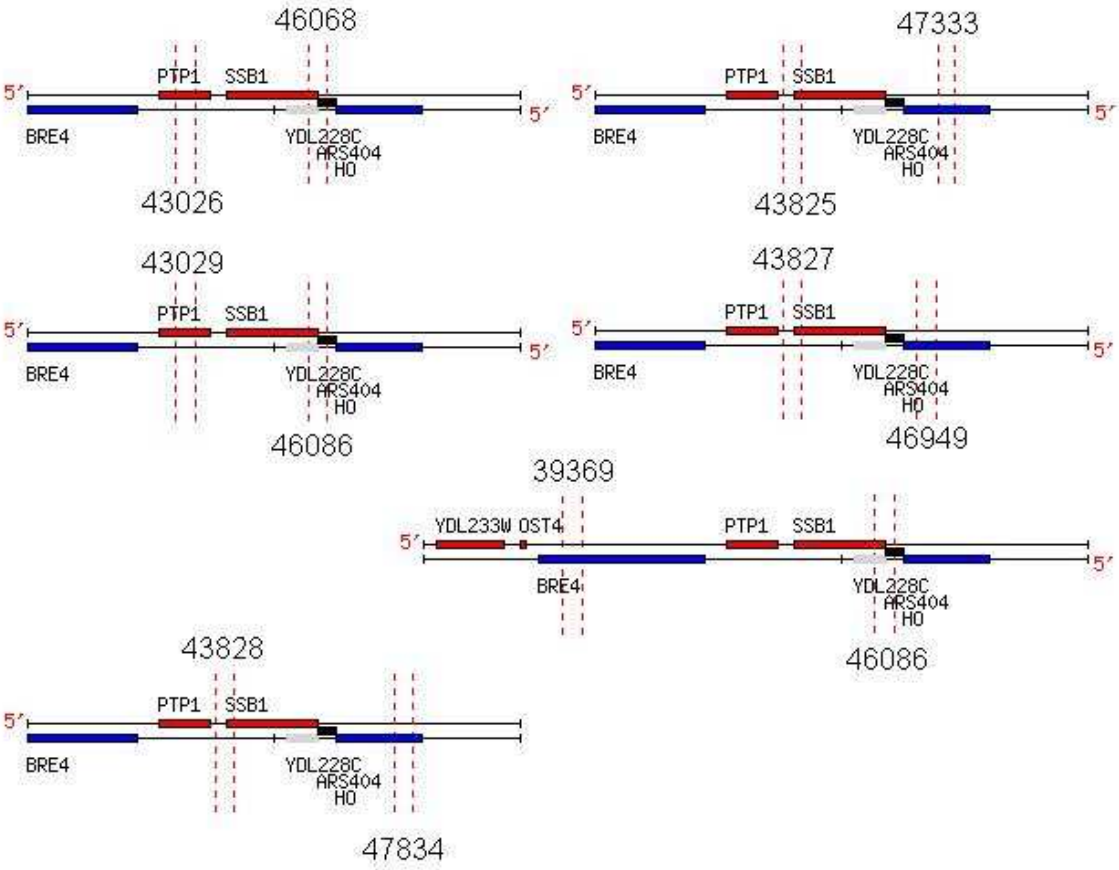
m*rs3/4*Δ cells were transformed with the first or the third library and incubated for 3 days. Transformation of the first library yielded about 100 000 transformants; in the screen with the third library even 400 000 of transformants were counted. Transformants were replica-plated on SD -leu selective media containing 80 µM of BPS and 5 µM of $FeCl_2$. After 2 days, the plates were replica plated on YPD media containing 100 µM BPS (without iron supplements). Colonies showed up after 3 days. The transformants of the third library were also replica-plated on YPD media containing 40 µM of cadmium-chloride. Suppressors were streaked out on YPD media containing BPS or cadmium to obtain single colonies. The candidates were treated like in the screen with the "Nasmyth" library, including restriction pattern analysis and PCR to exclude frequently occurring candidates in the proceeding screen as well as retransformation of the clones. All promising clones were sequenced.

The genomic locus matching the sequencing results of my clones was identified by BLAST search. Results of the BLAST search are shown on the next pages. Coordinates noted above the figures represent the first base of the matching region of the forward (M13) sequencing primer, coordinates noted below the figures represent the first base of the matching region of the reverse sequencing primer, respectively. Different isolates matching similar regions in the genome were clustered into groups. Clones of groups A-I were found as suppressors of the iron - dependent phenotype, clones belonging to groups K and L were found as suppressors of the cadmium - dependent phenotype of *mrs3/4*Δ cells, respectively.

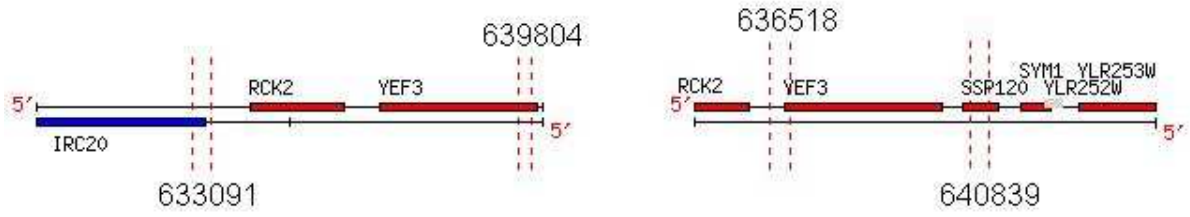
"Group A" – Chromosome XV



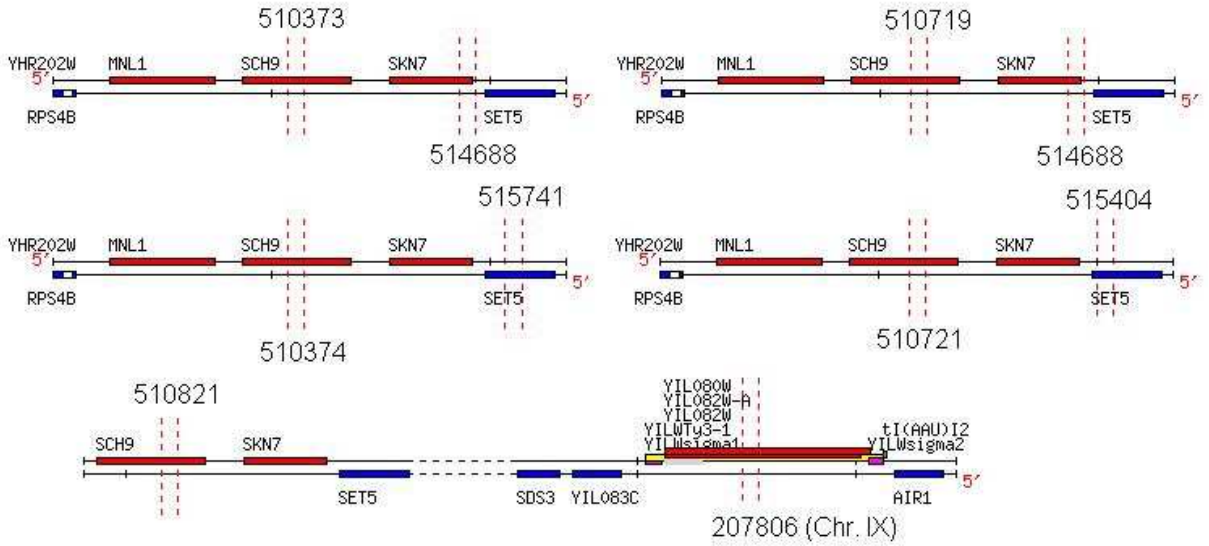
"Group B" – Chromosome IV



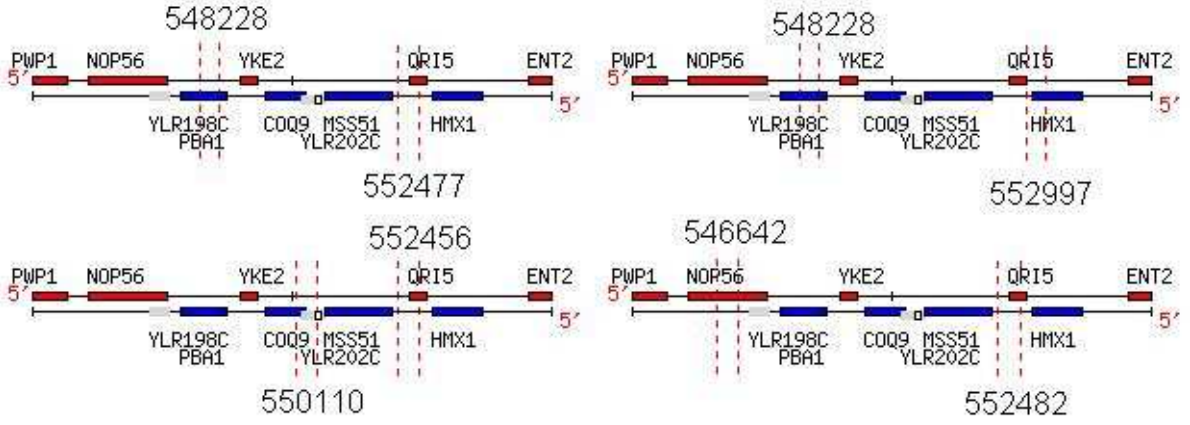
"Group C" – Chromosome XII



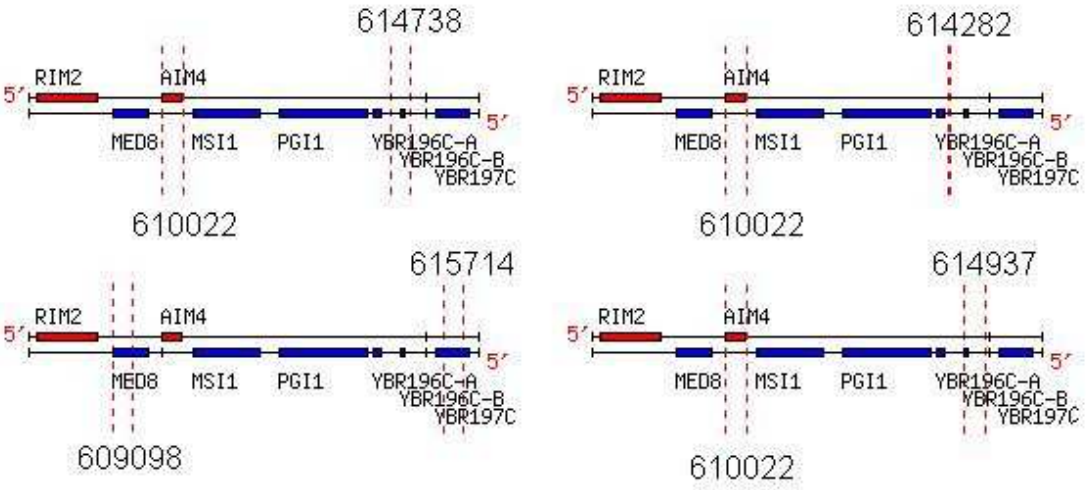
"Group D" – Chromosome VIII



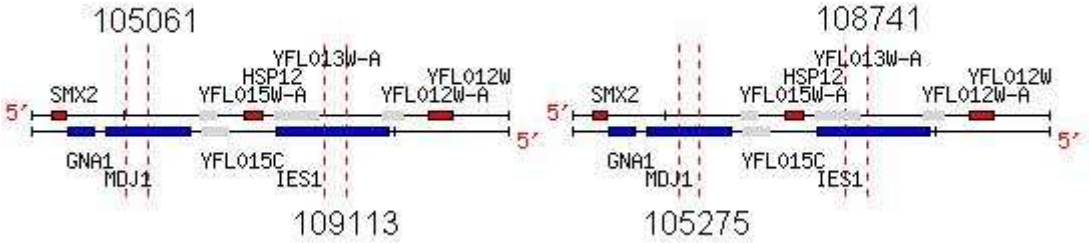
"Group E" – Chromosome XII



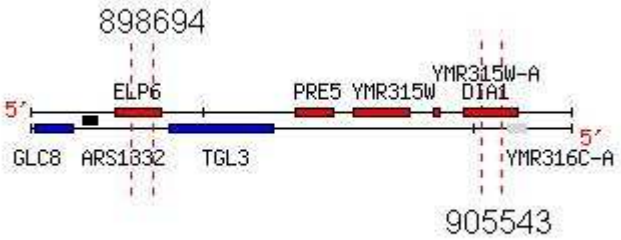
"Group F" – Chromosome II



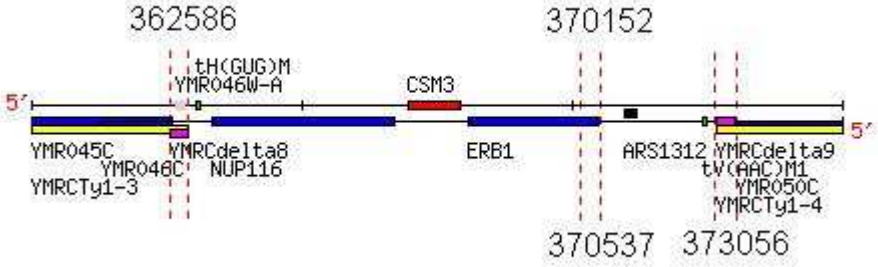
"Group G" – Chromosome VI



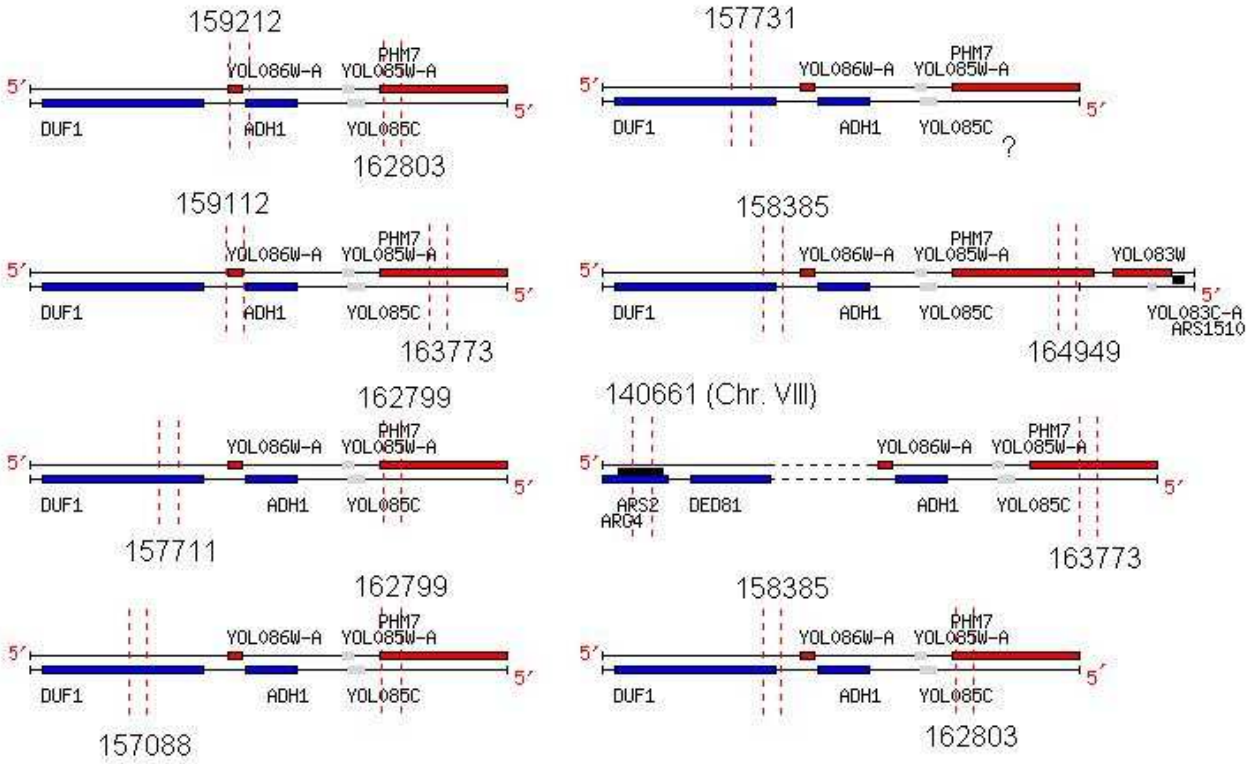
"Group H" – Chromosome XIII



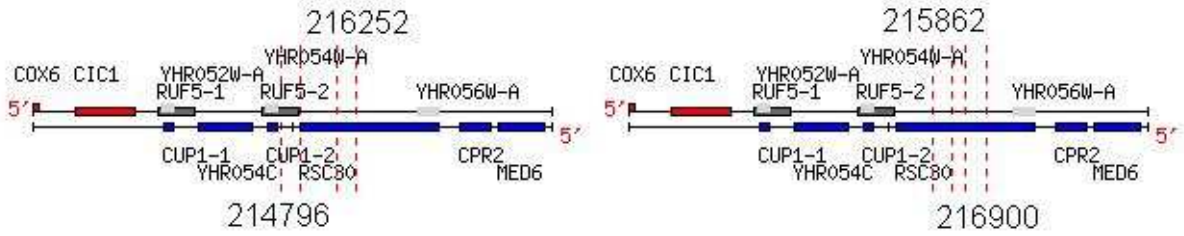
"Group I" – Chromosome XIII



"Group K" – Chromosome XV



"Group L" – Chromosome XIII



For both groups A and B, additional 5 clones were found by PCR (not sequenced).
In the end, I had analyzed 38 clones from iron deficient plates and 10 clones from the plates containing cadmium. It is a good sign that similar clones were found in both genomic libraries. Additionally, it seems that all clones are different, arguing for a good quality of the libraries and disproving the argument that some clones are over-represented. Only in rare cases some clones share one and the same sequence at one end, but this is not surprising as the ends are determined by Sau3A restriction sites. I was able to exclude certain genes which did not occur on all isolated fragments. Of course only genes separated by a Sau3A restriction site can be skipped this way. Subcloning of the fragments was not carried out, so it is not possible to predict which gene would be responsible for the suppression. The results for both libraries used are illustrated in figure 11. Common features of the genes found on the fragments are summarized in table 5.

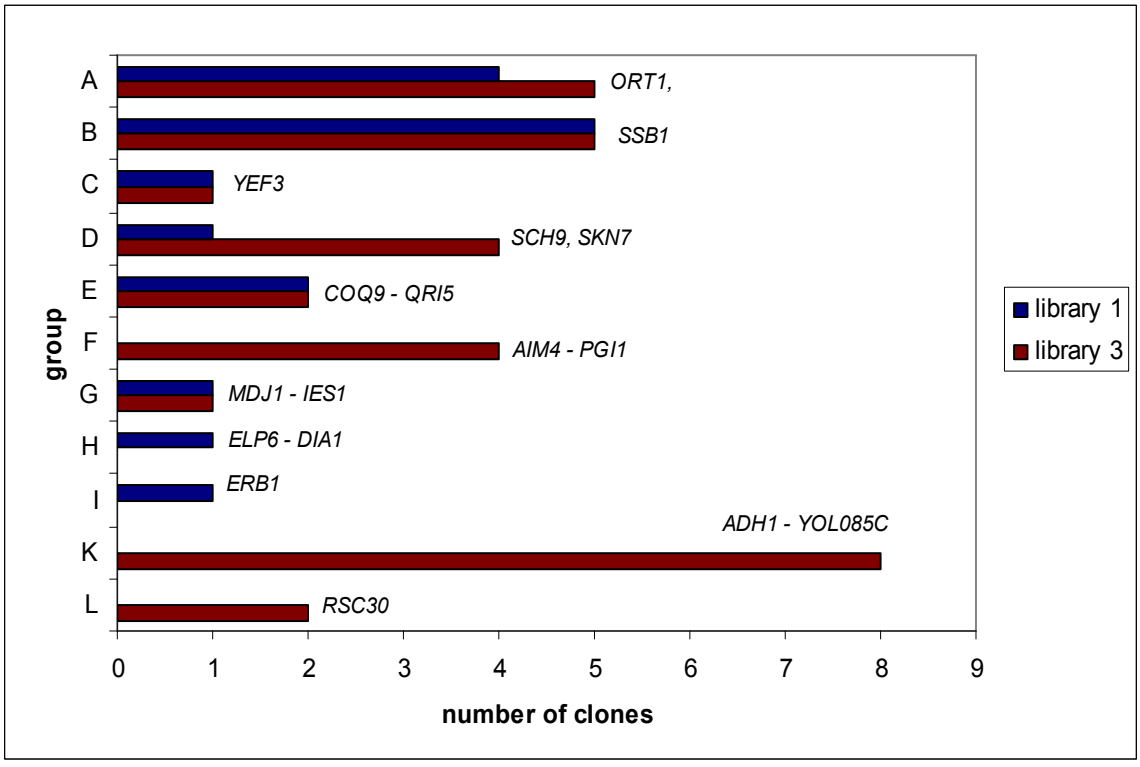


Figure 11: Different suppressors were found in the *mrs3/4/12*Δ libraries.
Results from both libraries used are presented. Please note that similar clones were found with both libraries.
Group A-I: isolated from iron deplete media
Group K and L: isolated from media containing cadmium

Gene	Description	molecular function	biological process	Cellular component
ORT1	ornithine transporter	L-ornithine transmembrane transporter activity	Arginine biosynthetic process / mitochondrial ornithine transport	mitochondrial envelope
SSB1	stress-seventy subfamily B	ATPase activity / unfolded protein binding	cotranslational protein folding, translation, translation fidelity, translational termination	Polysome / soluble fraction
YEF3	yeast elongation factor	translation elongation factor activity	Ribosome biogenesis / translational elongation	Ribosome
SCH9	Protein kinase; activation of osmostress-responsive genes	protein serine/threonine kinase	response to oxidative and osmotic stress / amino acid phosphorylation	cytoplasm / nucleus / vacuolar membrane
SKN7	suppressor of Kre Null (nuclear response regulator)	transcription factor activity / two component response regulator	response to oxidative stress, osmotic stress / regulation of cell size / transcription	Nucleus
COQ9	required for coenzyme Q biosynthesis	unknown	Respiration / ubiquinone biosynthetic process	mitochondrial inner membrane
MSS51	mitochondrial splicing suppressor (req. for translation of *COX1* mRNA)	unknown	positive regulation of translation	mitochondrial inner membrane / mitochondrial matrix
QRI5	required for accumulation of spliced *COX1* mRNA	unknown	mRNA processing	mitochondrial inner membrane
AIM4	altered inheritance rate of mitochondria	unknown	Unknown	cytoplasm
MSI1	multicopy suppressor of *IRA1* (subunit of *CAF-1*)	transcription regulator activity	Chromatin silencing / DNA repair / nucleosome assembly / signal transduction	*CAF*-1 complex / chromatin silencing complex
PGI1	glycolytic enzyme phosphoglucose-isomerase	glucose-6-phosphate isomerase activity	Glycolysis / gluconeogenesis / pentose-phosphate shunt	cytosol / mitochondrion
MDJ1	mitochondrial DnaJ (Co-chaperone, stimulates ATPase activity of Ssc1p	ATPase activator activity / Hsp70 protein regulator activity /	“de novo” protein folding / misfolded protein catabolism / mito. genome maintenence	mitochondrial matrix
IES1	subunit of the INO80 chromatin remod. complex	unknown	chromatin remodelling	INO80 complex / nucleus

ELP6	subunit of elongator complex; modification of wobble nucleosides in tRNA	unknown	protein urymlation / tRNA modification	Elongator holoenzyme complex
TGL3	triacylglycerol lipase of the lipid particle	triacylglycerol lipase activity	lipid metabolic process / triacylglycerol catabolic process	lipid particle
PRE5	proteinase yscE (alpha 6 subunit of the 20S proteasome)	endopeptidase activity	ubiquitin-dependent protein catabolic process	proteasome core complex / proteasome storage granule
DIA1	"digs into agar" (involved in invasive and pseudohyphal growth)	unknown	invasive growth in response to glucose limitation	cytoplasm
ERB1	required for maturation of the 25S and 5.8S ribosomal RNAs	unknown	rRNA processing	nucleus / nucleolus / preribosome
ADH1	alcohole dehydrogenase	alcohol dehydrogenase activity	ethanol biosynthesis / fermentation / NADH oxidation	Cytosol
RUF5-2	RNA of unknown function	unknown	unknown	Unknown
RSC30	"remodel the structure of chromatin" (component of the RSC chromatin remodelling complex)	DNA binding / contributes to DNA-dependent ATPase activity	ATP-dependent chromatin remodelling / double-strand break repair via NHEJ	RSC complex

Table 5: Common features of the genes found on the DNA fragments.
Information was obtained from the "Saccharomyces Genome Database" *(http://www.yeastgenome.org)*.

Comparison of the clones:

To compare the isolated fragments regarding their competence to suppress the phenotype, one candidate of each group was retransformed into yeast and spotted on iron-deficient or cadmium-containing media. The results of this retransformation assay are shown in figure 12. The growth test demonstrates that all isolated genomic fragments harbor a weak suppressor. All cells showed better growth than the *mrs3/4*Δ strain (negative control). The suppressing gene of "group E" or another gene on that fragment also mediates resistance to cadmium.

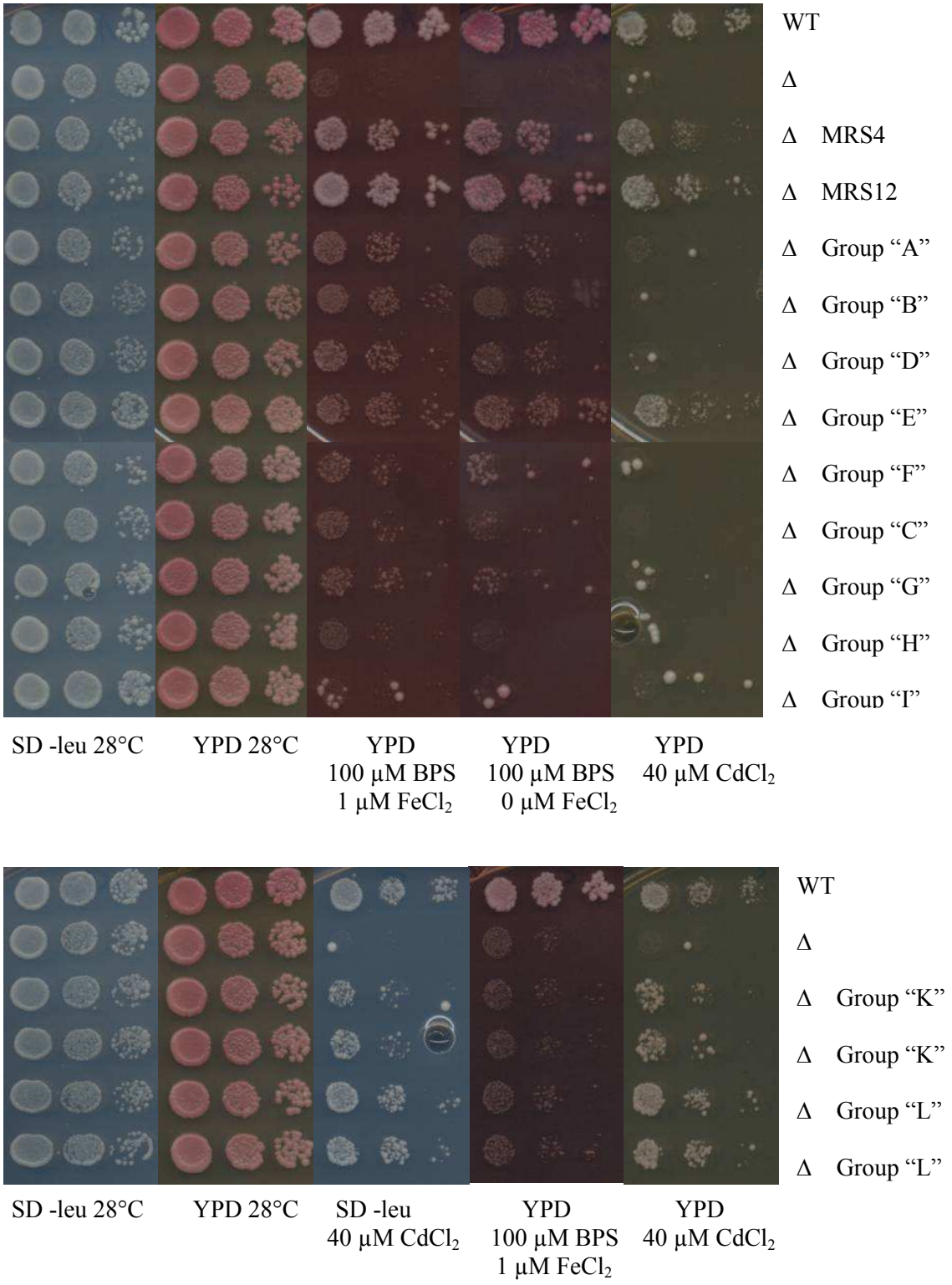


Fig. 11: Comparison of the clones found in the *mrs3/4/12*Δ library. W303 wild-type and *mrs3/4*Δ strains were transformed with emtpy vector or one clone of each group. Transformants were spotted on media containing BPS or cadmium. Assays with suppressors of the iron-related phenotype are depicted above. Below, suppressors of the cadmium-related phenotpye are shown.
"WT": W303; "Δ": GW400 (W303 mrs3/4Δ)

One gene which was found very often, *ORT1*, was particularly interesting. *ORT1* is the ornithine transporter of the inner mitochondrial membrane (Palmieri et al., 1997). *MRS12* is also a transporter of the inner mitochondrial membrane. Because I additionally found *ORT1*, one could imagine that every mitochondrial transporter is able to suppress the *mrs3/4*Δ phenotype to a low degree. To adress this question, *ORT1* and other transporters were transformed into an *mrs3/4*Δ strain and plated on media containing BPS or cadmium.

GW400 was transformed with different plasmids, containing one of the following mitochondrial transporters: *MRS12*, *MTM1* (a manganese trafficking factor; Luk et al., 2003), *PET8* (transporter for S-adenosylmethionine; Marobbio et al., 2003), *MIR1* and *PIC2* (two mitochondrial phosphate carriers; Schroers et al., 1998) and *ORT1*. The results are presented in figure 12. Aside from *MRS12*, only over-expression of *ORT1* allowed *mrs3/4*Δ cells to grow on iron restricted media. This result demonstrates that most mitochondrial transporters can not suppress the phenotype of an *mrs3/4*Δ mutant strain, but *ORT1* is capable to suppress the phenotype to a low degree.

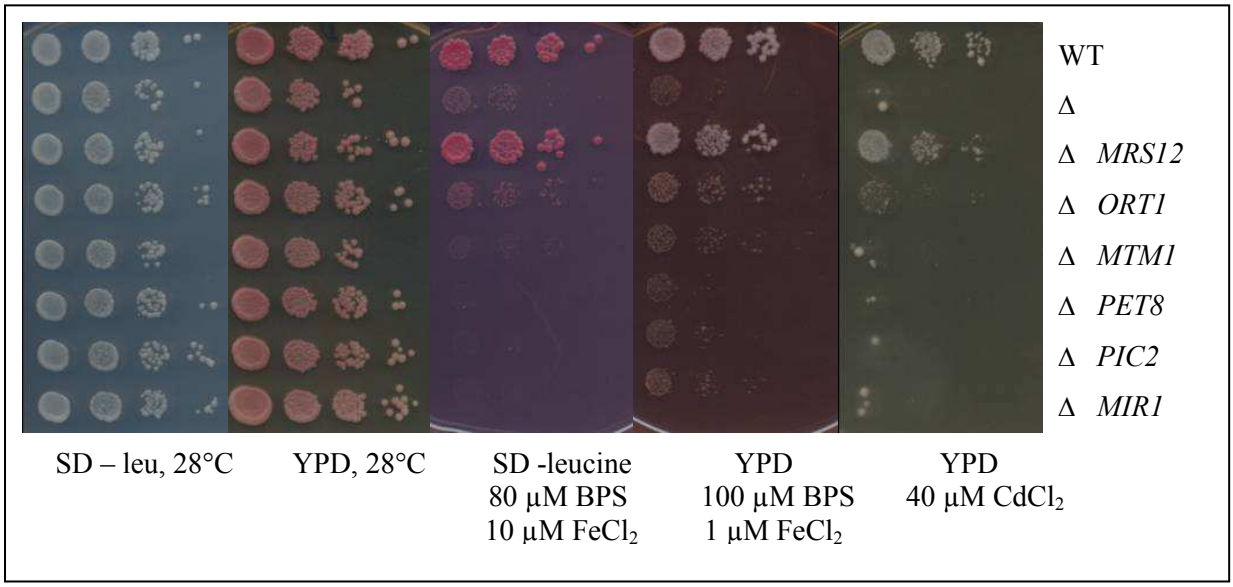


Figure 12: Only *ORT1* and *MRS12* are mitochondrial transporters competent to suppress the phenotype of an *mrs3/4*Δ strain. The suppression mediated by *ORT1* is very weak.
An *mrs3/4*Δ strain was transformed with pMB1 (*MTM1* in YEplac181), pLH1 (*PET1* in YEplac181), pBS1 (*PIC2* in YEplac181), pBS2 (*MIR1* in YEplac181) and a clone from the library containing *ORT1*. Transformants were spotted on media containing BPS or cadmium.
"WT": W303; "Δ": GW400 (W303 mrs3/4Δ)

3.2. Tests to identify the mechansim behind the cadmium-dependent phenotype

One of the phenotypes observable with *mrs3/4*Δ cells is the hypersensitivity to cadmium.

It has been proposed that iron starvation is associated with increased expression of broad specificity metal transporters in the plasma membrane, leading to and uptake of toxic heavy metals (Foury and Roganti, 2002). Details of this mechanism are not yet known.

I investigated whether over-expression of *MRS3*/*4*, on the other hand, leads to increased resistance to cadmium for some reason. For instance, expression of *MRS3*/*4* could lead to induction of genes mediating resistance to heavy metals. By expressing high levels of *MRS3* and *MRS4*, I might obtain additional information about the role of both proteins with respect to heavy metal - resistance. W303 wild-type and *mrs3/4*Δ strains were co-transformed with two multi-copy vectors expressing *MRS3* or *MRS4*, respectively, or with two vectors expressing both genes under the strong ADH promoter. Transformants were plated on media with varying cadmium concentrations. The results of this growth test are presented in figure 13.

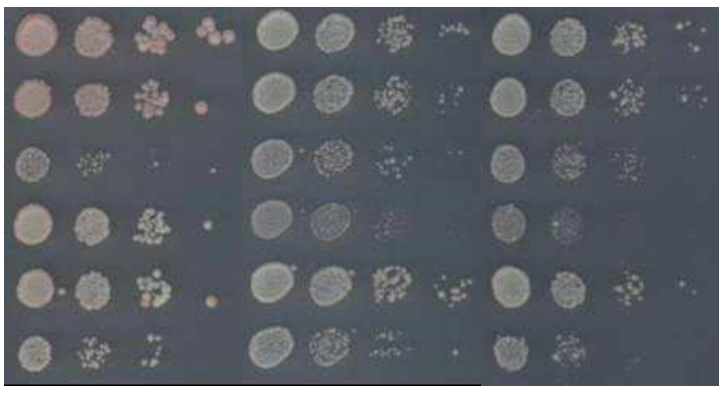


Figure 13: Strong over-expression of *MRS3/4* doesn't mediate resistance to cadmium.
Wild type and *mrs3/4*Δ strains were either co-transformed with empty vectors, with two multi-copy vectors expressing *MRS3* and *MRS4* or with two vectors expressing both genes under the strong ADH promoter.
"WT": W303; "Δ": GW400 (W303 *mrs3/4*Δ);
"*MRS3/4* (YEp)": pGW864 (*MRS3* in YEplac181) + pGW1091 (*MRS4* in pEMBLYe31);
"*MRS3/4* (pADH)": pGW822 (*MRS3* in pAAH5, pADH) + pGW821 (*MRS4* in pVT-U, pADH).

The results demonstrate that strains deleted for both *MRS3* and *MRS4* are very sensitive to cadmium – a phenotype which can be rescued when *MRS3* and *MRS4* are expressed on multi-copy plasmids. Strains over-expressing both genes on multi-copy plasmids do not show a growth advantage compared to the wild-type strain, however.

Strong over-expression under the ADH promoter leads to a growth defect. This reduced growth was observed on all media, not only on media containing cadmium, which points at a general growth defect of cells over-expressing *MRS3/4* under pADH.

According to these results, strong over-expression of *MRS3/4* is toxic to cells, and resistance to cadmium is not increased.

4. Discussion

4.1 *MRS12* was found in a screen with a common genomic library

The first screen revealed that *MRS12* (*RIM2*) is a very good suppressor of the phenotype caused by the deletion of *MRS3* and *MRS4*. It was the only candidate found aside from *MRS3*. *MRS4* was not found, because the conditions of the screen did not allow growth of cells over-expressing *MRS4*. As already mentioned, specific screening conditions might only give rise to colonies over-expressing *MRS3* or *MRS4*, but not both. Suppression of the *mrs3/4*Δ phenotype by over-expressing other members of the *mitochondrial carrier family* had been already investigated earlier (Wiesenberger G., unpublished data). *MRS12* was demonstrated to be a good suppressor of both the iron-related and the cadmium-related phenotype. Yet, it appears that the role of *MRS12* concerning iron homeostasis was underestimated. It was proposed that *MRS12* (*RIM2*) encodes a pyrimidine transporter which imports pyrimidine-nucleoside triphosphates in exchange to pyrimidine-nucleoside monophosphates (Marobbio, Di Noia, Palmieri, 2006). Deletion of *MRS12* results in rho^0 formation. *MRS12* is thus important for transport of pyrimidines and for the maintenance of the mitochondrial genome. Beside its annotated function, the protein is most likely involved in iron transport as well, as it is a good suppressor of the *mrs3/4*Δ phenotype.

Recent studies demonstrate that iron is co-transported with pyrimidines by Rim2p.
In our lab, a method was developed to measure the uptake of metal ions into yeast mitochondria indirectly (because direct measurement of iron uptake is not possible). Isolated mitochondria of the strain of interest were sonicated in presence of special fluorescent dyes to form submitochondrial particles. These particles practically contain all mitochondrial transporters which the strain expresses, in both orientations. The dye is enclosed in the particles ("loading" of the particles). The dye used depends on the ion; in the case of iron it is called "Phen-Green". Phen Green emits light with lower intensity when in complex with iron (quenching of fluorescent dyes).

In wild-type strains and cells over-expressing *MRS3/4*, quenching was detected without addition of nucleotides, which was expected, because uptake of iron into the particles was carried out by Mrs3/4p in this case. In an *mrs3/4*Δ strain over-expressing *MRS12*, addition of either iron or pyrimidines did not lead to quenching, whereas addition of both molecules at the same time resulted in strong quenching, suggesting transport of iron into the particles (Froschauer E., unpublished data). It can therefore be concluded that Rim2p is able to transport iron only in presence of nucleotides. In addition to the *MRS12*-mediated transport of iron, a transport activity for copper and zinc was measured, which also relies on the addition of nucleotides.

It was shown that expression of *FET3*, and other genes of the iron regulon, is up-regulated in an iron-starved *mrs3/4*Δ strain (Mühlenhoff et al., 2003). New experimental data provide evidence that *FET3* is not up-regulated in an *mrs12*Δ strain, but is even stronger induced in an *mrs3/4/12*Δ strain than in an *mrs3/4*Δ strain (Hassler M., diploma work). Two other genes of the iron regulon, *FIT2* and *FIT3*, were demonstrated to be even up-regulated in an *mrs12*Δ strain.
Apparently, contact site II of Rim2p is essential for the transporter to execute its function. It was demonstrated earlier, that contact site II is important for nucleotide carriers (Kunji et al., 2006). In the GTP/GDP carrier and the ATP/ADP carrier, a hydrophobic pocket is formed at contact site II, which interacts with the adenine or guanine ring. No transport of pyrimidine or iron was measured in submitochondrial particles of an *mrs3/4/12*Δ yeast strain expressing *MRS12* with a mutation in contact site II (Froschauer E. and Hassler M., diploma work). Furthermore, this mutant can neither complement the phenotype of an *mrs12*Δ strain nor suppress the phenotype of an *mrs3/4*Δ strain (Hassler M., diploma work). The mutation in contact site II does not lead to the degradation of *MRS12* mRNA or Rim2p proteins, though.

All these data suggest that Rim2p imports iron together with pyrimidines into mitochondria. The negatively charged pyrimidine bases might form a complex with iron(II) before being transported. Pyrimidine import into mitochondria might thus also arrange for iron import by a simple co-transport mechanism when the cation is available in sufficient amounts. In contrast, when iron is limited, a transport system consisting of Mrs3/4p might adopt that task.

4.2 A screen with lower stringency did apparently not reveal interesting candidates.

In this screen, stringency was decreased to find weaker suppressors. A genomic library was constructed from an *mrs3/4/12*Δ yeast strain just for this purpose and allowed the application of less stringent conditions. The second attempt to reveal novel transporters with iron-uptake activity did apparently not reveal interesting candidates. I expected that I would also find genes encoding iron chelators or genes of the iron regulon like *AFT1* or *FET3*. When iron is limited, cellular signaling causes the transcription factor *AFT1* to bind to the promoter region of a number of genes that constitute the *iron regulon*, promoting their expression. The resulting proteins contribute to efficient cellular iron uptake. While exerting their role mainly on the plasma membrane, it is still imaginable that some of these proteins have the potential to support growth of yeast strains with a defect in mitochondrial iron transport. However, I did not find genes of the iron regulon. To test whether genes of the iron regulon support growth of an *mrs3*/*4*Δ strain, these genes could be over-expressed using a proper vector. An *mrs3/4*Δ strain transformed with these constructs should still show reduced growth, comparable to the untransformed strain.

At least 7 clones were found in the screen with less stringent conditons. The isolated fragments contained a number of different genes and I did not perform subcloning to identify the gene responsible for the suppression. For all fragments however, I investigated the annotated gene functions. Unfortunately, no genes found on the isolates were clearly associated with transport function, and I was not able to figure out which gene would probably be responsible for the suppression. I assume that the capability to support growth on low iron is only a side effect of the clones. Perhaps it would be interesting to investigate, how the gene products contribute to groth on low iron. On the other hand, the main goal of this work was to discover new transporters and further experiments with the isolates might not have yielded important information.

4.3 Suppressors found in a screen with lower stringency are most likely not directly involved in iron transport.

The genes that emerged in the screen are virtually not involved in the regulation of metal ions. In addition, analysis of microarray data implicated no important connection between candidates found in this screen.
ORT1 and *SSB1* were found very often on media containing BPS. *ORT1* was interesting to me because it encodes another mitochondrial transporter. The ornithine transporter of the mitochondrial inner membrane exports ornithine from mitochondria and is essential for arginine biosynthesis. Ornithine itself is positively charged in aquaeous solution, so it is unlikely that it is transported together with iron. It is implausible how *ORT1* could manage iron uptake. It's rather conceivable that over-expression of *ORT1* causes metabolic changes, not influencing iron homeostasis but allowing cells to grow upon iron depletion. *SSB1* (Chromosome IV) is a cytoplasmic ATPase that is associated with ribosomes. It has the function of a chaperone and is important for the folding of newly formed polypeptide-chains. I found no explanation why such a gene supports growth on iron deficient media. *IES1* and *MSI1* are involved in chromatin remodelling, a mechanism which is often activated when a cell has to adapt to new conditions. *SKN7*/*SCH9*, two genes involved in stress signalling were found, too. That over-expression of genes involved in chromatin remodelling or stress signalling might enhance growth of cells on iron-restricted media is imaginable.

COQ9 is important for ubiquinone biosynthesis and thus for respiratory growth. On the same fragment, *MSS51* and *QRI5* were found. Both gene products are required for translation and accumulation of *COX1* mRNA. Cox1p is a subunit of cytochrome C oxidase, which is also part of the respiratory chain. Irrespective to the gene that is particularly responsible for the suppression, all genes of this genomic fragment are involved in respiratory growth of yeast, arguing that this process is influenced upon iron starvation. The importance of iron for respiration is well known, because iron is an important component of Fe/S cluster proteins.

Many genes found in the screen are involved in metabolic or catabolic processes. Except for the genes mentioned above, which are required for aerobic respiration, I found *PGI1*, which is essential for glycolysis, *TGL3*, which is an important player in lipid metabolism and *ADH1*, which is important for fermentation.
I can not predict why I found genes involved in metabolism. Maybe a change in metabolism supports growth when iron is limited. I propose that genes isolated in my screen enhance the growth of an *mrs3/4*Δ yeast strain on iron-deficient media by epigenetic changes, stress signalling or metabolic adaptations. However, unless the exact suppressor of each fragment is defined, all predictions are very insecure.

4.4 *MRS3*/*4* over-expression does not increase resistance to cadmium

It has been proposed that iron starvation is associated with increased expression of broad specificity metal transporters in the plasma membrane, leading to an uptake of toxic heavy metals. This explains the hypersensitivity of *mrs3*/*4*Δ strains to cadmium. I additionally hypothesized that over-expression of *MRS3*/*4* could induce genes mediating resistance to heavy metals, or that resistance to heavy metals is directly linked to mitochondrial iron content. In the present work, I demonstrated that over-expression of *MRS3*/*4* does not mediate resistance to cadmium. However, low over-expression of *MRS3*/*4* in multi-copy vectors can complement the hypersensitivity. Strong over-expression of *MRS3*/*4* under the ADH promoter results in a growth defect. It was shown that both deletion and over-expression of MRS3/4 causes induction of genes of the iron regulon (Mühlenhoff et al., 2003). Probably strong over-expression of *MRS3/4* leads therefore to increased expression of the broad specificity metal transporters in the plasma membrane, too. On the plates, a growth defect is also visible on synthetic media not containing cadmium, though. I therefore assume that strong over-expression rather leads to a general growth defect.

4.5 Arguments undermining or confirming the significance of my results

After *MRS12* was found and the stringency was decreased, only very weak suppressors with no apparent function in iron homeostasis were found. In the screen with decreased stringency, only a few candidates were analysed, so it might be possible that the screen did not cover all suppressors. Nearly all discovered genes recurred several times, however, and were detected in separately created libraries, suggesting that no more suppressors can be found. An important precondition for the screen was the creation of a genomic library with a good quality. The third library I created, I obtained about 80 000 transformants. According to the quality assay, vector religation occurred in less than 20% of the clones. All other clones contained different inserts. Because the library was created with Sau3A-fragmented genomic DNA, genes containing multiple Sau3A restriction sites might have been degraded more likely and might thus not be found in the screen. The chance however, that this event occurred exactly in the transporter I was looking for, is very low. I already claimed that potential suppressors might remain undiscovered, because their affinity to iron might be too low or their over-expression might inhibit cell growth. This is the major argument against my screen, preventing a definite statement on whether more mitochondrial transporters with specificity for iron can be found in the yeast genome.

4.6 Conclusion

My studies aimed at the detection of novel or already-known transporters of the inner mitochondrial membrane with so far unknown specificity for iron. Although I found many suppressors, only one candidate shows a well-detectable transport activity for iron. No strong suppressors were found except for *MRS12* and all other suppressors are apparently not involved in iron homeostasis. Despite the concerns remarked above, I dare to state that no mitochondrial transporters with affinity to iron are present in yeast except for Rim2p and Mrs3/4p. Rim2p might co-transport iron with pyrimidines when available in sufficient amounts, whereas Mrs3/4p mediated high-affinity transport is only turned on when iron is short. Ongoing experiments tend to reveal how exactly *MRS12* manages iron import. As soon as this work is done, it would be proper to figure out whether other transporters have similar features.

5. Appendix

5.1 Additional plasmid constructs

Name	**background**	**sel. marker**	**gene**	**promoter**	**creator**
pBS1	Yeplac181 (yeast 2 micron)	*LEU2*	*PIC2*	endogenous	Barbara Sladek
pBS2	Yeplac181 (yeast 2 micron)	*LEU2*	*MIR1*	endogenous	
pLH1	Yeplac181 (yeast 2 micron)	*LEU2*	*PET8*	endogenous	Lizz Heaver
pLH3	YCplac111 (CEN4-ARS1)	*LEU2*	*MRS3*	endogenous	
pLH4	YCplac111 (CEN4-ARS1)	*LEU2*	*MRS4*	endogenous	
pGW821	pVT-U	*URA3*	*MRS4*	ADH	Gerlinde Wiesenberger
pGW822	pAAH5	*LEU2*	*MRS3*	ADH	
pGW864	Yeplac181 (yeast 2 micron)	*LEU2*	*MRS3*	endogenous	
pGW866	Yeplac181 (yeast 2 micron)	*LEU2*	*MRS4*	endogenous	
pGW1091	pEMBLYe31	*URA3*	*MRS4*	endogenous	

5.2 Abbreviations

BPS bathophenanthroline-disulfonate (an iron chelating agent)

TEAE tri-ethyl-aminoethyl (DNA binding compound, covalently bound to cellulose)

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