Lipid – membrane protein interaction in yeast

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KURZBESCHREIBUNG / ABSTRACT

Um die Aktivität der *Sacchraromyces cerevisiae* Sphingomyelinase Isc1p in verschiedenen Membranzusammensetzungen, insbesondere in Gegenwart von unüblichen Sterolen, zu testen, wurden unterschiedliche Varianten dieses integralen Membranproteins in *S. cerevisiae* Stämmen exprimiert und deren Aktivität charakterisiert. Die verwendeten Hefestämme weisen knockouts im Lipidmetabolismus auf, welche wiederum unterschiedlich zusammengesetzte Lipidmembranen bedingen. Um Rückschlüsse auf die zelluläre Lokalisation des Proteins zu erlauben, wurden Isc1p unterschiedliche tags angehängt, das FLAG-tag Epitop zur Immunodetektion auf Western blots und GFP für fluoreszenzmikroskopische Untersuchungen. Außerdem wurde die für Isc1p codierende Nukleotidsequenz mit dem Ziel mutagenisiert, eine umfangreiche Bibliothek an mutierten Sequenzen zu erhalten, welche anschließend Einblick in die strukturellen Bedingungen der Lipid-Membranprotein Interaktion geben soll. Es wird vermutet, dass Isc1p in Gegenwart von unüblichen Sterolen aktiver ist als in Membranen mit üblicher Zusammensetzung, dies wirft einige interessant Fragen zur Interaktion von Lipiden und Membranproteinen auf.

The *S. cerevisiae* sphingomyelinase Isc1p is an integral membrane protein and is suspected to be interacting specifically with sterols. Sterols and sphingolipids have been shown to interact specifically with each other in membranes. The sphingolipid inositol phosphorylceramide is the natural substrate of Isc1p in *S. cerevisiae* and it is a tempting thought that these participants interact within membranes. Available data suggests that Isc1p activity is altered in strains that feature knockouts of sterol biosynthesis genes and that this altered activity is part of a mechanism that allows yeast cells to adjust their membrane composition. Various Isc1p variants, including N-terminally tagged variants with FLAG-tag epitope and GFP, were expressed in *S. cerevisiae* strains with distinct membrane composition. Especially the *erg3 erg6* double knockout strain was of central interest. Literature suggests that Isc1p activity is increased in these strains, raising some questions about the interaction of lipids and membrane proteins. The tags make for localization studies possible. The ISC1 coding sequence also was mutagenized to yield insights into the structural conditions of lipid- membrane protein interaction.

INTRODUCTION

LIPIDS AND THEIR PHYSIOLOGICAL APPEARANCES

Whatever life there is on earth, it disconnects its cell's interior from the environment by means of hydrophobic membranes. These membranes consist to a very large degree of all kinds of lipids. These amphipathic molecules possess, due to their physicochemical properties, an inherent drive for forming layered sheets in aqueous solution and on the liquid – gaseous interphase. In these sheets the lipids will align in a way that hydrophilic headgroups are pointing towards the aqueous phase and aliphatic tails are aligned next to each other, hidden from water. Depending on the ratio of polar headgroup to apolar tail diameter and various other parameters, these lipids form level sheets, warped structures, mono and bilayers, vesicles or lipid droplets. Depending on the kingdom, cells incorporate various different species of lipids into their membranes, whether ingested or synthesized. Whereas the archaeal membrane lipids are structurally quite distinct from the eubacterial and the eucaryotic main lipids, the latter two share a common basic

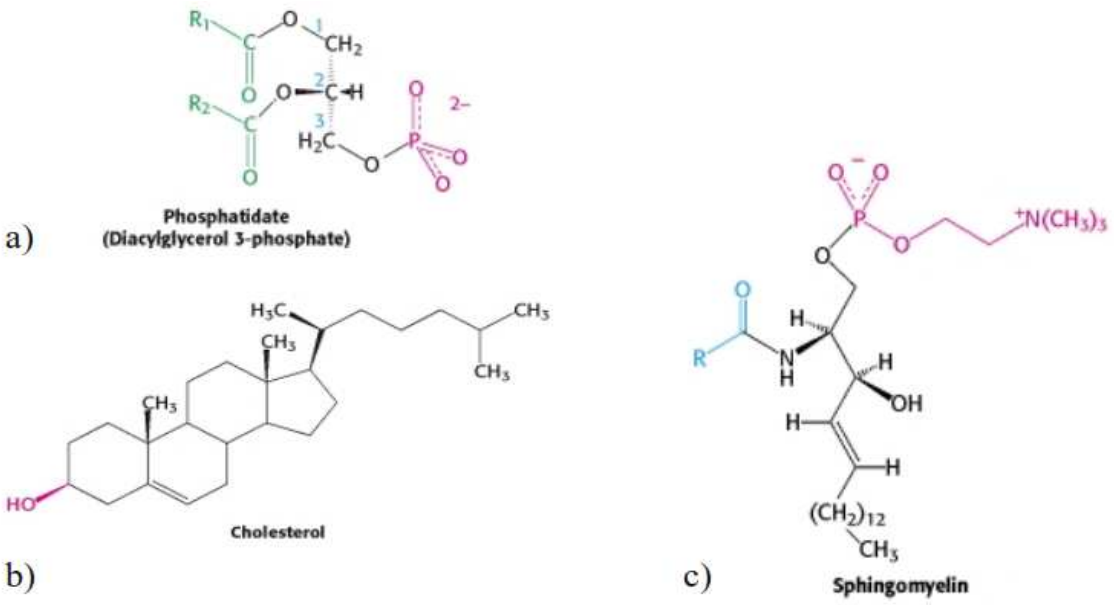


Figure 1: a) General chemical structure of the simplest glycerophospholipid, phosphatidate, where R1 and R2 denote the acyl chains of the fatty acids esterified with glycerol and the headgroup consists of a single esterified phosphate. The acyl chains are usually saturated and unbranched. b) The structure of the important mammalian sterol cholesterol comprises four homocyclic rings, various substitutions and a double bond between Carbon atoms 5 and 6. c) Structure of sphingomyelin, with phosphocholine as headgroup and a double bond at position 4 in the long chain base. *(Stryer, et al., 2002)*

structure, namely fatty acids esterified to a glycerol backbone. In eukaryotes these glycerophospholipids appear in membranes in combination with other lipid classes, mainly sphingolipids and sterols. Upkeep, constant renewal and turnover of these membranes constitute a major metabolic burden, especially in fast growing cells. Although an important role, the separation from cell interior and exterior as well as formation of organelles is not the only role that lipids fulfill. From single celled organisms all the way through to mammals, e.g. sphingolipids do also play key roles in various signal transduction and cell cycle regulation pathways and their turnover is well regulated (Hannun, 1994) (Spiegel, 1996).

Sphingolipids, including sphingosine, sphingosine-1-phosphate, sphingomyelin and complex glycosphingolipids, constitute a major part of eukaryotic membranes only. Sphingolipids are based on a highly modular blueprint, very much like glycerophospholipids. A grand combinatorial diversity emerges from the possibility of combining various long chain bases, fatty acids and headgroups to form a sphingolipid species. This large diversity in turn gives rise to complicated metabolic pathways and intricate regulation schemes. And beyond that structural diversity, different lipid membranes also feature different constituents, for example the inner mitochondrial membranes consist to a large extent of cardiolipin which is absent in the plasma membrane. The polar headgroups can consist of a single phosphate group linked via a phosphoester bond to the long chain base in the simplest case and can feature alternating phosphates, sugars and sugar alcohols in complex glycosphingolipids.

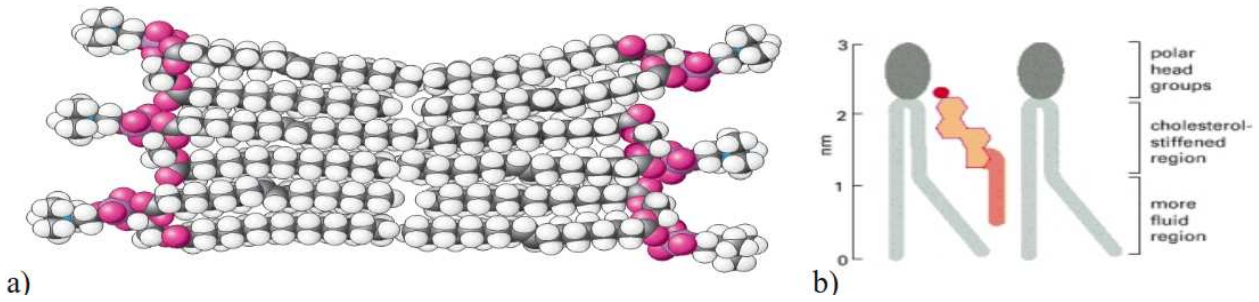


Figure 2: a) Lipids align to one another and thereby effectively form a bilayered aggregate that features a rather hydrophobic interior that is separated from the aqueous exterior by the layer of head groups. *(Stryer, et al., 2002)* **b) Double bonds in the hydrophobic chain regions of lipids and sterols result in more disorder in lipid membranes, especially when compared to the closely fitting chains in a).** *(Alberts, et al., 4th Edition)*

This complexity also leads to innumerable possibilities of steric interactions in membranes, involving all glycerophospholipids, sphingolipids and sterols. To make matters more complex, membranes are also home to even more hydrophobic compounds and many a protein. By introduction of double bonds or substitutions even as minute as methyl groups, a whole different interaction pattern can emerge, as data from Guan et al (Guan, 2009) suggests for sterols and membrane proteins in the yeast *S. cerevsiae*. Their work showed that *Saccharomyces cerevisiae* cells adjust the membrane composition in reaction to sterol synthesis defects primarily by adjusting sphingolipid composition. This sphingolipid adjustment may happen via means of headgroup turnover. Based on their work, the integral membrane protein Isc1p was chosen to star in this thesis'. The protein Isc1p was identified as a phospholipase C (Sawai H, 2000) some 10 years ago and it is still a viable assumption that Isc1p is the only Inositol phosphoryl ceramide phospholipase C in *S. cerevisiae*. Identifying the enzymes involved in sphingolipid metabolism was of great importance to the efforts of deciphering intracellular signal transduction pathways and means of cell cycle regulation. Sphingolipids and related compounds assume crucial functions as first and second messengers. Research for revealing the biosynthetic pathways of sphingolipids, their assembly and degradation as well as their signaling roles and trafficking was to a certain extent performed in the single celled yeast *S. cerevisiae* (Dickson, 1997) (Jenkins, 1997) . Most principles discovered are directly applicable also to mammalian systems that disqualify for research because of their inherent complexity and because of ethical implications.

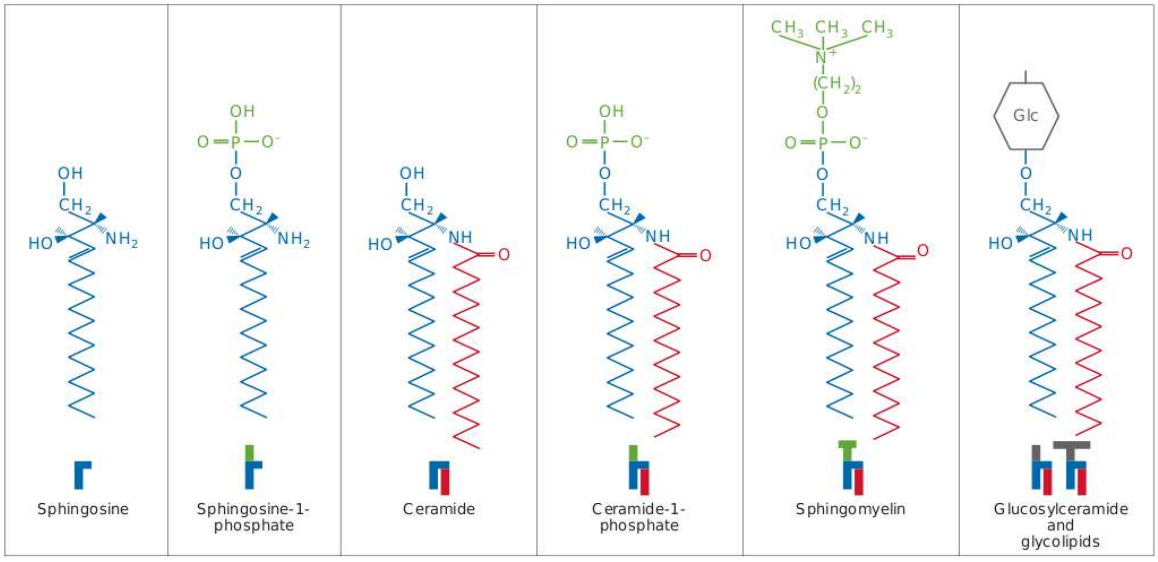


Figure 3: The modular building scheme of sphingolipids is exemplified by sphingomyelin and some precursors, as well as glycosylceramide, that bears glucose attached as hydrophilic, yet uncharged head group. The long chain base part is depicted in blue, the fatty acid moiety in red and the head groups in green and grey, respectively. (Hannun, et al., 2004)

Being the only enzyme in yeast exhibiting this hydrolytic activity makes Isc1p an ideal candidate for further characterization, as isoforms and redundancy in the proteome often make research much more complicated. Isc1p is rather promiscuous as far as the headgroup is regarded, since as its name indicates it was characterized as Inositol phosphosphingolipid phospholipase C. It hydrolyses inositol phosphoryl ceramide to ceramide and the soluble phosphoinositol. Similarly this enzyme hydrolyses sphingomyelin by cleaving the phosphoester bond liberating phosphocholine (see Figure 3). *S. cerevisiae* membranes do not contain sphingomyelin but rather inositolphosphoryl ceramide.

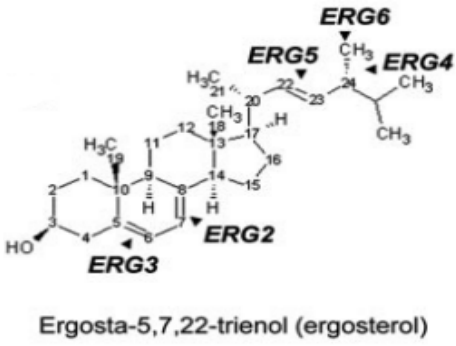
Sterols and sphingolipids are shown to interact preferentially with each other and are claimed to form rafts drifting in a sea of glycerophospholipids. Convincing biophysical data suggests so, but the membrane systems in which these measurements are being conducted do not exactly mirror the reality, but rather use simplified membranes with limited numbers of components. This dynamic clustering of sphingolipids and sterols, be it cholesterol in mammals, ergosterol in fungi or other sterols, attracted a great deal of attention and is suggested to be playing important roles in assembly of these micro domains (Klose, 2010). Sphingolipids and sterols can associate spontaneously into clusters, which has been shown repeatedly and is generally accepted. This concept of lateral organization within membranes has been the subject of many a discussion (Munro, 2003) ever since its first proposal, though. Lipid rafts are suspected to enable protein sorting within and between membranes, thereby playing an important role in the formation of signal transduction complexes by integral membrane proteins as well as glycosylphosphatidylinositol-anchored soluble proteins (Simons, et al., 1997). But the fact that these observations were made in vitro does not necessarily mean that these interactions are of biological significance. These rafts may also be an artifact of biochemical and biophysical investigations. Nevertheless, potential interactions of membrane-bound and integral membrane proteins with membrane lipids and interactions of membrane lipids with each other are under scrutiny by a vivid academic community.

It is even suggested that the different membrane compositions of the various subcellular organelles are being reflected in the amino acid distribution of the transmembrane domains of proteins localized to the respective subcellular membrane structures, like for example the endoplasmic reticulum or the Golgi apparatus (Sharpe, et al., 2010). Integral membrane proteins

are being considered as unhandy because of their low solubility in aqueous systems. With the advent of new techniques, proteins featuring transmembrane domains have become increasingly attractive targets of research. As are the lipids which were characterized cumbersome and slowly in the beginning, but are now targets for holistic approaches like lipidomics analyses in order to compare changes in overall membrane composition under varying conditions (Ejsing, et al., 2009).

Ergosterol biosynthesis, starting from squalene, is an interesting, multi step enzymatically catalyzed process that is mainly localized in the membranes of the endoplasmic reticulum. Although sterol biosynthesis was not of central interest to this work, some enzymes involved are to be mentioned in order to describe the experimental setup. A total of 50 *S. cerevisiae* strains, which already featured various erg - knockouts, have been received and cultivated during the work for this thesis and a few thereof were selected to be employed in this research directly. The knockout of sterol biosynthesis genes concerned (i) *ERG2* which encodes an enzyme that catalyzes the isomerization of a double bond from the Δ-8 position to the Δ-7 position, (ii) *ERG3* which encodes C-5 desaturase, an enzyme that introduces a double bond between C-5 and C-6 positions, (iii) *ERG4* the C-24(28) sterol reductase, that reduces a double bond, (iv) *ERG5*, a sterol desaturase that catalyzes the formation of the C-22(23) double bond and (v) *ERG6* the Δ-24-sterol C-methyltransferase which converts for example zymosterol to fecosterol.

ERG knockout	Isc1p activity change
erg2	=
erg2 erg3	=
erg2 erg6	↗
erg3 erg6	↗
erg6	↘
erg4 erg5	↘



Ergosta-5,7,22-trienol (ergosterol)

Figure 4: Representation of ergosterol with indicated points of action of selected enzymes involved in its synthesis and suggested changes in activity of Isc1p in the respective single or double knockout *S. cerevisiae* strains. Isc1p activity levels appeared to change with the background of sterol synthesis knockouts, leading to the notion that this enzyme may constitute a major part in response to unusual sterols by adjusting the sphingolipid parts of the membranes accordingly (Guan, 2009).

ISC1P

Although the metabolic pathways of sphingolipid biosynthesis have largely been elucidated, there still exists much obscurity regarding molecular interactions of lipids in vivo and the regulation of these biosynthetic pathways. The Mg^{2+} dependent sphingomyelin phosphodiesterase Isc1p (EC 3.1.4.12) is homologous to mammalian neutral sphingomyelinase and has already been characterized to some extent. It also is homologous to bacterial sphingomyelin phosphodiesterase C from *Bacillus cereus*. The structure of *B. cereus* sphingomyelinase was already solved (Ago, et al., 2006). That renders Isc1p a viable candidate for elucidating interaction patterns of proteins with lipid membrane components like sterols, glycerophospholipids and sphingolipids, especially since mammalian neutral sphingomyelinase, which plays important roles in differentiation and development, is poorly characterized. As mentioned above, it was shown that alterations in the membrane composition can lead to altered Isc1p activity (Guan, 2009). This work suggested that the activity of enzymes involved in sphingolipid headgroup turnover is being influenced by changed sterol composition. Ergosterol is the dominating sterol species in yeasts, it even is the most abundant lipid in *S. cerevisiae* amounting to 12 mol% of the assayed lipids (Ejsing, et al., 2009).

Because Isc1p was identified as being the only enzyme that hydrolyzes these complex sphingolipids, it became the candidate for further research into this area. With a whole array of *S. cerevisiae* strains with sterol synthesis knockouts at our hands in combination with suitable activity assays, revealing some further details of protein – lipid interaction seemed feasible. Isc1p was shown to be localized to the endoplasmic reticulum during early growth phase and suggested to translocate to the mitochondria in the post-diauxic growth phase (Vaena de Avalos, et al., 2004). Although it is possible that the shift from a fermentable carbon source like glucose to a non-fermentable one, like ethanol or acetate, results in extensive reshaping of metabolic networks, this still raises some questions. For one thing, this translocation would call for means of substantial trafficking from the endoplasmic reticulum to the mitochondria. Furthermore, yeast mitochondrial membranes almost completely lack complex sphingolipids, which are the substrate for Isc1p, and sterol content in endoplasmic reticulum membranes also is much higher than in mitochondria. These facts prompted us to envisage localization studies with Isc1p in *S. cerevisiae*. Another interesting finding was that Isc1p activity heavily depends on the presence of

anionic lipids like phosphatidylserine, phosphatidylglycerol or cardiolipin. Stripping of these lipids results in no detectable activity (Okamoto, 2002) (Sawai H, 2000), and the presence of the phosphatidylcholine does not stimulate Isc1p activity.

Set against the existing knowledge, this thesis' work was intended to represent a starting point for further investigations by (i) performing localization studies of Isc1p in live *S. cerevisiae* cells or localize the protein after subcellular fractionation, (ii) assaying Isc1p activity in various live *S. cerevisiae* strains that feature knockouts of genes encoding enzymes involved in sterol biosynthesis, thereby obtaining information about sterol and sphingolipid interaction, (iii) establishing in vitro Isc1p activity assays involving radioactively labeled substrate for reliable quantitation as well as an in vivo assay and (iv) performing low frequency random mutagenesis of the two predicted transmembrane domains in order to identify the amino acids involved in the interaction with the membrane lipids. Similar mutagenesis experiments lead to the identification of the amino acids necessary for catalytic activity and binding of the substrates (Okamoto, et al., 2003).

MATERIALS

CHEMICALS, PROTEINS, PRIMERS, MICROORGANISMS, PLASMIDS AND APPLIANCES.

Table 1: Low molecular weight compounds used

Substance name	**Supplier**
Acetic acid	Carl Roth GmbH
Agar Agar	Carl Roth GmbH
Agarose LE	Biozyme
α-D-(+)-Glucose	Carl Roth GmbH
Ampicillin	Sigma-Aldrich
Bio-Rad Bradford Dye Reagent concentrate	Bio-Rad
Bovine serum albumin (BSA)	Carl Roth GmbH
Chloroform	Carl Roth GmbH
Dimethylsulfoxid (DMSO)	Finnzymes
Dithiothreitol (DTT)	Bio-Rad
Ethanol	Carl Roth GmbH
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH
Galactose	Carl Roth GmbH
Glycerol	Carl Roth GmbH
Glycin	Carl Roth GmbH
Hydrochloric acid (HCl)	Carl Roth GmbH
Isopropyl-ß-D-thiogalactopyranosid (IPTG)	Peqlab Biotechnologie
Lithium acetate	Fluka
Magnesiumchloride ($MgCl_2$)	Carl Roth GmbH
Magnesiumsulfate ($MgSO_4$)	Carl Roth GmbH
Methanol	Carl Roth GmbH
NBD-C6-Sphingomyelin	Invitrogen
Phenol red	Carl Roth GmbH
Phosphatidylcholine	Sigma-Aldrich
Phosphatidylserine	Sigma-Aldrich
Pierce 1-Step TMB blotting	Pierce
Pierce SuperSignal West Pico Chemiluminescent substrate for HRP	Pierce
Polyethylenglycol (PEG) 3350	Sigma-Aldrich
Ponceau S	Sigmna-Aldrich
Potassium chloride (KCl)	Carl Roth GmbH
Potassium dihydrogen phosphate	Carl Roth GmbH
Potassium hydrogen phosphate trihydrate	Carl Roth GmbH
Protein 90 protein preparation	DM Drogerie Markt
QIAprep® Spin Miniprep	QIAGEN

Roth Roti-Blue	Carl Roth GmbH
Roti®-Prep Plasmid Kit	Carl Roth GmbH
Sodium chloride (NaCl)	Carl Roth GmbH
Sodium hydroxide (NaOH)	Carl Roth GmbH
Sphingomyelin	Sigma-Aldrich
Sphingomyelin ^{14}C labeled [methyl-^{14}C-phosphorylcholine] sphingomyelin	PerkinElmer
Trichloroacetic acid	Carl Roth GmbH
Tris(hydroxymethyl)-aminomethane	Carl Roth GmbH
Triton X100	Sigma-Aldrich
Tryptone	Bacto Laboratories Pty Ltd Carl Roth GmbH Oxoid
Tween 20	Carl Roth GmbH
Whey powder	Regional dairy
Yeast extract	Bacto Laboratories Pty Ltd Carl Roth GmbH

Table 2: *E. coli* strains used for expression and cloning work

Strain	**Specific genotype**	Supplier
E. coli BL21 Star (DE3)	F - ompT hsdS B (r B - m B -) gal dcm rne131 (DE3)	Invitrogen
E. coli Origami B (DE3)	F– ompT hsdSB(rB– mB–) gal dcm lacY1 ahpC (DE3) gor522::Tn10 trxB	Novagen
E. coli Top10F'	F´ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG	Invitrogen

Table 3: Used E. coli, S. cerevisiae and shuttle plasmids

Plasmid	**Description**	**Supplier**
pCM_dsbC_linker	pMS470 with chloramphenicol instead of ampicillin resistance cassette and additionally expressing DsbC	(Kietzmann, 2009)
pFA6a-GFP(S65T)-kanMX6	4882 bp plasmid constructed by Longtine et al for facilitating *S. cerevisiae* research	(Longtine, 1998)
pMS470_d8	standard *E. coli* expression vector with and ampicillin resistance and inducible tac promoter	(Balzer, et al., 1992)
pYES2	5,9 kb *S. cerevisiae* expression vector, *URA3* for selection of transformants, inducible GAL1 promoter and Ampicillin resistance for *E. coli*	Invitrogen

Table 4: S. cerevisiae strains received from Howard Riezman's laboratory during thesis work

New Name	Old Number	Short description	Mating Type	Markers
PH1	RH448	WT(a)	a	MATa his4 leu2 ura3 lys2 bar1
PH2	RH449	WT(α)	alpha	MATα his4 leu2 lys2 ura3 bar1
PH3	RH1201	WT(a/α)	diploid	MATa/α his4/his4 leu2/leu2 ura3/ura3 lys2/lys2 bar1/bar1
PH4	RH2635	WT(α)	alpha	MATα his4 leu2 ura3 bar1
PH5	RH2947	WT(a)	a	MATa his1
PH6	RH2948	WT(α)	alpha	MATα his1
PH7	RH2897	erg2	a	MATa erg2-1Δ::URA3
PH8	RH3616	erg2 erg6	a	MATa leu2 bar1 ura3 erg2-1Δ::URA3 erg6Δ
PH9	RH3622	erg6	a	MATa his4 leu2 ura3 bar1 erg6Δ::LEU2
PH10	RH4213	erg3	a	MATa erg3Δ::LEU2 his4 ura3 lys2 bar1 leu2
PH11	RH5225	erg3 erg6	a	MATa erg3Δ::LEU2 erg6Δ::LEU2 bar1 ura3 his4 leu2 lys2
PH12	RH5228	erg2 erg3	a	MATa erg3Δ::LEU2 erg2Δ::URA3 bar1 ura3 lys2 his4 leu2
PH13	RH5233	erg4 erg5	a	MATa erg4Δ::URA3 erg5Δ::kanMX4 bar1 ura3 lys2 his4 leu2
PH14	HPY134	erg4 erg5 isc1	a	MATa erg4::LEU2 erg5::LEU2 isc1::kanMX his4 leu2 ura3 lys2 bar1
PH15	HPY139	erg3 erg6	a	MATa erg3::LEU2 erg6::LEU2 his4 leu2 ura3 lys2 bar1
PH16	HPY141	erg2 erg3	a	MATa erg2::LEU2 erg3::LEU2 his4 leu2 ura3 lys2 bar1
PH17	HR5933	erg2 erg3 isc1	a	MATa erg2::LEU2 erg3::LEU2 isc1::kanMX his4 leu2 ura3 lys2 bar1
PH18	HPY121(RH	isc1	a	MATa isc1::kanMX his4 leu2 ura3 lys2 bar1
PH19	HPY131 (RH	erg3 erg6 isc1	a	MATa erg3::LEU2 erg6::LEU2 isc1::kanMX his4 leu2 ura3 lys2 bar1
PH20	HPY161	erg3 erg6 ISC1-3HA heterozygous diploid	diploid	MATa/α erg3::LEU2/ERG3 erg6::LEU2/ERG6 ISC1-3HA-kanMX/ISC1 his4/his4 leu2/leu2 ura3/ura3 LYS2/lys2 bar1/bar1
PH21	HPY162	WT ISC1-GFP	a	MATa ISC1-GFP-kanMX his4 leu2 ura3 bar1
PH22	HPY166	erg3 ISC1-GFP	a	MATa erg3::LEU2 ISC1-GFP-kanMX his4 leu2 ura3 lys2 bar1
PH23	HPY168	erg3erg6 ISC1-GFP	a	MATa erg3::LEU2 erg6::LEU2 ISC1-GFP-kanMX his4 leu2 ura3 lys2 bar1
PH24	HPY170 (RH	WT ISC1-HA3	a	MATa ISC1-HA3-kanMX his4 leu2 ura3 bar1
PH25	HPY172	erg3erg6 ISC1-HA3	a	MATa erg3::LEU2 erg6::LEU2 ISC1-HA3-kanMX his4 leu2 ura3 bar1
PH26	HPY173 (RH	erg3erg6 ISC1-HA3	a	MATa erg3::LEU2 erg6::LEU2 ISC1-HA3-kanMX his4 leu2 ura3 lys2 bar1
PH27	HPY174 (RH	erg6 ISC1-HA3	a	MATa erg6::LEU2 ISC1-HA3-kanMX his4 leu2 ura3 lys2 bar1
PH28	HPY176 (RH	erg3 ISC1-HA3	a	MATa erg3::LEU2 ISC1-HA3-kanMX his4 leu2 ura3 bar1
PH29	HR123		a	MATa sst1sst2 leu1 trp5 ade2 can1
PH30	RH5918	erg3 erg6 isc1	a	MATa erg3::LEU2 erg6::LEU2 isc1::kanMX his4 leu2 ura3 lys2 bar1
PH31	RH5917	erg5 isc1	a	MATa erg5::LEU2 isc1::kanMX his4 leu2 ura3 lys2 bar1
PH32	RH5916	erg4 isc1	a	MATa erg4::LEU2 isc1::kanMX his4 leu2 ura3 lys2 bar1
PH33	RH5915	erg2 isc1	a	MATa erg2::LEU2 isc1::kanMX his4 leu2 ura3 lys2 bar1
PH34	RH5912	isc1	a	MATa isc1::kanMX his4 leu2 ura3 lys2 bar1
PH35	RH5911	erg4 erg5 isc1 heterozygous diploid	diploid	MATa/α erg4::LEU2/ERG4 erg5::LEU2/ERG5 isc1::kanMX/ISC1 his4/his4 leu2/leu2 ura3/ura3 lys2/lys2 bar1/bar1
PH36	RH5910	erg3 erg6 isc1 heterozygous diploid	diploid	MATa/α erg3::LEU2/ERG3 erg6::LEU2/ERG6 isc1::kanMX/ISC1 his4/his4 leu2/leu2 ura3/ura3 LYS2/lys2 bar1/bar1
PH37	RH5909	erg2 erg5 isc1 heterozygous diploid	diploid	MATa/α erg2::LEU2/ERG2 erg5::LEU2/ERG5 isc1::kanMX/ISC1 his4/his4 leu2/leu2 ura3/ura3 lys2/lys2 bar1/bar1
PH38	RH5908	erg2 erg3 isc1 heterozygous diploid	diploid	MATa/α erg2::LEU2/ERG2 erg3::LEU2/ERG3 isc1::kanMX/ISC1 his4/his4 leu2/leu2 ura3/ura3 lys2/lys2 bar1/bar1
PH39	RH6949	erg5 sur2 PDR12::CFP::HygB	a	MATa erg5::KanMX sur2::LEU2 PDR12::CFP::HygB his4 leu2 ura3 lys2 bar1
PH40	RH6037	erg3 erg6 ISC1-HA3	alpha	MATα erg3::LEU2 erg6::LEU2 ISC1-HA3-kanMX his4 leu2 ura3 bar1
PH41	RH6029	erg3 erg6 ISC1-GFP-kanMX	alpha	MATα erg3::LEU2 erg6::LEU2 ISC1-GFP-kanMX his4 leu2 ura3 bar1
PH42	RH6005	erg6 ISC1-GFP-kanMX	alpha	MATα erg6::LEU2 ISC1-GFP-kanMX his4 leu2 ura3 lys2 bar1
PH43	RH6003	ISC1-GFP-kanMX	alpha	MATα ISC1-GFP-kanMX his4 leu2 ura3 lys2 bar1
PH44	RH5977	erg3 erg6 ISC1-3HA-kanMX heterozygous diploid	diploid	MATa/α erg3::LEU2/ERG3 erg6::LEU2/ERG6 ISC1-3HA-kanMX/ISC1 his4/his4 ura3/ura3 LYS2/lys2 bar1/bar1
PH45	RH5976	erg3 erg6 ISC1-GFP-kanMX heterozygous diploid	diploid	MATa/α erg3::LEU2/ERG3 erg6::LEU2/ERG6 ISC1-GFP-kanMX/ISC1 his4/his4 ura3/ura3 LYS2/lys2 bar1/bar1
PH46	RH5975	erg3 erg6 ISC1-GFP-kanMX heterozygous diploid	diploid	MATa/α erg3::LEU2/ERG3 erg6::LEU2/ERG6 ISC1-GFP-kanMX/ISC1 his4/his4 ura3/ura3 LYS2/lys2 bar1/bar1
PH47	RH5974	erg2 erg3 ISC1-3HA-kanMX heterozygous diploid	diplod	MATa/α erg2::LEU2/ERG2 erg3::LEU2/ERG3 ISC1-3HA-kanMX/ISC1 his4/his4 ura3/ura3 lys2/lys2 bar1/bar1
PH48	RH5973	erg2 erg3 ISC1-GFP-kanMX heterozygous diploid	diploid	MATa/α erg2::LEU2/ERG2 erg3::LEU2/ERG3 ISC1-GFP-kanMX/ISC1 his4/his4 ura3/ura3 lys2/LYS2 bar1/bar1
PH49	RH5539	erg3 erg6	diploid	MATa/α erg3::LEU2/ERG3 erg6::LEU2/ERG6 his4/his4 ura3/ura3 lys2/lys2 bar1/bar1
PH50	RH6940	isc1	alpha	MATa/α isc1::kanMX his4 leu2 ura3 lys2 bar1

Table 5: Strains obtained by transformation of *S. cerevisiae* strains with pYES2 plasmid constructs

Name	Short Description	Markers
PH51	PH2 + pYES2 plasmid w/o	MATα his4 leu2 lys2 URA3 bar1
PH52	PH2 + pYES2 plasmid with ISC1	MATα his4 leu2 lys2 URA3 bar1
PH53	PH2 + pYES2 plasmid with flag_ISC1	MATα his4 leu2 lys2 URA3 bar1
PH54	PH2 + pYES2 plasmid with GFP_ISC1	MATα his4 leu2 lys2 URA3 bar1
PH55	PH11 + pYES2 plasmid w/o	MATa erg3Δ::LEU2 erg6Δ::LEU2 bar1 URA3 his4 leu2 lys2
PH56	PH11 + pYES2 plasmid with ISC1	MATa erg3Δ::LEU2 erg6Δ::LEU2 bar1 URA3 his4 leu2 lys2
PH57	PH11 + pYES2 plasmid with flag_ISC1	MATa erg3Δ::LEU2 erg6Δ::LEU2 bar1 URA3 his4 leu2 lys2?
PH58	PH11 + pYES2 plasmid with GFP_ISC1	MATa erg3Δ::LEU2 erg6Δ::LEU2 bar1 URA3 his4 leu2 lyss2
PH59	PH18 + pYES2 plasmid w/o	MATa isc1::kanMX his4 leu2 URA3 lys2 bar1
PH60	PH18 + pYES2 plasmid with ISC1	MATa isc1::kanMX his4 leu2 URA3 lys2 bar1
PH61	PH18 + pYES2 plasmid with flag_ISC1	MATa isc1::kanMX his4 leu2 URA3 lys2 bar1
PH62	PH18 + pYES2 plasmid with GFP_ISC1	MATa isc1::kanMX his4 leu2 URA3 lys2 bar1
PH63	PH49 + pYES2 plasmid w/o	MATa/α erg2::LEU2/ERG2 erg3::LEU2/ERG3 ISC1-GFP-kanMX/ISC1 his4/his4 leu2/leu2 ura3/ura3 lys2/lys2 bar1/bar1 URA3
PH64	PH49 + pYES2 plasmid with ISC1	MATa/α erg2::LEU2/ERG2 erg3::LEU2/ERG3 ISC1-GFP-kanMX/ISC1 his4/his4 leu2/leu2 ura3/ura3 lys2/lys2 bar1/bar1 URA3
PH65	PH49 + pYES2 plasmid with flag_ISC1	MATa/α erg2::LEU2/ERG2 erg3::LEU2/ERG3 ISC1-GFP-kanMX/ISC1 his4/his4 leu2/leu2 ura3/ura3 lys2/lys2 bar1/bar1 URA3
PH66	PH49 + pYES2 plasmid with GFP_ISC1	MATa/α erg2::LEU2/ERG2 erg3::LEU2/ERG3 ISC1-GFP-kanMX/ISC1 his4/his4 leu2/leu2 ura3/ura3 lys2/lys2 bar1/bar1 URA3
PH67	PH2+ flag cassette	MATα his4 leu2 lys2 ura3 bar1 isc1::flag_ISC1-kanMX6
PH68	PH2+ GFP cassette	MATα his4 leu2 lys2 ura3 bar1 isc1::GFP_ISC1-kanMX6
PH69	PH49+ flag cassette	MATa/α erg3::LEU2/ERG3 erg6::LEU2/ERG6 his4/his4 ura3/ura3 lys2/lys2 bar1/bar1 isc1::flag_ISC1-kanMX6
PH70	PH49+ GFP cassette	MATa/α erg3::LEU2/ERG3 erg6::LEU2/ERG6 his4/his4 ura3/ura3 lys2/lys2 bar1/bar1 isc1::GFP_ISC1-kanMX6

Table 6: *E. coli* strains bearing plasmids for expression purposes as well as simply for storing the plasmids.

Name	Strain	Plasmid insert
PHE1	OrigamiB (DE3)	pMS470_ISC1
PHE2	OrigamiB (DE3)	pCm_dsbC_ISC1
PHE3	OrigamiB (DE3)	pMS470_flag_ISC1
PHE4	OrigamiB (DE3)	pCm_dsbC_flag_ISC1
PHE5	OrigamiB (DE3)	pMS470_flag_ISC1_370stop
PHE6	OrigamiB (DE3)	pCm_dsbC_flag_ISC1_370stop
PHE7	OrigamiB (DE3)	pMS470_flag_ISC1_417stop
PHE8	OrigamiB (DE3)	pCm_dsbC_flag_ISC1_417stop
PHE9	OrigamiB (DE3)	pMS470_linker
PHE10	OrigamiB (DE3)	pCm_dsbC_linker
PHE11	BL21 star	pMS470_ISC1
PHE12	BL21 star	pCm_dsbC_ISC1
PHE13	BL21 star	pMS470_flag_ISC1
PHE14	BL21 star	pCm_dsbC_flag_ISC1
PHE15	BL21 star	pMS470_flag_ISC1_370stop
PHE16	BL21 star	pCm_dsbC_flag_ISC1_370stop
PHE17	BL21 star	pMS470_flag_ISC1_417stop
PHE18	BL21 star	pCm_dsbC_flag_ISC1_417stop
PHE19	BL21 star	pMS470_linker
PHE20	BL21 star	pCm_dsbC_linker
PHE21	Top10F'	pYES2
PHE22	Top10F'	pYES2_ISC1
PHE23	Top10F'	pYES2_flag_ISC1
PHE24	Top10F'	pYES2_GFP_ISC1

Table 7: Nucleotide sequences used as templates for constructs

Name	**Description**	**Source**
CYC1 TT	Transcription terminator region from pYES2 plasmid, used for constructing integration cassettes	Invitrogen
GFP	Green fluorescent protein variant, used to construct N terminally tagged Isc1p, from pFA6a-GFP(S65T)-kanMX6 plasmid	(Longtine, 1998)
ISC1	1434 bp *S. cerevisiae* gene coding for Isc1p, located on Chromosome 5, 19th open reading frame right of the centromere on the Watson strand, YER019W	
kanMX6	Geneticin resistance cassette, complete with promoter, ORF and terminator region, from pFA6a-GFP(S65T)-kanMX6 plasmid	(Longtine, 1998)

Table 8: Primers employed for obtaining ISC1 coding sequence, further cloning steps and integration cassette construction

Primer	Sequence
E. coli expression	indicates the part annealing to the ISC1 gene
ndeI_flag_ISC1_fw	5'AGATATACATATGGACTACAAGGACGACGATGATAAGATGTACAACAGAAGACAGA 3'
ndeI_ISC1_fw	5'AGGAGATATACATATGTACAACAGAAAAGACAGAGATGTTCACGAGAG 3'
notI_ISC1_rv	5' CTTAATAGCGGCCGCTTATCATTTCTCGCTCAAGAAAGTTTG 3'
notI_370_stop_rv	5' AGTAAGGCGGCCGCTTATCACTGCAAGATCAGCTCTCTATC 3'
notI_417stop_rv	5' GATTAGGCGGCCGCTTATCATGCAGTAAACGTTGTCA 3'
linker_fw Braun	5' TATGTATCTGCGGCCGCTTTATTA 3'
linker_rv Braun	5' AGCTTAATAAAGCGGCCGCAGATACA 3'
Strain verification	
ERG2_fw	5' ATGGTATATAAGAGAAAGAAGCG 3'
ERG2_rv	5' AAAGCTAAAGAGAATATAAACAAATAG 3'
ERG3_fw	5' CTTTACATTTGTCTTGTTCTTTG 3'
ERG3_rv	5' GCGTATCTTAAAACATGTATGTG 3'
ERG4_fw	5' GAAAAATTACCAAACAGTGG 3'
ERG4_rv	5' CTTTTCAAGCCCTTTTGTC 3'
ERG5_fw	5' CTTATTCTTACTCATTTCTTTCTTG 3'
ERG5_rv	5' AAGGGAAGAAAATAAAAGTATTC 3'
ERG6_fw	5' CTGTTAATTTTCATTCAAGTTTC 3'
ERG6_rv	5' GCATCGGACAGTCTGTTTG 3'
kanMX6_fw	5' GATTCCTGTTTGTAATTGTCC 3'
kanMX6_rv	5' GATTGCCCGACATTATCG 3'
URA3_fw	5' GGCGGAAGAAGTAACAAAG 3'
URA3_rv	5' TTTGTTTGCTTTTCGTGC 3'
Leu2_fw	5' ACCCAACCCACCTAAATG 3'
Leu2_rv	5' GAACGAACATCAGAAATAGC 3'
ISC1 mutagenesis	
ISC1_TM_mut_fw	5'AGATACTCCAACTACGAAACC 3'
ISC1_TM_mut_rv	5' GAACCTCTTGTTCGACTTCG 3'
ISC1_mut_fw	5' GCCAGTGTGCTGGAATTC 3'
ISC1_mut_rv	5' CGCTTATCATTTCTCGCTC 3'
Primers for constructing the integration cassettes and the pYES2 constructs	
pYES2_FLAG_ISC1_fw	5' CCTAGAATTACATGGACATACAAGGACGACGATG 3'
pYES2_noflag_ISC1_fw	5' CAGCGAATTCATGTACAACAGAAAAGACAGAG 3'
ISC1_oe_rv	5' GACATAACTAATTACATGATTCATTTCTCGCTCAAGAAG 3'
pYES2_GFP_fw	5' AATCAGAATTCATGGAAGAACTTTTCACTGGAG 3'
flag_hom_fw	5' TCCGCGTAAAAAGGGAAAAAAAGCAGATATATGGACTACAAGGACGAC 3'
GFP_hom_fw	5'TCCGCGTAAAAAGGGAAAAAAAGCAGATATATGGAAGAACTTTTCACTGGA 3'
GFP_rv	5' CTGTCTTTTCTGTTGTACATTAGTTCATCCATGCCATG 3'
ISC1_oe_fw	5' ATGTACAACAGAAAAGACAGAG 3'

cyctt_fw	5' ATCATGTAATTAGTTATGTCACG 3'
cyctt_rv	5' GCAATTAAAGCCTTCGA 3'
kanMX6_fw	5' GCTCGAAGGCTTTAATTTGCGATCTGTTTAGCTTGCCTC 3'
kanMX6_hom_rv	5' CATATGCTAAAGAAAATCGATAATATTAGAAAAACTCATCGAGC 3'
Primers for sequencing	
GAL1_seq_fw	5' GATCGGACTACTAGCAGC 3'
CYC1_seq_rv	5' GGTTGTCTAACTCCTTCC 3'
ISC1_mid_seq_rv	5' CCAGGCCCCGTCAGATACC 3'
kanMX6_mid_seq_fw	5' CGTATTTCGTCTCGCTCAGG 3'

Table 9: Buffers and solutions

Name	**Composition**
Ampicillin stock	1000x stock solution 100 mg/ml: 100 mg of Ampicillin dissolved in 1 ml of ddH_2O
Chloramphenicol stock	1000x stock solution 10 mg/ml: 10 mg of Chloramphenicol dissolved in 1 ml of Ethanol abs.
Geneticin, G418 stock	1000x stock solution 100 mg/ml: 100 mg dissolved in 1 ml of ddH_2O
IPTG Stock 100 mM	1000x stock solution:1.19 g IPTG in 50 ml of ddH_2O
Ponceau S solution	0.1% Ponceau S in 5% acetic acid
Potassium phosphate buffer, pH 8.0	214.54 g KH_2PO_4, 8.17 g K_2HPO_4 * $3H_2O$, filled to 1000 ml with ddH_2O
TBST	999.5 ml 1x TBS, 0.5 ml Tween 20
TBST-Milk	10 g whey powder or 5 g of DM Protein dissolved in 100 ml TBST
TE buffer	Tris 10 mM (0.121 g in 100 ml), EDTA 1 mM (0.372 g in 100 ml), adjust pH with HCl to 7,5 and fill ddH_2O to 100 ml; 10x TE : 0,1 M Tris, 10 mM EDTA and pH adjusted with HCl
Tetracycline stock	1000x stock: 12,5 mg/ml dissolved in 1 ml of 1:1 ddH_2O:Ethanol
Tris buffer saline (TBS)	10x stock: 30.3 g Tris (0.25 M), 87.6 g NaCl (1.5 M), pH adjusted with 1 M HCl to desired pH, e.g. pH 7.5, to 1000 ml with ddH_2O
Tris-HCl	Respective molarity, pH adjusted with HCl e.g. 100 mM, pH 7.0: 12.1 g Tris diluted in ddH_2O, pH adjusted with 1 M HCl

Table 10: Liquid media and plates

Name	**Composition**
LB medium	10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, autoclaved together
LB plates	LB medium + 20 g/l agar agar, all autoclaved together
YPD medium	10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose, glucose solution autoclaved separately to prevent Maillard reactions
YPD plates	YPD medium + 20 g/l agar agar
SOC medium	20 g/l tryptone, 0.58 g/l NaCl, 5 g/l yeast extract, 2 g/l $MgCl_2$, 0.16 g/l KCl, 2.46 g/l $MgSO_4$, 3.46 g/l glucose
SD-URA plates	6.7 g/l yeast nitrogen base without amino acids, 2 g/l dropout powder mix without uracil, 0.1 g NaOH, 20 g/l agar agar, 20 g/l glucose; tryptophan should not be autoclaved: add 4 ml of 250x stock 10 mg/ml sterile filtered after some cooling

	10g SD-Ura dropout mix: The desired selection marker uracil is to be left out. **Substance** **g** **Substance** **g** Adenine 2 Leucine 2 Uracil - Lysine 2 Histidine 2 Tyrosine 2
	For plates and liquid media that require addition of antibiotics: Antibiotics were added under sterile conditions inside a laminar flow working bench after the other media components had been cooled to a temperature that allowed easy touching with the hands.

Table 11: Enzymes and other proteins

Protein	**Supplier**
DreamTaq DNA polymerase	Fermentas
Goat-anti-mouse IgG conjugated with Horseradish Peroxidase	Invitrogen
Goat-anti-rabbit IgG conjugated with Horseradish Peroxidase	Invitrogen
Mouse-anti-Flag M2 monoclonal antibody	Sigma-Aldrich
Mutazyme II DNA Polymerase	Stratagene
PfuUltra™ Hotstart DNA Polymerase	Stratagene
Phusion DNA Polymerase	Finnzymes
Rabbit-anti-Isc1p polyclonal antibody	Eurogentec
Restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Nde*I, *Not*I, *Pae*I, *Sac*I, *Dpn*I	Fermentas
T4 DNA Ligase	Fermentas

Table 12: Employed kits

Amplex Red Sphingomyelinase Assay	Invitrogen
Gene Jet Plasmid Miniprep Kit	Fermentas
GeneMorph®II Random Mutagenesis Kit	Stratagene
Promega Wizard SV Gel and PCR Clean-Up System	Promega
Roti-Prep Plasmid Isolation Kit	Carl Roth GmbH

Table 13: Appliances used during the work for this thesis

Use	Appliance
Absorption measurement, OD_{600}	DU® 800 Spectrophotometer, Beckman Coulter Cuvettes, same as for OD measurement BioPhotometer, Eppendorf Cuvettes (10x4x45mm), Sarstedt
Agarose gel electrophoresis	PowerPacTM Basic + Sub-Cell GT, Bio-Rad
Centrifuge	AvantiTM Centrifuge J-20XP + JA-14 rotor and JA-25,50 rotors, Beckman CoulterTM
Culture incubation	HT MiltronII, Infors AG Certomat® BS-1, Sartorius
Electro-transformation	MicroPulserTM, Bio-Rad Electroporation Cuvettes (2mm gap) Molecular BioProducts Inc.
Imaging	Syngene G:Box
Liquid scintillation counter	Packard TRI-CARB 2900TR
Microcentrifuge	Centrifuge 5415R Eppendorf
OD measurement	BioPhotometer, Eppendorf Cuvettes (10x4x45mm), Sarstedt
PCR reaction	GeneAmp®PCR System 2700, Applied Biosystems
Protein gels + Western Blotting	NuPAGE Novex 4-12% Bis-Tris Gels 1.0 mm, 17 well, Xcell SureLock Mini-Cell and PowerEase® 500 Power Supply, all Invitrogen Nitrocellulose membrane, Hybond-ECLTM, Amersham Biosciences
Sonication	Sonifier250, Branson
Tabletop Centrifuge	Centrifuge 5810, Eppendorf
Thermomixer	Thermomixer comfort, Eppendorf
TLC plate	Merck silica gel 60
Vortexer	Vortex-Genie, Scientific Industries Inc

METHODS

EXPRESSION OF ISC1P IN *E. COLI*

Expression of Isc1p in *E. coli* was planned to serve one main goal. This goal was the production of enough Isc1p protein to produce antibodies for detecting the protein on Western blots. Since sphingolipids and sterols are highly uncommon in prokaryotic membranes, expression in *E. coli* was not meant to yield activity data, even if expression was successful. Production of antibodies towards the whole protein in rabbits (by G. Daum, Graz University of Technology) was one option, however, antibodies can also be ordered commercially. In the letter case, immunization of rabbits will occur with synthetic peptides. Expression in *E. coli* was only a quick assessment of its feasibility and was meant to be disregarded if it did not work well right away. Expression plasmids, suitable bacterial strains and the necessary expertise were easily accessible. Construction of *E. coli* expression vectors proved to be a straightforward task, once the plasmids were chosen. Expressing in two *E. coli* strains and with two distinct plasmid backbones simultaneously seemed to be the way to tackle this task efficiently. Since the bacterial hosts feature membranes very different from the yeast *S. cerevisiae,* no adverse effects were to be expected from the introduction of a sphingomyelinase activity into the bacterial systems. The protein Isc1p is an integral membrane protein in the yeast *S. cerevisiae.* Thus production in *E coli*, especially in larger amounts, may lead to problems associated with solubility and aggregation of the proteins. We decided to construct various truncated versions of the gene for expression. The bioinformatics tool Phyre (Kelley, et al., 2009) digests the results from as much as three independent secondary structure prediction tools to provide a comprehensive prediction. This allowed for the identification of two transmembrane spanning helixes and for the construction of truncated Isc1p that featured one transmembrane helix and no transmembrane helix at all, respectively. These constructs and the whole Isc1 protein of *S. cerevisiae* chromosome V were N-terminally tagged with the eight amino acid FLAG-tag epitope against which antibodies are commercially available. Since bioinformatics tools are available for almost any imaginable desire, there also exist tools to predict signal peptides and intracellular localization, which were employed to no avail (Bendtsen, et al., 2004) (Horton, et al., 2007). The absence of signal peptides at least allowed for N-terminal tagging, which would be problematic if this were not the case. N-terminal tags appeared to be more desirable, because the C-terminus of the protein is in

proximity of the transmembrane helixes which we suspect to be responsible for interaction with membrane components. Hence, we did not want to disturb the C-terminal structures by the introduction of tags. Antibodies raised against truncated protein versions are not less specific. The soluble domain still presented plenty of epitopes and obtaining a soluble protein for immunization was a major advantage. Supported by bioinformatics and available literature, several PCR primers were designed, to obtain *ISC1* by colony PCR from the haploid *S. cerevisiae* strain PH2 (see Table 4). Subsequently, *ISC1* was cloned into E. coli expression vectors pMS470_Δ8_linker and pCm_dsbC_linker. These plasmids have been used before and featured only a limited amount of restriction sites, but the restriction endonucleases *Hin*dIII and *Pae*I would also cut within the *ISC1* gene. So the restriction site for *Not*I was appended before the genes were cloned into the plasmid. The material for this "linker" region was already at hand (Braun, 2008). Two *E. coli* strains optimized for heterologous expression were selected as hosts. The *E. coli* strain BL21 provides an increased mRNA half-life due to elimination of an RNAse. The specialized Origami B strain features an oxidizing cytosolic environment that enables efficient formation of disulfide bonds, which may enhance the chances of successful heterologous expression of Isc1p with a total of 10 cysteine residues. Overall stability of proteins overexpressed heterologously in Origami B should be enhanced, because this strain not only lacks two reductases but also two proteases. In addition, coexpression of the disulfide bond isomerase dsbC from pCm_dsbC_linker plasmid was also tested. This enzyme helps proteins attaining the correct fold by cleaving improperly formed disulfide bonds.

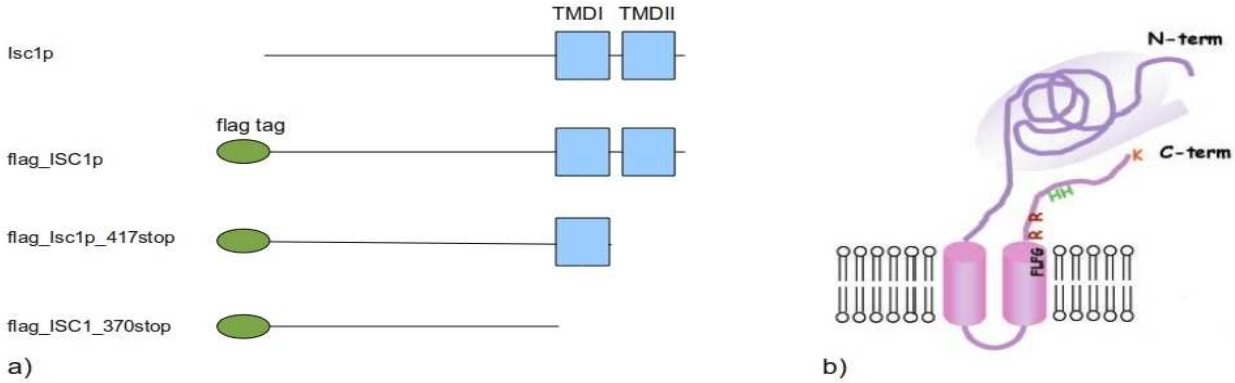


Figure 5: a) Schematic representation of the complete, the truncated and tagged Isc1p versions for *E. coli* expression. The transmembrane domains were clipped off in order to achieve enhanced solubility since Isc1p is an integral membrane protein which may result in poor expression. b) Proposed Isc1p structure. The N-terminal catalytic domain is soluble and it is suggested that this domain gets pulled towards its substrate in the membranes when being activated by phosphatidylserine, phosphatidylglycerol or cardiolipin (Okamoto, 2002).

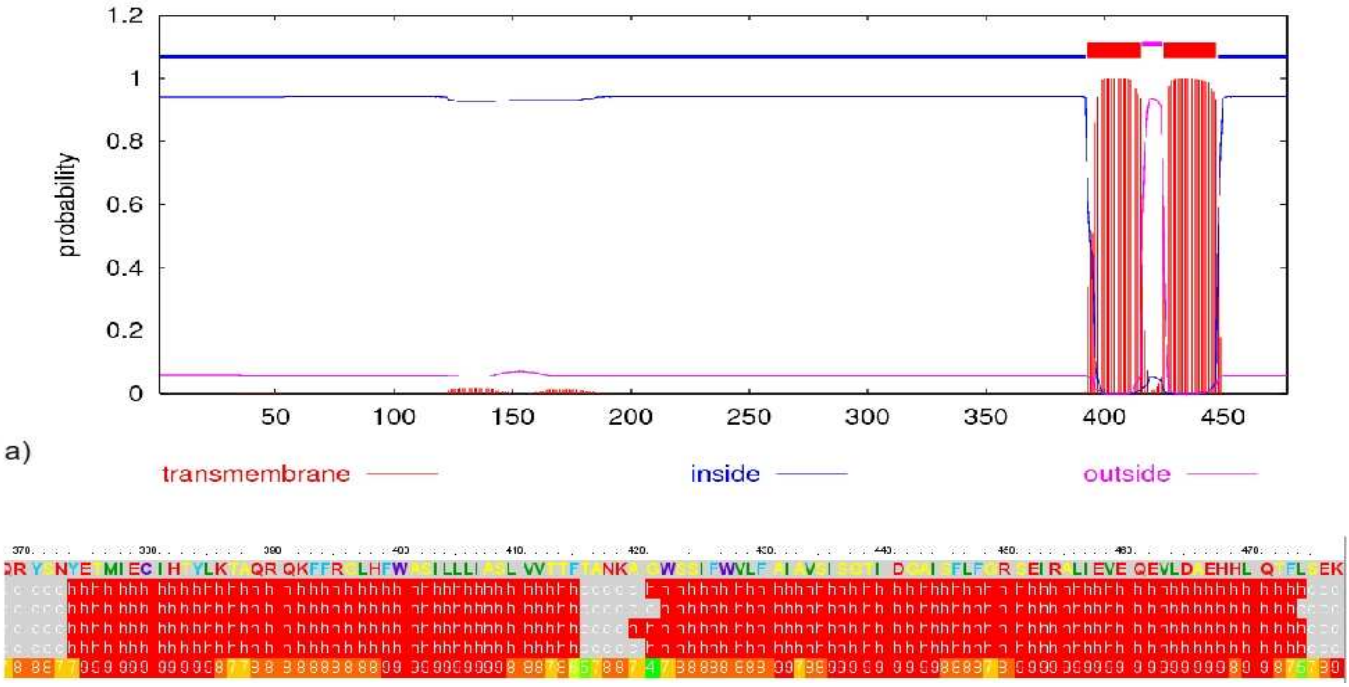


Figure 6: a) Transmembrane topology prediction using TMHMM web server and Isc1p amino acid sequence (Krogh, et al., 2001), (Sonnhammer, et al., 1998). Interestingly, the probability scale reaches to 1.2, but the sections between 393-415 and 425- 447 are predicted to be transmembrane helices with probability of 1. b) Detail of the secondary structure prediction using the Phyre web server. (Kelley, et al., 2009) The helices suspected to be representing the transmembrane moieties are located between amino acids 375-415 and 421-473, respectively. The constructs for *E. coli* expression were designed on the basis of the Phyre results, as not to cripple the helices, but rather amputate them as whole and cut in the coil regions.

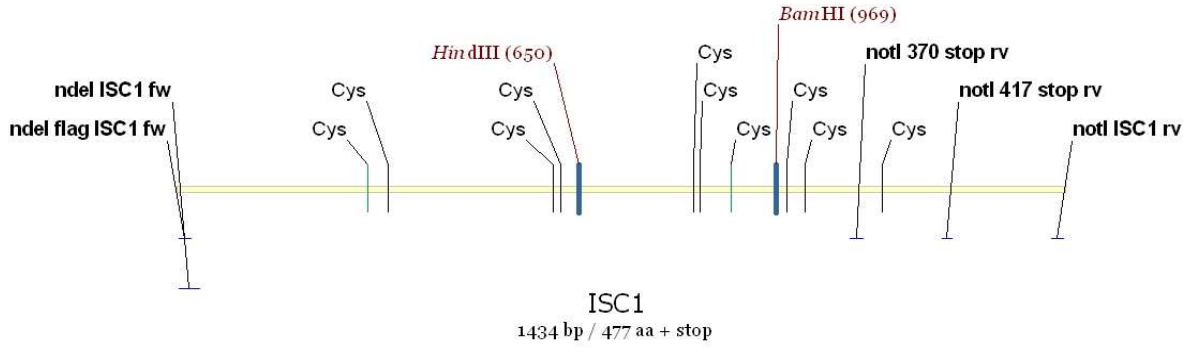


Figure 7: Layout of the primer annealing sites within the *ISC1* gene. With the help of these primers, the PCR products can be cloned into the plasmid via restriction enzymes and ligase. The reverse primers were designed to include two stop codons at the 5' end. The two indicated restriction sites, *Hin*dIII and *Bam*HI, sadly couldn't be used for cloning work, since the enzymes would cut within the coding sequence. Arithmetically there are five disulfide bonds possible with ten cysteine residues, which is why a disulfide bond isomerase was coexpressed.

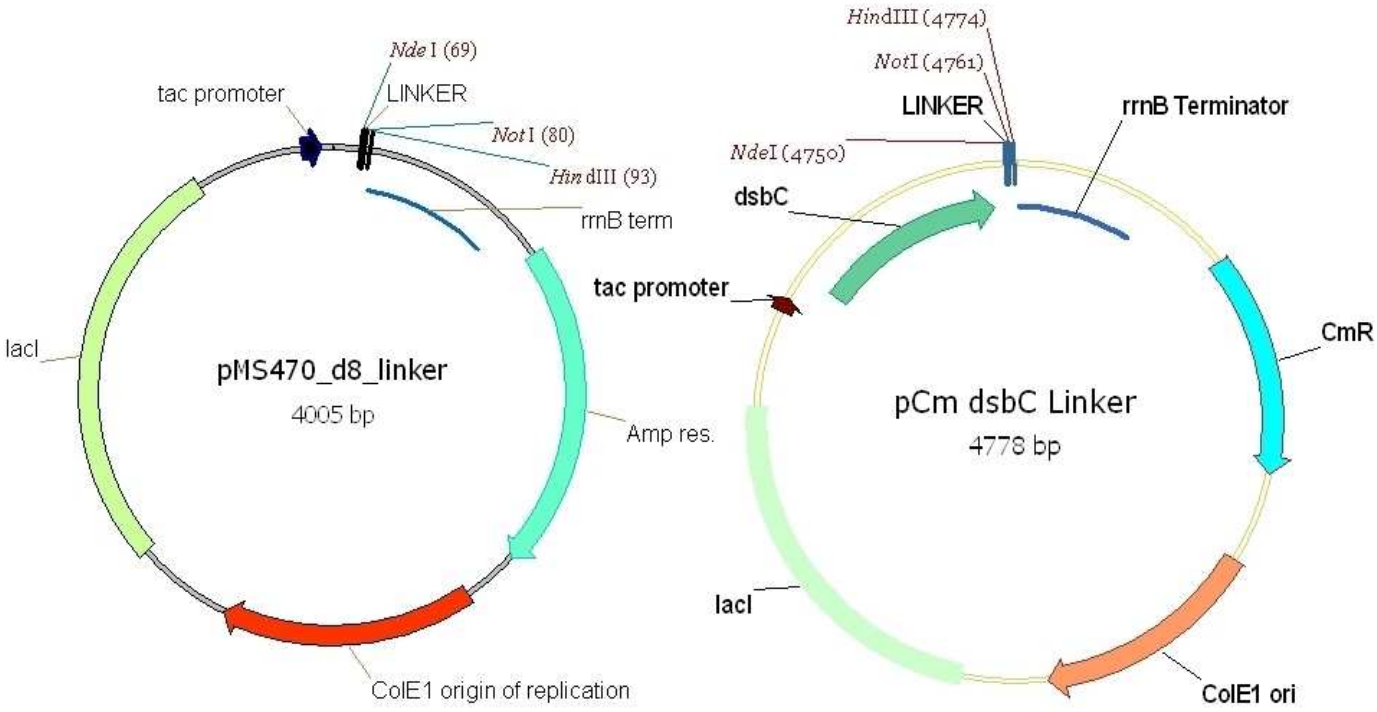


Figure 8: Graphic representation of the *E. coli* expression plasmids pMS470_d8_linker and pCm_dsbC_linker. The indicated features include the inducible *tac* promoter, endonucleases sites, plasmid origin of replication and ampicillin and chloramphenicol resistance markers. To limit the expression from *tac* promoter without induction, the plasmids additionally encode a repressor protein, *lacI*, that binds to the operator region in order to inhibit transcription based upon the *E. coli* lac operon.

The overall plan for expression in *E. coli* cells was as follows. The *ISC1* sequences were obtained by colony PCR of wild type *S. cerevisiae* strains and primers that feature restriction sites in order to allow for convenient cloning into the plasmids. Once the plasmids and the *ISC1* fragments were digested with *Nde*I and *Not*I, the parts were ligated and transformed into *E. coli* cells to amplify the vectors. After performing control digests of the ligated plasmids and sequencing of the insert regions for verification, the plasmids were transformed into the expression strains. Protein expression in these strains yielded enough material to perform SDS-PAGE with subsequent Western blotting.

S. CEREVISIAE COLONY PCR

Standard PCRs were performed with the respective primer combination in order to obtain the sequences for cloning into the expression plasmids. The haploid *S. cerevisiae* wild type strain PH2 served as template, whereas the strain PH18, *isc1::kanMX6,* was included to check whether the primers only lead to *ISC1* specific product (Table 14). A small amount of the colonies, corresponding to about 1 mm diameter of a single colony was picked with sterile toothpicks and resuspended in 50 µl of zymolyase (2.5 mg/ml ~50 U) in a PCR reaction tube and incubated for 15 min at 37°C. After incubation, the cells were collected by centrifugation at 5000 rpm for 1 min and the supernatant was withdrawn. The cells were incubated for 5 min at 92°C for inactivation of DNAses and afterwards resuspended in PCR reaction mixture.

Table 14 : Primer combinations, annealing temperatures and negative controls for colony PCR

Strain	Construct obtained	Forward primer	Reverse primer	T_m °C	Product length [bp]
WT	ISC1	ndeI_ISC1_fw	notI_ISC1_rv	55	1462
WT	flag_ISC1	ndeI_flag_ISC1_fw	notI_ISC1_rv	50	1483
WT	flag_370stop_ISC1	ndeI_flag_ISC1_fw	notI_370stop_rv	50	1165
WT	flag_417stop_ISC1	ndeI_flag_ISC1_fw	notI_417stop_rv	50	1309
isc1	-	ndeI_ISC1_fw	notI_ISC1_rv	55	(none)
isc1	-	ndeI_flag_ISC1_fw	notI_ISC1_rv	50	(none)
isc1	-	ndeI_flag_ISC1_fw	notI_370stop_rv	50	(none)
isc1	-	ndeI_flag_ISC1_fw	notI_417stop_rv	50	(none)

PCR reaction mix:

26.5	µl	deion. H_2O (fill up to 50 µl)
10	µl	Phusion HF buffer (5X)
5	µl	dNTPmix of 2 mM each (final conc.: 200 µM each)
2.5	µl	Primer fw of 10 µM (final conc.: 0.5 µM)
2.5	µl	Primer rv of 10 µM (final conc.: 0.5 µM)
3	µl	DNA template. pelleted cells
0.5	µl	Phusion- Polymerase
50µl		

PCR program: 98°C/30 sec – (98°C/10 sec- 50/55°C/20 sec - 72°C/25 sec) x30 – 72°C/10 min – 4°C/∞

PREPARATIVE AND ANALYTICAL AGAROSE GELS

Mostly agarose gels with 1% (w/v) agarose were cast (2.5 g in 250 ml TAE buffer) and supplemented with ethidium bromide for detection of DNA bands under UV light. For preparative purposes, 10x75 µl combs were used and separation occurred at 90V. Forpurely analytical gels the applied voltage seldom was below 120 V. The samples were loaded onto the gel after addition of stained loading buffer.

The DNA fragments resulting from colony PCR were purified on agarose gels, excised and extracted from the gel by use of Wizard SV Gel and PCR Clean-Up System. These fragments were then cut with restriction enzymes, as were the plasmids.

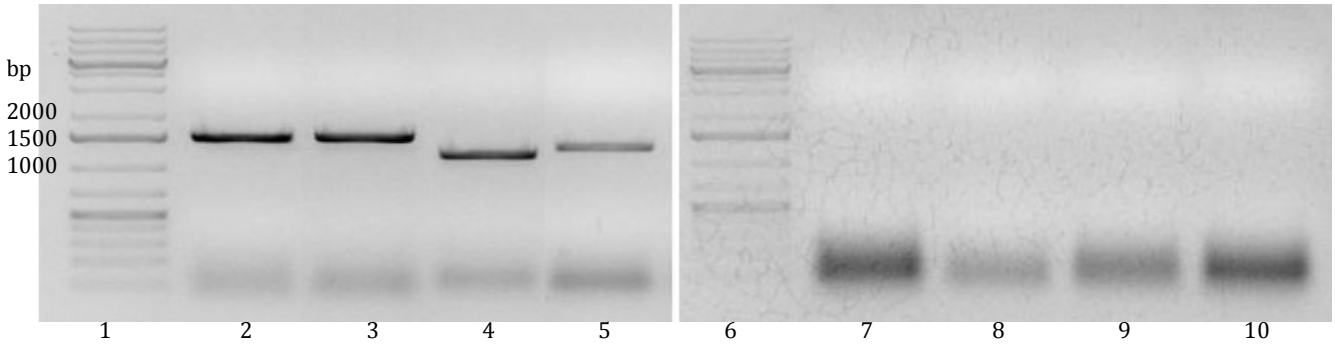


Figure 9: Images taken from the preparative agarose gels of the colony PCR. The bands on the left hand side correspond neatly to the anticipated sizes of 1462 (2), 1483 (3), 1165 (4) and 1309 (5) base pairs. Although the difference between the largest two fragments cannot be seen on this gel photo, the sequence coding for the FLAG-tag is included in the forward primer for product 2 (Tables 8 and 14, respectively). The right panel shows the PCR product of using the same primers and conditions with the *isc1* knockout strain PH18. These reactions (7-10) did not yield any product since no *ISC1* gene is present in this strain.

ELECTROCOMPETENT E . COLI CELLS

To obtain cells that are receptive for plasmids and DNA fragments, dubbed competent cells, these cells were grown, washed and stored at -80 °C according to standard protocol. Thirty ml of LB medium were inoculated with a single colony of the desired strain, preferably from a relatively young agar plate. If desired and possible, antibiotics can be added to the media for the overnight cultures, for example 12.5 µg/ml tetracycline for Origami B cells. After shaking at 110 rpm and at 37°C over night, 500 ml of LB media were inoculated with 5 ml of the overnight culture. The

cultures were harvested when OD_{600} reached 0,7-0,8, typically after about three h but depending on the strain. Culture medium is removed by centrifugation and after multiple washing steps and resuspension in sterile 10% glycerol, the cells were stored in small aliquots at -80 °C until needed.

ELECTROTRANSFORMATION

Electroporation of competent cells provides a convenient way of introducing plasmids into *E. coli*. The competent Top10F' cells were thawed on ice and transferred into prechilled electrotransformation cuvettes. The solution with plasmid that is going to be introduced should be desalted beforehand in order to prevent short-circuiting of the cuvettes which would result in severely decreased viability and transformation efficiency.

Typically 0.5-10 µl plasmid solution (10-100 ng DNA) from a minipreparation or 20 µl from a ligation step were blended with the electrocompetent cells. Upon placing the cuvettes in the electroporator device the cells were electro shocked (program EC2). Fast addition of 920 µl SOC medium results in increased viability and after incubation for 1 h at 37 °C and 650 rpm, the cells were plated onto selective media. In the case of Origami B and BL 21 Star cells transformed with pMS470_d8_linker and pCm_dsbC_linker such plates would be LB-ampicillin and LB-chloramphenicol plates, respectively. If desired, the cells were pipetted into an overnight culture directly and cultivated in a liquid media. This was done for obtaining the necessary amount of pMS470_d8_linker and pCm_dsbC_linker with Top10F' cells, since only very little of both plasmids was available.

DOUBLE DIGEST WITH NDEI AND NOTI

After plasmid extraction with a miniprep isolation kit, the necessary amount of plasmids was on hand. The colony PCR products and the plasmids were digested with *Nde*I and *Not*I restriction enzymes for cloning. Digests occurred over night at 37 °C in buffer O, as suggested by the manufacturer. A subsequent purification step by agarose gel guaranteed purity and the correct size of the digestion products.

50 µl **plasmid solution** from miniprep ~50 ng/µl
7 µl buffer O (10x)
8 µl deionized water
3 µl *Not*I
2 µl *Nde*I
70 µl total volume

30 µl ISC1 constructs from **colony PCR** ~20 ng/µl
6 µl buffer O (10x)
12 µl deionized water
1 µl *Not*I
1 µl *Nde*I
50 µl total volume

LIGATION OF PLASMID BACKBONES WITH ISC1 CONSTRUCTS

T4 DNA ligase was used to join the plasmid backbones and inserts.

14.7 µl ddH_2O
1 µl Vector backbone (100 ng)
1-2 µl ISC1 construct (10 ng)
2 µl T4 DNA ligase buffer 10x
0.3 µl T4 DNA ligase
20 µl total volume

Ligation was performed at 22°C for 2 h. Afterwards the solution was heated to 65 °C for 10 min. The solutions were desalted on membrane filters drifting in ddH_2O for 30 min and then as much of the solution as could be recovered from the filters was transformed into Top10F' *E. coli* cells to amplify the plasmids.

VERIFICATION OF CORRECT INSERTS AND TRANSFORMATION INTO EXPRESSION STRAINS

Control digests of the various plasmids with insert were performed and after being deemed correct on basis of the digests, two of each plasmid kind were sent for sequencing. After examining the resulting sequence data that included information from three sequencing reactions per plasmid to cover the whole inserts, the verified plasmids were transformed into the BL21 star and Origami B expression strains. Electrotransformation happened according to the description above.

Table 15: List of the generated plasmids with inserts for expression in BL21 star and Origami B

Plasmid	Insert	Size [bp]
pCm_dsbC	ISC1	6207
pCm_dsbC	flag_ISC1	6234
pCm_dsbC	flag_ISC1_370stop	5910
pCm_dsbC	flag_ISC1_417stop	6054
pCm_dsbC_linker	-	4778
pMS470_Δ8	ISC1	5434
pMS470_Δ8	flag_ISC1	5461
pMS470_Δ8	flag_ISC1_370stop	5137
pMS470_Δ8	flag_ISC1_417stop	5281
pMS470_Δ8_linker	-	4005

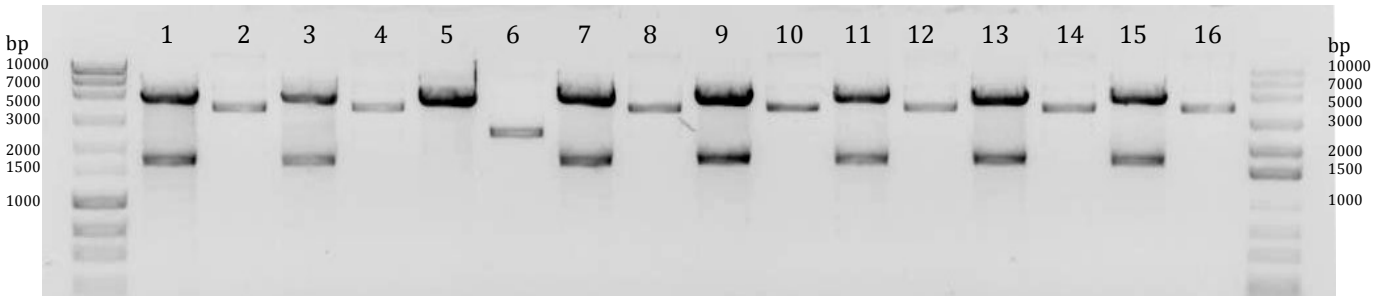


Figure 10: Photograph of an agarose gel with pCm_dsbC_ISC1 and pCm_dsbC_flag_ISC1 plasmid, digested with *Sac*I and *Pae*I (*Sph*I) to yield 4570 bp + 1637 bp and 4570bp + 1664 bp fragments, respectively, to exemplify the control digests. The difference between the tagged and the untagged constructs cannot be determined on basis of the gel, but this information was used to select plasmids for sequencing. Lanes 1-8 represent four plasmids with *ISC1* coding sequence, whereas the odd numbered lanes are the digested samples and the even lanes are undigested plasmid. Similarly, four plasmids with FLAG-tagged *ISC1* can be seen digested and undigested on lanes 9-16. Interestingly the plasmid on lane 6 appears to be much smaller and also lacks the smaller band on lane 5. The plasmids represented in lanes 2, 4, 10 and 12 were chosen to be sequenced. The other plasmids with inserts listed in table 15 were checked in a similar fashion.

E. COLI FERMENTATION AND HARVESTING

Overnight cultures in LB medium with 100 µg/ml ampicillin or 10 µg/ml chloramphenicol, respectively, were used to inoculate the 500 ml main cultures with 10 ml of overnight culture each. After incubation for roughly 6 h at 100 rpm and 28 °C, an OD_{600} of 0.6-0.7 was reached. Expression from the *tac* promoter was induced by addition of 0.1 mM IPTG to the culture media and the fermentation continued over night, at 100 rpm and 28 °C . On the next day a final OD_{600}

measurement was followed by cell harvesting via centrifugation and cell pulping using sonification. The first centrifugation step was 10 min at 4000x g in a precooled centrifuge. After removal of the supernatant, the pellets were resuspended in 30 ml of 20 mM potassium phosphate buffer, pH 8.0, and transferred into 50 ml Greiner tubes. After centrifugation at 4000 rpm for 10 min, again in a cooled centrifuge, the supernatant was discarded and the pellets weighed and resuspended in 25 ml of potassium phosphate buffer. Ultra sonic pulping proceeded for 5 min in a pulping beaker that was placed in ice cooled water in order to prevent excess heating of the samples, the sonifier was set to 80 % duty cycle and output control 8. The resulting cell homogenate was transferred to 50 ml centrifugation tubes and spun for 1 h at 10 °C and 75,600 x g. It was unclear whether Isc1p was to be found in the supernatant or in the pellet, so the supernatants were sterile filtered with 0.2 µm filters and the pellets resuspended in 4x the weight (in gram) of 20 mM potassium phosphate buffer, pH 8.0 (in ml), with the help of Dounce homogenizers. Both fractions were stored at -20 °C.

PROTEIN CONCENTRATION DETERMINATION

The total protein concentrations of the supernatants and the pellet fractions were determined with Bio-Rad Bradford reagent. One hundred and sixty µl of ddH_2O and 40 µl Bradford reagent were mixed per sample and 200 µl of this mix were pipetted into each well of a 96 well micro plate. The use of micro plates allows for convenient multiple determinations of the protein concentrations. Ten µl of the sample solution, properly diluted, were added to the dilute reagent. After 10 min of thorough mixing with the lid on the plate, the absorption at 595 nm was determined with a plate reader. The samples were measured at least threefold at and the calibration function was determined with BSA 1.0/0.5/0.25/0.125/0.0625 mg/ml in the same manner.

Table 16: Calibration function data for protein concentration determination

Sample	A-595-1	A-595-2	A-595-3	Average
BSA 1,0mg/ml	1.16	1.17	1.14	1.16
BSA 0,5mg/ml	0.80	0.85	0.85	0.83
BSA 0,25mg/ml	0.63	0.60	0.57	0.61
BSA 0,125mg/ml	0.50	0.49	0.47	0.50
BSA 0,0625mg/ml	0.41	0.42	0.42	0.41
blank	0.35	0.35	0.38	0.36

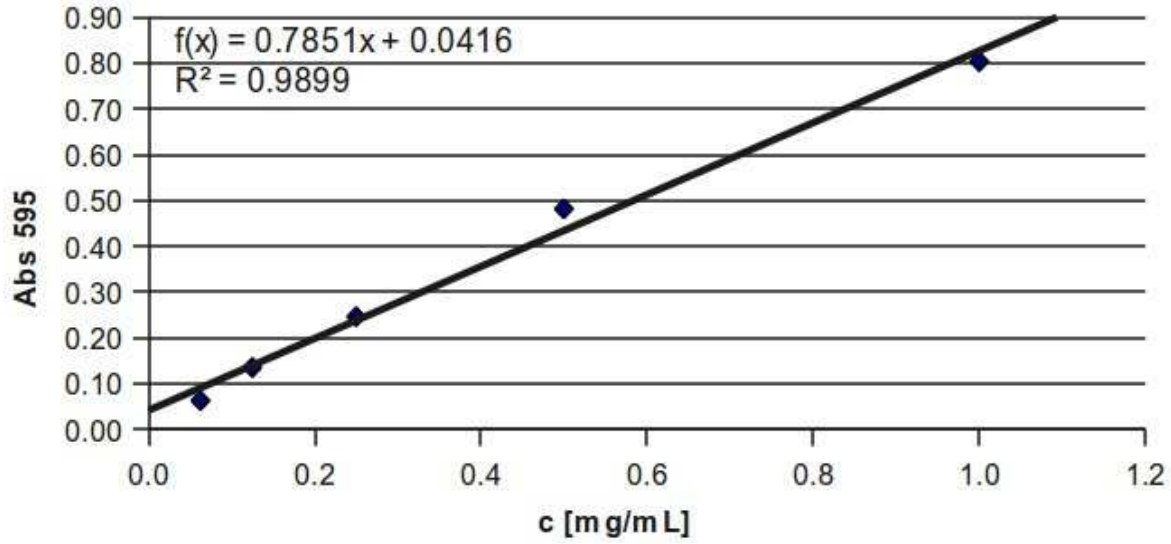


Figure 11: Calibration function for protein concentration determinations. The function f(x)= 0,7851x + 0,0416 was used for all further calculations.

SDS-PAGE AND WESTERN BLOTTING

After the total protein concentration in the samples had been determined, the samples were analyzed via sodium dodecyl sulfate – polyacrylamide gel electrophoresis, which separates the proteins according to their size. Invitrogen's NuPAGE system was used, which comprises precast polyacrylamide gels and a handy device for letting the gels run, the Mini-cell. The samples were treated according to the NuPAGE manual, which is similar to the standard protocol for protein gel electrophoresis published in 1970 (Laemmli, 1970). The samples were diluted as to load a maximum of 5 µg protein per slot. Sample preparation included the addition of lithium dodecyl sulfate sample buffer and reducing agent. After incubating the samples for 10 min at 40°C, a total of 10 µl was pipetted into the wells and separation occurred for 50 min by applying 200 V constant and limiting the current to 110 mA/gel. The gels were either stained using the Roth Roti-Blue staining solution or were subjected to Western blotting. Roti-Blue dye binds primarily to protein and delivers less background color in the gel compared to Coomassie blue, which decreases the time necessary for washing steps. Since the objective was to specifically detect Isc1p, Western blotting and immunodetection was performed primarily.

Blotting of proteins onto nitrocellulose membranes occurred again using the NuPAGE Mini-cell and the associated blotting module. Within the blotting module, the blotting membrane, the gel, filter paper sheets and blotting pads were assembled like depicted in Figure 12. For blotting the proteins onto the membrane, the conditions were as follows: 120 V and 250 mA for 1 hour.

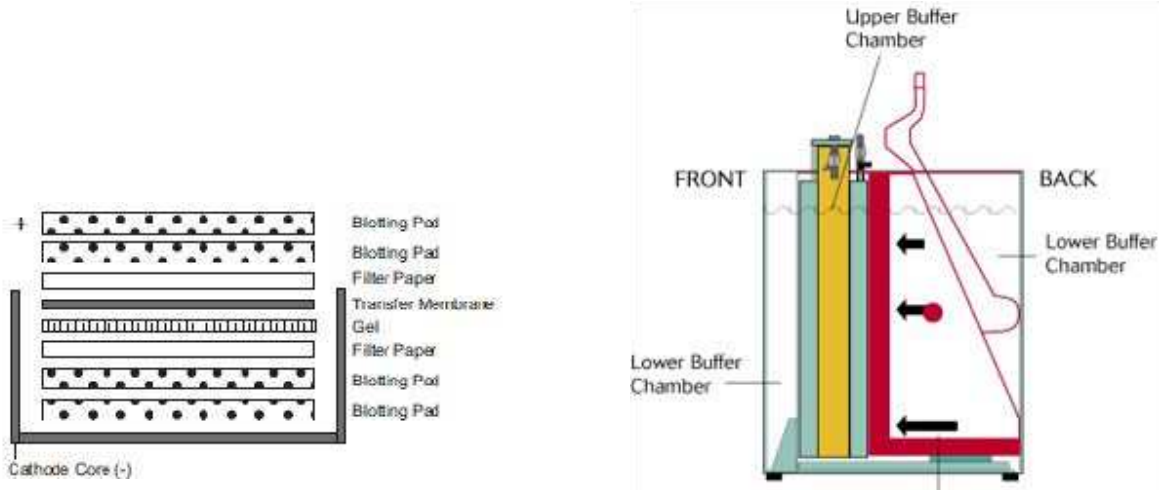


Figure 12: This figure illustrates how the sponges, filter paper, gel and nitrocellulose membrane were arranged for Western blotting. The right panel shows a schematic representation of how the electrophoresis chamber looks like and how it is assembled.

After completing the blotting procedure, the nitrocellulose membranes were stained using Ponceau S to detect proteins. After photographing the stained membranes, they were subjected to blocking and simultaneous decoloring with TBST-milk buffer for 1 h at 37 °C or over night at 4 °C, respectively. After washing with TBST, the primary antibody solution was applied for 1 h at 37 °C and under constant moderate shaking. This step was followed by 5x 5 min washing with TBST and incubation with the secondary antibody again for 1 h at 37 °C. After another series of washing with TBST 3x 3 min, the detection reaction with Pierce SuperSignal West Pico Chemiluminescent substrate for HRP was performed and imaged with the G:Box. Alternatively, the presence of secondary antibody with horseradish peroxidase was detected using Pierce 1-Step TMB blotting substrate solution.

OVEREXPRESSION IN *S. CEREVISIAE* WITH PLASMID PYES2

Three different Isc1p constructs were inserted into the *S. cerevisiae* expression plasmid pYES2. One construct was without an attached tag, one featured the eight amino acid N-terminal FLAG-tag (Hopp, 1988) and one construct featured N-terminal GFP tag. The GFP sequence was from the plasmid pFA6a-GFP(S65T)-kanMX6 (Longtine, 1998).

In order to obtain information on the localization of Isc1p within *S. cerevisiae* cells, the planning involved two distinct approaches: The first involving Western blotting and immunodetection of cell fractionations with suitable antibodies and the second approach was based on the fusion of Isc1p and green fluorescent protein, thereby elegantly allowing tracking of the protein in live cells via fluorescence microscopy techniques. Unfortunately the extent of all these experiments went beyond the scope of a Master's thesis. The constructs' activity in various yeast strains was assayed and the localization studies were postponed. The GFP tagging approach hinges mainly on (mis-)localization of the hybrid protein as well as improper folding as a result of the tagging with a roughly 230 amino acid green fluorescent protein tag. Bioinformatics' tools did not predict a signal sequence encoded within the *ISC1* gene, hence N-terminal tagging was no issue. Clean sample preparation without carryover of contamination is key to performing cell fractionation.

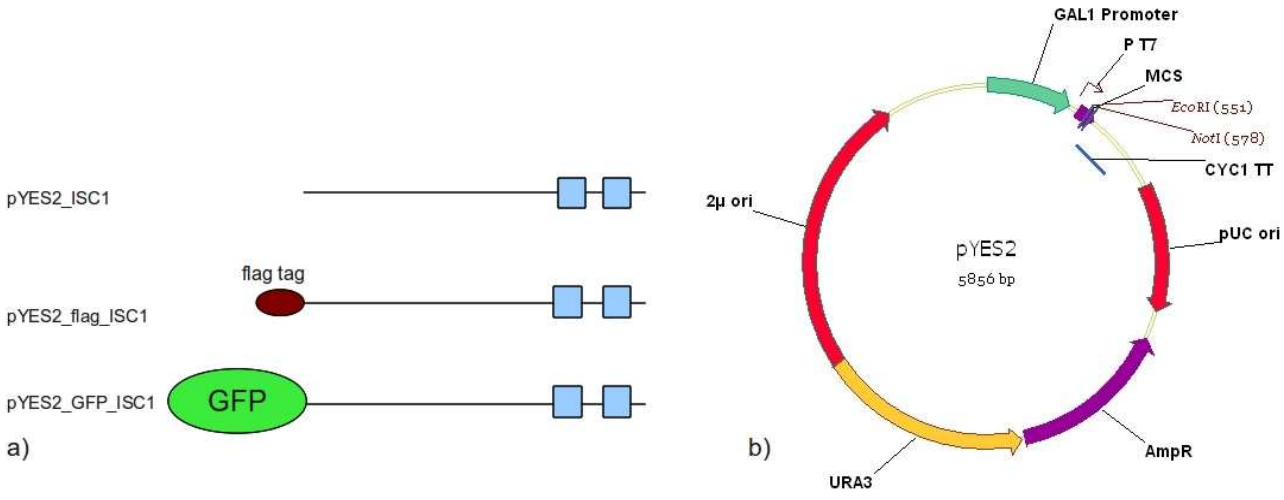


Figure 13: a) Schematic depiction of the inserts for pYES2 plasmid. b) The shuttle vector pYES2 can conveniently be transformed into *E. coli* for amplification, since it features the pUC origin of replication in addition to the yeast 2µ ori and an ampicillin resistance marker. Selection for successful transformation in *S. cerevisiae* happens via *URA3* marker and uracil auxotrophy of the strains used. The galactose inducible GAL1 promoter in combination with the CYC1 transcription terminator provide efficient transcription of the gene inserted via *Eco*RI and *Not*I restriction enzymes.

OBTAINING THE SEQUENCES FOR CLONING

To generate the fragments for cloning into the pYES2 backbone with *Eco*RI and *Not*I the following PCRs were performed:

***ISC1* for pYES2**		
28.5	µl	dd H_2O (fill up to 50 µl)
10	µl	Phusion HF buffer (5X)
5	µl	dNTPmix of 2 mM each (final: 200 µM each)
2.5	µl	pYES2_noflag_ISC1_fw 10 µM
2.5	µl	notI_ISC1_rv 10 µM
1	µl	pMS470_flag_ISC1 10 ng/µl
0.5	µl	Phusion- Polymerase
50 µl		

flag_*ISC1* for pYES2		
28.5	µl	dd H_2O (fill up to 50 µl)
10	µl	Phusion HF buffer (5X)
5	µl	dNTPmix of 2 mM each
2.5	µl	pYES2_flag_ISC1_fw 10 µM
2.5	µl	notI_ISC1_rv 10 µM
1	µl	pMS470_flag_ISC1 10 ng/µl
0.5	µl	Phusion- Polymerase
50 µl		

PCR program: 98°C/30 s – (98°C/10 s- 60°C/20 s - 72°C/30 s) x30 – 72°C/10 min – 4°C/∞

The sequence GFP_ISC1 for pYES2 cloning was obtained by performing overlap PCR with GFP and *ISC1* cassettes that were generated using overlapping primers.

***ISC1* for overlap**		
28.5	µl	dd H_2O (fill up to 50 µl)
10	µl	Phusion HF buffer (5X)
5	µl	dNTPmix of 2mM
2.5	µl	ISC1_oe_fw 10 µM
2.5	µl	notI_ISC1_rv 10 µM
1	µl	pMS470_flag_ISC1 10 ng/µl
0.5	µl	Phusion- Polymerase
50µl		

GFP for overlap		
28.5	µl	dd H_2O (fill up to 50 µl)
10	µl	Phusion HF buffer (5X)
5	µl	dNTPmix of 2 mM each
2.5	µl	pYES2_GFP_fw 10 µM
2.5	µl	GFP_rv 10 µM
1	µl	pMS470_flag_ISC1 10 ng/µl
0.5	µl	Phusion- Polymerase
50µl		

PCR program: 98°C/30sec – (98°C/10sec- 50°C/20sec - 72°C/30sec) x30 – 72°C/10min – 4°C/∞

Overlap extension PCR		
21.5	µl	dd H_2O (fill up to 50 µl)
10	µl	Phusion HF buffer (5X)
5	µl	dNTPmix of 2 mM each
5	µl	ISC1 for overlap 50 ng/µl
5	µl	GFP for overlap 50 ng/µl
2	µl	pYES2_GFP_fw 10 µM
2	µl	not!_ISC1_rv 10 µM
0.5	µl	Phusion- Polymerase
50 µl		

PCR program: 98°C/30 s – (98°C/10 s- 45°C/30 s - 72°C/30 s) x25 – 72°C/10 min – 4°C/∞

The increasing sizes of the products from the overlap PCRs were monitored by agarose electrophoresis. Fragments of the correct size were excised for the next step.

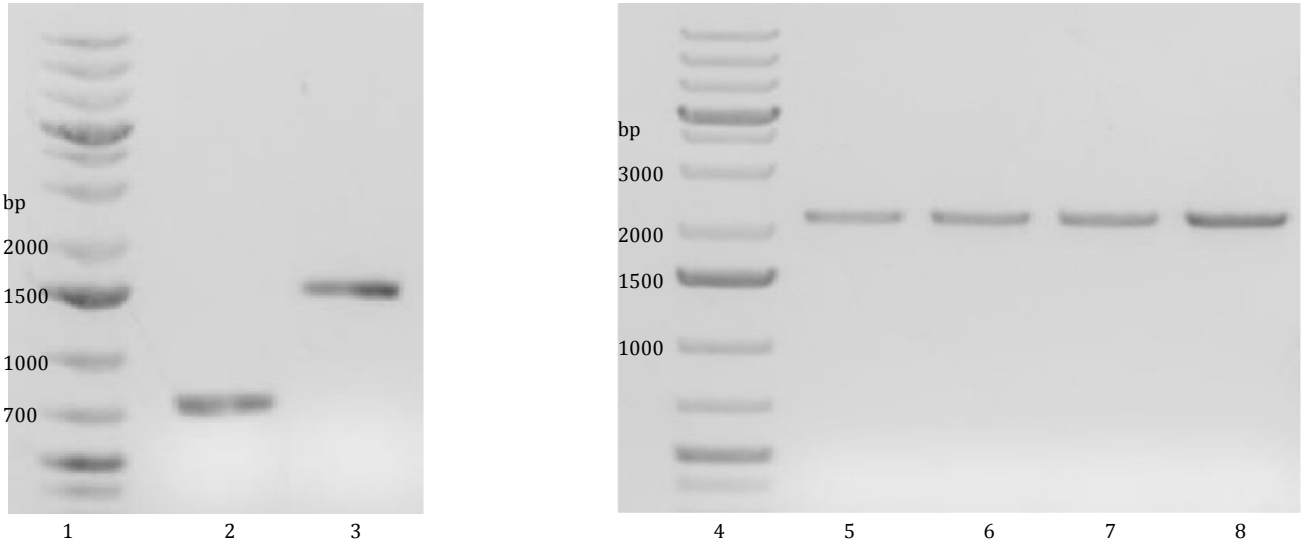


Figure 14: Fragments, GFP (2) and *ISC1* (3), for overlap PCR and subsequent cloning into pYES2. The GFP band is in accordance with the expectations at ~720 bp and the ISC1 band with overlapping regions and restriction site can be found at ~1500 bp. The panel on the right hand side shows the product of the overlap extension PCR ~2200 bp (5-8).

Cloning into pYES2 and controls

After a digest with *Eco*RI and *Not*I, the pYES2 backbone was joined with the inserts in a ligation reaction as described above. Subsequent control digests assured the quality of the constructed plasmids. Control digests of the pYES2 plasmid with insert were performed with *Bam*HI (two restriction sites) and with *Eco*RI and *Not*I (Figure 15 and Table 17). After the plasmid sizes were deemed correct, they were sent sequencing with the following primers: gal1_seq_fw, ISC1_mid_seq_rv, cyctt_seq_rv and the plasmid with the GFP tag was sequenced with an additional primer, ISC1_mid_seq_fw, to cover the whole construct. The verified plasmids were transformed into Top10F' cells to store and replicate them.

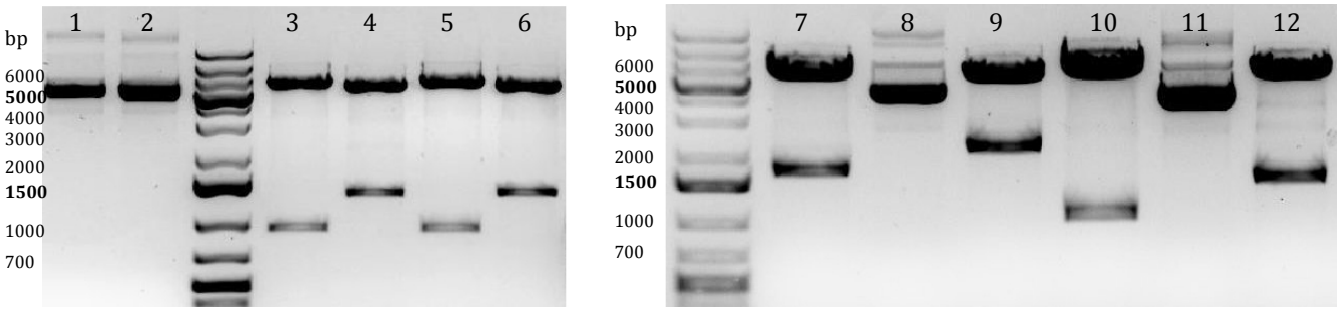


Figure 15: Left panel: lanes 1+2 show undigested pYES2_ISC1 plasmid, lanes 3+5 the fragments resulting from *Bam*HI digest and the lanes 4+6 fragments from *Eco*RI and *Not*I digest. The fragment sizes correspond neatly to the predicted sizes listed in Table 17. The lanes designated 7+8+9 show the pYES2_GFP_ISC1 plasmid digested with BamHI, undigested and digested with EcoRI and NotI, respectively. Lanes 10+11+12 show the corresponding FLAG-tagged plasmid, again digested as well as undigested. The actual size of the plasmids can be obtained by addition of the fragment sizes, the undigested plasmids do not run at the correct size.

Table 17: pYES2 constructs: total size and control digest fragments

Plasmid	Restriction digest	Expected fragments [kbp]	Total size [bp]
pYES2_ISC1	*Bam*HI	~6.2 + ~1.1	7273
pYES2_ISC1	*Eco*RI + *Not*I	~5.8 + ~1.5	7273
pYES2_GFP_ISC1	*Bam*HI	~6.3 + ~1.7	7972
pYES2_GFP_ISC1	*Eco*RI + *Not*I	~5.8 + ~2.2	7972
pYES2_flag_ISC1	*Bam*HI	~6.3+ ~1.05	7300
pYES2_flag_ISC1	*Eco*RI + *Not*I	~5.9 + ~1.5	7300

CHEMICALLY COMPETENT S. CEREVISIAE AND TRANSFORMATION

5 ml of liquid YPD medium were inoculated with a single colony from a plate and incubated under constant shaking at 30°C over night. The *erg3 erg6* double knockout strain PH11 was incubated at 24°C, because it did grow very slowly at 24°C but not at all at 30°C. The main culture was inoculated with the according volume of overnight culture to an OD_{600} of 0.1. After reaching OD_{600} of 0.3-0.5 the main cultures were harvested by centrifugation form 10 min at 2500 rpm in a 50 ml centrifuge tube. Afterwards, the cells were resuspended once in 25 ml ddH_2O and once in 5 ml LiOAc mix. LiOAc mix was prepared by mixing 1 ml 10x TE buffer, pH 7.5, with 1 ml of 1 M lithium acetate solution and 8 ml of ddH_2O. After pelleting the cells again, they were resuspended in 0.5 ml of LiOAc mix and divided into 100 µl aliquots. For a transformation, 10 µl of single stranded salmon DNA 2 mg/ml and 0.5-10 µg of the DNA to be transformed were added to the aliquots. For transforming large plasmids, rather 10 µg DNA was used and for inserting cassettes for homologous integration only 0.5 µg were taken. After pipetting 500 µl PEG mix into the tubes, the contents were mixed. PEG mix comprised 1 ml 10x TE buffer, 1 ml of 1 M LiOAc and 8 ml of 50 % (w/v) PEG 3350. After incubation for 30 min at 30°C and addition of 50 µl DMSO, the transformation mixes were incubated for 15 min at 37°C or 42°C, respectively. The less viable strain PH11 was incubated at 37°C. Following a quick spin in a microcentrifuge for 10 s, the supernatant was removed and the cells were resuspended in 500 µl YPD medium and regenerated for 2 h. Pelleting in a microcentrifuge, resuspension in 200 µl TE buffer pH 7.5 and plating out on selective plates completed the procedure. Since the pYES2 backbone features the *URA3* selection marker for *S. cerevisiae*, the cells were plated on SD-Ura.

Table 18: Transformations into *S. cerevisiae*

Host strain	**Transformed DNA**
PH2 haploid WT	pYES2, pYES2_ISC1, pYES2_flag_ISC1, pYES2_GFP_ISC1 flag_ISC1 integration cassette, GFP_ISC1 integration cassette
PH11 haploid *erg3 erg6*	pYES2, pYES2_ISC1, pYES2_flag_ISC1, pYES2_GFP_ISC1
PH18 haploid *isc1*	pYES2, pYES2_ISC1, pYES2_flag_ISC1, pYES2_GFP_ISC1
PH49 diploid *erg3*/*ERG3 erg6*/*ERG6*	pYES2, pYES2_ISC1, pYES2_flag_ISC1, pYES2_GFP_ISC1 flag_ISC1 integration cassette, GFP_ISC1 integration cassette

Figure 16: Growth of the PH49 strain with and without transformation on YPD containing 200 µg/ml G418. Selection on SD-Ura plates worked similarly well, but 0,2 % yeast extract allowed for slow growth of untransformed cells. Initially, the G418 concentration used was 100 µg/ml but that proved insufficient. Even on the plate depicted, the diploid *erg3/ERG3 erg6/ERG6* strain PH49 can grow, very slowly though (between 5 and 7 o'clock). The haploid double knockout PH11, *erg3 erg6*, had difficulties simply growing at 28 °C on YPD plates.

After transformation with the integration cassettes carrying the kanMX6 cassette, the selection occurred on YPD plated containing G418. For a negative control in expression experiments, the empty pYES2 plasmid was transformed as well (Table 18).

To confirm the presence of the correct plasmids in the cells, a series of colony PCRs was performed. With the help of various primer combinations it was possible to distinguish between the empty plasmid, FLAG-tagged plasmid and GFP tagged plasmid. Colony PCR was performed as stated above and was found to yield the best results when the plates never were refrigerated and not older than three days. When fresh cells are employed, it is sufficient to slightly touch a colony with a pipette tip and resuspend the cells in the PCR mixture, which then will be slightly opaque.

Table 19: Primers for transformation verification, expected fragment size and annealing temperature

Plasmid	Forward primer	Reverse primer	Expected fragment [bp]	T_m
pYES2 empty	Gal1_seq_fw	Cyc1_seq_rv	~280	40
pYES2_ISC1	Gal1_seq_fw	ISC1_mid_seq_rv	~450	40
pYES2_flag_ISC1	pYES_flag_ISC1_fw	ISC1_mid_seq_rv	~430	55
pYES2_GFP_ISC1	pYES2_GFP_fw	GFP_rv	~700	55

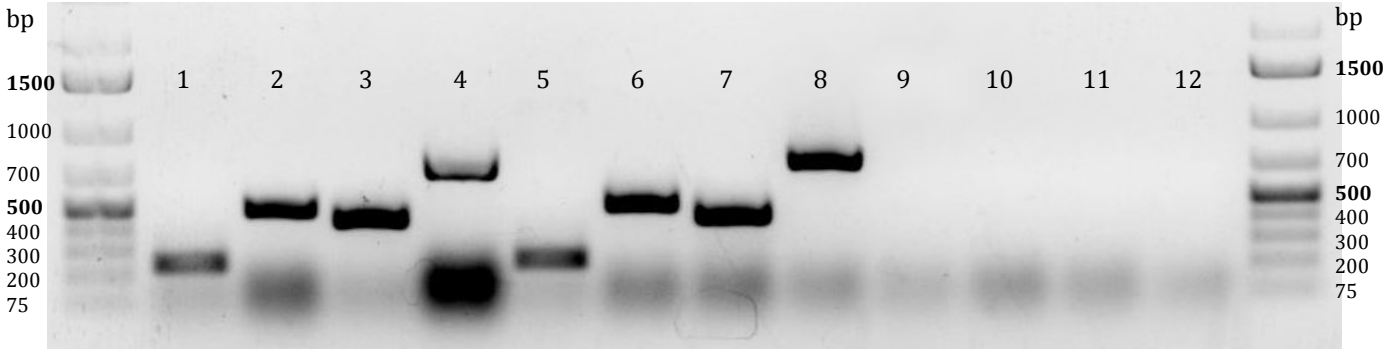


Figure 17: Colony PCR of strains carrying plasmids pYES2 (1, 5), pYES2_ISC1 (2, 6), pYES2_flag_ISC1 (3, 7) and pYES2_GFP_ISC1 (4,8). Primer and expected fragment size are listed in Table 19. Lanes 9-12 contain negative control with WT strain PH 2 and the same forward and reverse primer combinations.

S. CEREVISIAE FERMENTATION

Several strains were grown in shaking flasks in order to perform various sphingomyelinase activity assays in vivo and in vitro. Beyond the activity assays, a couple of Western blot experiments with the different Isc1p constructs were performed in order to verify the presence of the proteins, tagged and untagged. The fermentation in shaking flasks was performed in accordance to the pYES2 manual and started with small cultures in 30 ml SD-Ura liquid medium containing 2 % glucose and 0.2 % yeast extract that were incubated under constant shaking at 150 rpm overnight at 30°C, except for strain PH11 at 24°C. After determining the OD_{600}, the main cultures were inoculated to an OD_{600} of 0.2. The main culture medium was 150 ml of SD-Ura, 2 % raffinose and 0,2% yeast extract. Incubation occurred under unchanged conditions. After four hours, galactose was added to a final concentration of 2%. Samples were taken immediately after induction and at several time points until the end of fermentation.

The use of raffinose is reasonable, because it does not repress the GAL1 promoter as the presence of glucose does. Raffinose is expensive, though. This is why for the following fermentations I did not use raffinose as carbon source, but rather overnight cultures with 2% glucose and main cultures with 2% galactose, separated by pelleting and resuspending the cells in order to remove the medium with residual glucose.

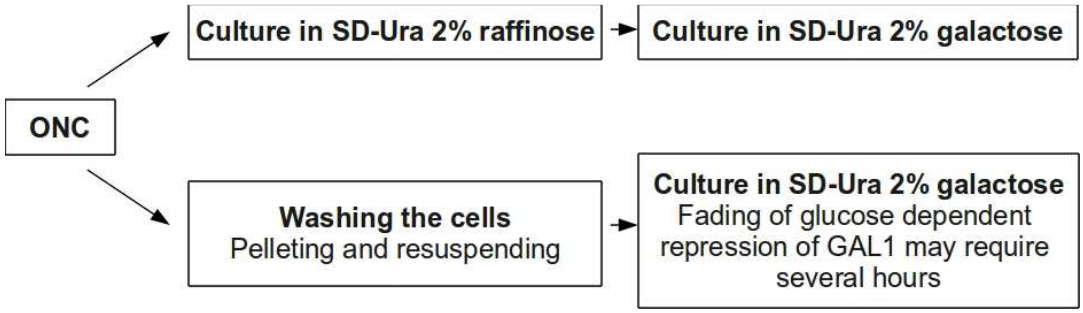


Figure 18: This figure illustrates two ways of dealing with the repression of GAL1 promoter by presence of glucose in the fermentation medium. As raffinose does neither induce nor repress GAL1 transcription and addition of galactose to the medium leads to immediate induction of transcription, growing cells in medium containing raffinose as carbon source would be the way to go. Unfortunately, it is expensive. Galactose and glucose are commonplace in molecular biology laboratories and so the lower path in the scheme was preferred, although main culture fermentation lasted longer in order to factor the time for de-repression into the whole fermentation duration.

RAPID PREPARATION OF TOTAL PROTEIN EXTRACT

Total protein extracts from *S. cerevisiae* were performed by the following protocol. Three OD_{600} equivalents of late exponential cells were centrifuged and the supernatants were discarded, leaving only about 0.5 ml of medium in the tube. After addition of 50 µl of 1.85 M NaOH, the mix was left on ice for 10 min. Following the addition of 50 µl of 50 % w/v trichloroacetic acid, the mix was again incubated for 20 min on ice. Centrifugation at 12000 x g for 5 min in a microcentrifuge allowed for removal of the supernatant and resuspension of the pellet in 70 µl of 1 part NuPAGE sample buffer, 1 part 1 M Tris base and 2 parts ddH_2O with 2 % 2-mercaptoethanol. After 10 min at 40 °C, the samples were loaded onto SDS-PAGE gels and further analyzed.

ASSEMBLY OF CASSETTES FOR HOMOLOGOUS INTEGRATION

For studying localization and activity without encountering the effects of overexpression on the whole system, it was decided that the expression of tagged Isc1p variants with the intrinsic promoter of the *ISC1* gene might be useful. To reach this goal, a cassette featuring the desired gene variant and a selection marker were transformed into the strains PH2 and PH49. Both are wild type strains, distinct by their ploidy. Two cassettes were generated with N-terminal FLAG-tag and GFP tag, respectively. Transcription from *ISC1* promoter to the *ISC1* transcription terminator would also include the whole kanMX6 resistance marker module for the antibiotic G418 (geneticin), unless there is another terminator region inserted in between.

To prevent this unintentionally long transcript, the CYC transcription terminator from the pYES2 plasmid was included in the cassettes after the sequence coding for Isc1p constructs. The building blocks were generated via PCR. The GFP sequence and the kanMX6 module originated from the plasmid pFA6a-GFP(S65T)-kanMX6. The CYC transcription terminator and *ISC1* sequences were amplified from the plasmid pYES2 and pMS470_flag_ISC1, respectively (Figure 19).

The fragments were purified from agarose gels and subjected to overlap extension PCR. The final products of the correct size were sequenced, with the primers flag_hom_fw, GFP_hom_fw, GFP_rv, ISC1_mid_seq_rv, ISC1_mid_seq_fw, ISC1_oe_rv, ISC1_oe_fw cyctt_fw, cyctt_rv, kanMX6_fw, kanMX6_mid_seq_fw and kanMX6_hom_rv. Integration cassettes with the correct sequences were transformed as described above.

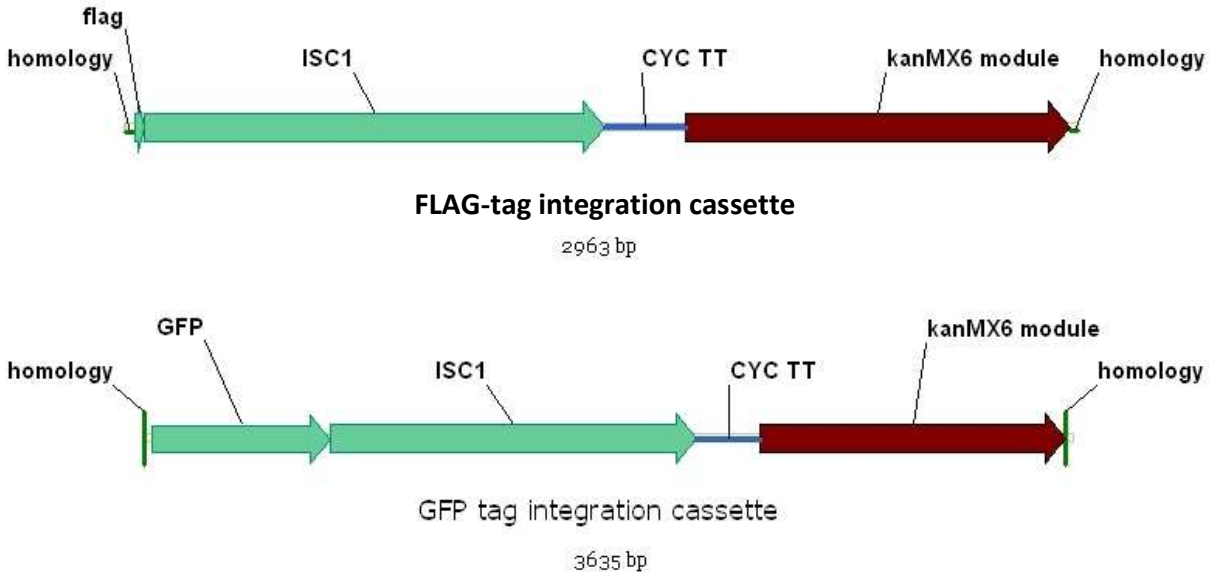


Figure 19: Layout of two integration cassettes. The cassettes feature relatively short regions flanking the actual insert. These regions are homologous to the sequences 5' and 3' of *ISC1* gene on *S. cerevisiae* chromosome V. Thus, the cassettes will be inserted into the chromosome effectively replacing the *ISC1* gene. The homologous regions had been added by the primers flag_hom_fw, GFP_hom_fw and kanMX6_hom_rv. The kanMX6 module includes all elements necessary for transcription of its gene. Therefore, an additional transcription terminator region from the plasmid pYES2 was included between tagged Isc1p genes and kanMX6 module.

Table 20: Conditions and ingredients for the overlap extension PCRs

GFP + ISC1			**CYC TT + kanMX6**		
23.5	µl	ddH_2O	18.5	µl	ddH_2O
10	µl	Phusion HF Buffer 5x	10	µl	Phusion HF Buffer 5x
5	µl	dNTP Mix (2 mM each)	5	µl	dNTP Mix (2 mM each)
2	µl	oe ISC1 from (100ng/µl)	10	µl	oe cyc tt from (20ng/µl)
5	µl	oe GFP from (40 ng/µl)	2	µl	oe kanMX6 from (100 ng/µl)
2	µl	GFP_hom_fw	2	µl	cyctt_fw
2	µl	ISC1_oe_rv	2	µl	kanMX6_hom_rv
0.5	µl	Phusion Polymerase	0.5	µl	Phusion Polymerase
50	µl		50	µl	
flag-ISC1 + CYC TT					
18.5	µl	ddH_2O			
10	µl	Phusion HF Buffer 5x			
5	µl	dNTP Mix (2 mM each)			
10	µl	oe cyc tt from (20ng/µl)			
2	µl	oe flag ISC1 from (100 ng/µl)			
2	µl	flag_hom_fw			
2	µl	cyctt_rv			
0.5	µl	Phusion Polymerase			
50	µl				
25 cycles: 98°C/30 s - (98°/10 s - 45°/30 s - 72°/45 s)x25 - 72°/10 s - 4°/ ∞					

Flag-tagged cassette 3kb			**GFP-tagged cassette 3.6kb**		
22.5	µl	ddH_2O	22.5	µl	ddH_2O
10	µl	Phusion HF Buffer 5x	10	µl	Phusion HF Buffer 5x
5	µl	dNTP Mix (2 mM each)	5	µl	dNTP Mix (2 mM each)
4	µl	flag-ISC1-cycTT from previous PCR (50 ng/µl)	4	µl	GFP-ISC1 from previous PCR (50 ng/µl)
4	µl	kanMX6 from 30.07.09 (50 ng/µl)	4	µl	cycTT-kanMX6 from previous PCR (50 ng/µl)
2	µl	flag_hom_fw	2	µl	GFP_hom_fw
2	µl	kanMX_hom_rv	2	µl	kanMX_hom_rv
0.5	µl	Phusion Polymerase	0.5	µl	Phusion Polymerase
50	µl		50	µl	
25 cycles: 98°C/30 s - (98°/10 s - 45°/30 s - 72°/80 s)x25 - 72°/10 s - 4°/ ∞					

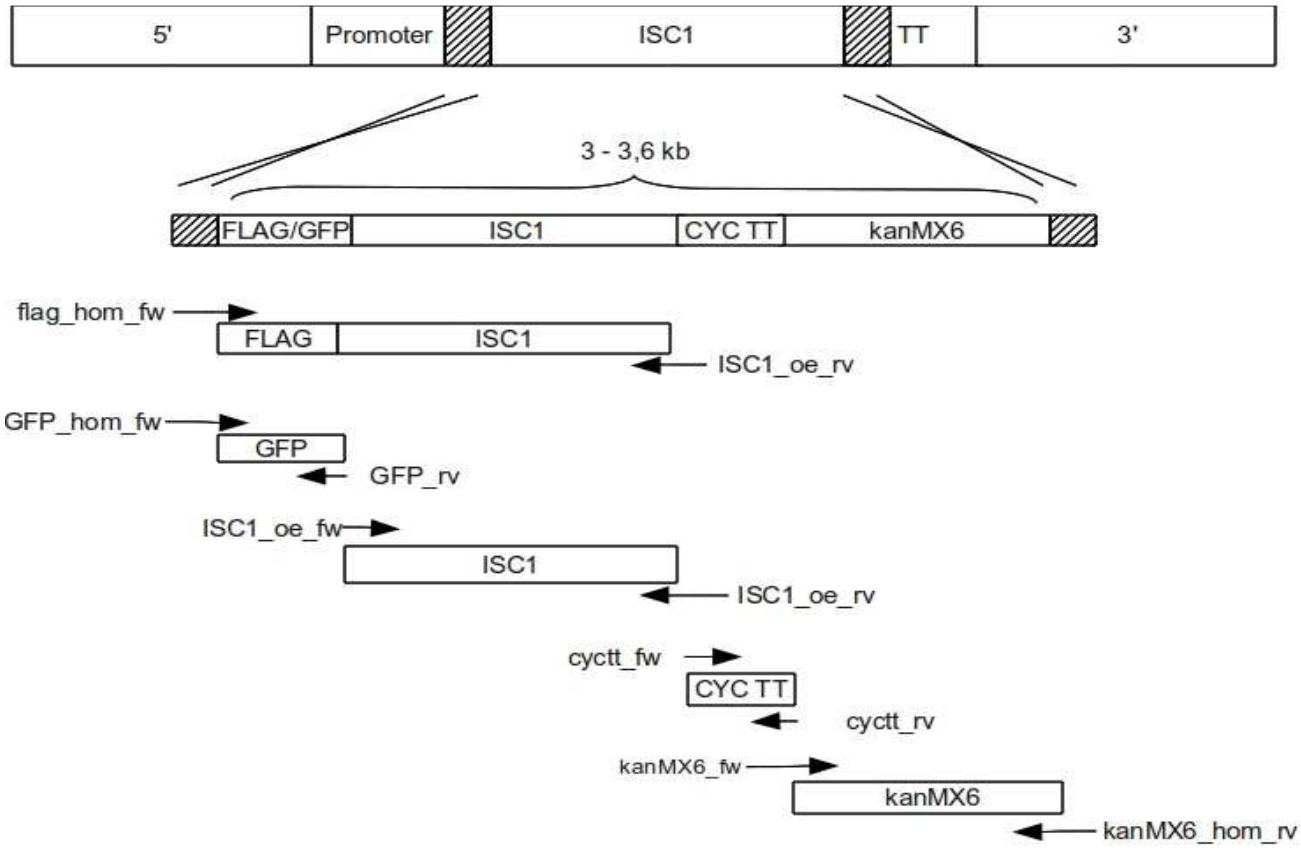


Figure 20: This scheme indicates how the various overlapping fragments were sequentially assembled into larger building blocks. Flag_ISC1 sequence and CYC terminator were joined and afterwards the kanMX6 module was attached to yield the overall 2963 bp FLAG-tag insertion cassette. The GFP tag cassette was generated similarly, only that GFP constituted an additional building block. GFP and *ISC1* sequences were joined, as were CYC tt and kanMX6, before the resulting fragments were again joined in an overlap extension PCR to yield 3635 bp GFP tag cassette. The reactions included the respective end primers and the extension time was adjusted to the resulting fragment length.

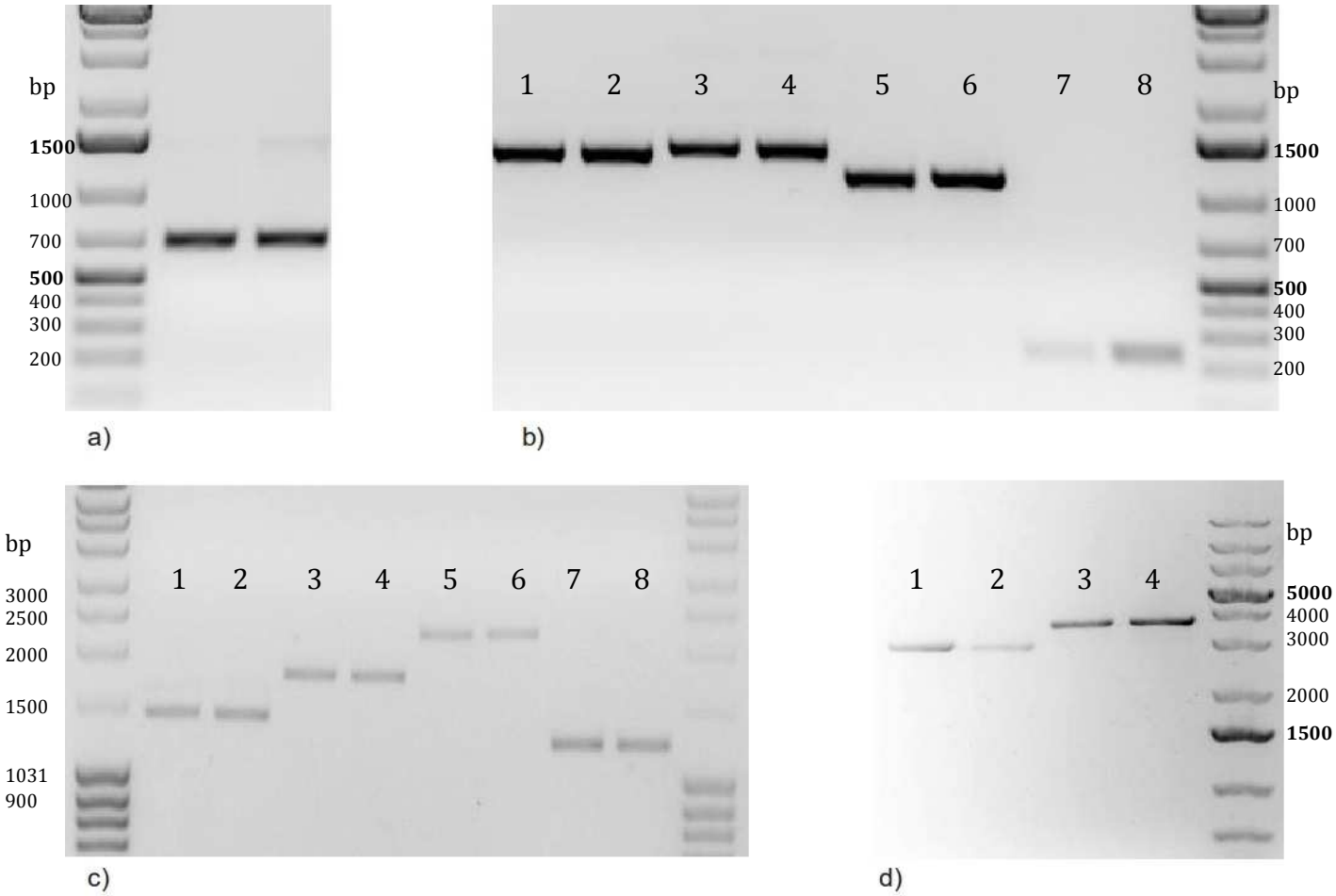


Figure 21: This collection of agarose gel images documents the gradual assembly of the integration cassettes. a) Roughly 700 bp fragments of GFP, two reactions in parallel. b) Lanes 1+2, *ISC1* for overlap extension ~1450 bp; 3+4, flag_ISC1 for overlap extension ~1500 bp; 5+6, kanMX6 ~1240 bp and 7+8, CYC terminator region ~250 bp. c) Overlap PCR results as described in table 20. Lanes 1+2, kanMX6+cyctt ~1470 bp; 3+4, flag_ISC1+cyctt ~1730 bp; 5+6, GFP+ISC1 ~2190 bp and 7+8, kanMX6 ~1240 bp. d) This gel image presents the complete integration cassettes of ~3000 and ~3600 bp, respectively. For detailed descriptions, see figures 19 and 20.

MUTAGENESIS OF TRANSMEMBRANE DOMAINS AND WHOLE CODING SEQUENCE

A minor part of this thesis dealt with random mutagenesis of the whole sequence coding for *ISC1* on the one hand and the mutagenesis of the two predicted transmembrane helices on the other hand. A large collection of mutagenized *ISC1* genes in combination with high efficiency transformation and a suitable *S. cerevisiae* strain would allow screening. A synthetic defect strain that survives certain conditions, e.g. cultivation at certain temperature, only when functional/dysfunctional Isc1 protein is present is necessary for screening. A strain that survives at the given temperature only when the *ISC1* copy does not yield a functional protein will help identify the amino acid exchanges that yield a defunct enzyme. Hot spots for such fateful amino acid exchanges are expected to be the active site and substrate binding pocket, as well as the parts of the protein that are involved in activating interactions with other membrane components.

Mutagenesis occurred via error prone PCR with Gene Morph random mutagenesis kitto amplify the selected regions and thereby introducing point mutations. Subsequently, quick change PCR was performedto incorporate the mutagenized sequences into functional *S. cerevisiae* expression plasmid. The plasmid pYES2_ISC1 with the coding sequence for the untruncated and untagged Isc1p was used as template in the mutagenesis reactions as well as in the quick change reaction. The mutagenized parts annealed to the plasmid and DNA synthesis along the templates ensured the incorporation of these parts into the plasmids. The nicks were repaired after transformation into *E. coli*. The mutagenesis reactions aimed for (i) a mutation rate of 5 point mutations per whole gene and (ii) 3 single nucleotide exchanges for the ~242 bp region that contains the transmembrane helices. This goal was achieved by tweaking the parameters for the Mutazyme II polymerase PCR, namely by increasing the cycle number for the PCR and by decreasing the amount of template. Quite the opposite effect can be achieved by a decrease in cycle number and an increase in amount of template DNA. Whether the desired mutation rate was acchieved was checked by sequencing a couple of plasmids and calculating an average mutation rate. After optimizing of the parameters the desired rates were obtained and the plasmids were stored away at -80 °C. After the mutagenic PCR, the fragments were purified on agarose gels. After the Q-PCR, the products were digested with *Dpn*I, to cut the methylated restriction sites in order to get rid of the template plasmids prior to electrotransformation.

Table 21: Mutagenesis and Quickchange PCR conditions

Mutagenesis PCR whole coding sequence			**Q-PCR**		
3	µl	pYES2_ISC1 100 ng/µl	1	µl	pYES2_ISC1 100 ng/µl
5	µl	Mutazyme II Buffer 10x	15	µl	Fragment from Mut. PCR
5	µl	dNTP Mix (2 mM each)	5	µl	dNTP Mix (2 mM each)
2.5	µl	ISC1_mut_fw 10 µM	5	µl	Pfu Ultra buffer 10x
2.5	µl	ISC1_mut_rv 10 µM	1.6	µl	DMSO
31	µl	dd H_2O	21.4	µl	dd H_2O
1	µl	Mutazyme II Polymerase	1	µl	Pfu Ultra Polymerase
50	µl		50	µl	
95°C/2 min - (95°/30 s - 42°/30 s - 72°/2 min)x20 - 72°/10 s - 4°/ ∞			95°C/2 min - (95°/50 s - 60°/30 s - 68°/16 min)x25 - 72°/10 s - 4°/ ∞		

2x Mutagenesis PCR transmenbrane regions			**PCR Q**		
1	µl	pYES2_ISC1 1 ng/µl or 1 µl of 1:100 dilution from PCR 1 for the second PCR	1	µl	pYES2_ISC1 100 ng/µl
5	µl	Mutazyme II Buffer 10x	15	µl	Fragment from Mut. PCR
5	µl	dNTP Mix (2 mM each)	5	µl	dNTP Mix (2 mM each)
2.5	µl	ISC1_TM_mut_fw 10 µM	5	µl	Pfu Ultra buffer 10x
2.5	µl	ISC1_TM_mut_rv 10 µM	1.6	µl	DMSO
33	µl	dd H_2O	21.4	µl	dd H_2O
1	µl	Mutazyme II Polymerase	1	µl	Pfu Ultra Polymerase
50	µl		50	µl	
95°C/2 min - (95°/30 s - 42°/30 s - 72°/50 s)x35 - 72°/10 s - 4°/ ∞			95°C/2 min - (95°/50 s - 60°/30 s - 68°/16 min)x25 - 72°/10 s - 4°/ ∞		
1 µl of a 1:100 dilution of the resulting fragments from the first mutagenesis reaction of the transmembrane regions was used to perform another step of mutagenesis using the same conditions to meet the desired mutation frequency.					

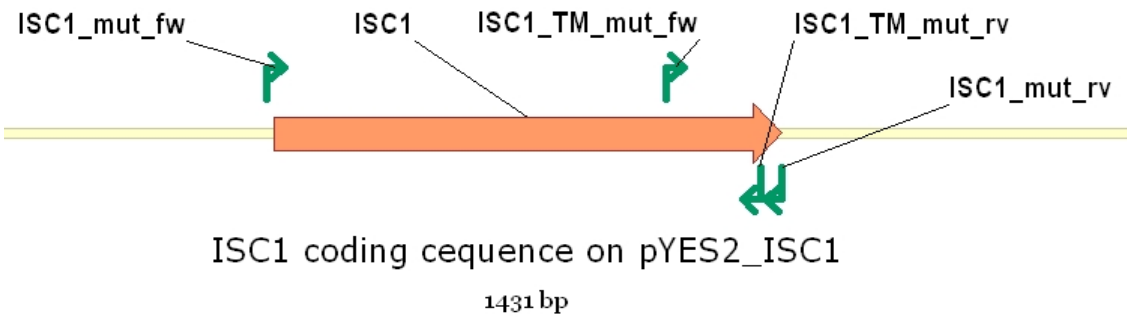


Figure 22: Mutagenesis primer binding sites on the *ISC1* coding sequence and on the vector backbone outside the coding sequence.

For digest, 1 µl of the *Dpn*I enzyme solution was added directly to the PCR reaction solution, which suits the enzyme well. Incubation occurred for 3 h at 37 °C and inactivation happened by incubation for 30 min at 70°C. Two µl of each reaction were transformed into *E. coli* Top10F' cells like described above. Single clones were picked and sent for sequencing and mutation rate analysis.

Table 22: Mutation rates

Whole *ISC1* gene, 1431 bp

	Mutations counted
cloneI	9
cloneII	3
cloneIII	4
cloneIV	2
average	4,5

Transmembrane region, 242 bp

	Mutations counted
cloneI	4
cloneII	0
cloneIII	3
cloneIV	2
cloneV	6
cloneVI	0
average	2,5

RADIOASSAY FOR ISC1P

Based upon the differing hydrophobicity characteristics of apolar ceramide backbones and polar headgroups, this assay yields quantitative data for phospholipase C activity (i.e. Isc1p activity). Besides Isc1p, *S. cerevisiae* do not possess additional enzymes exhibiting phospholipase C activity on complex sphingolipids like inositolphosphate ceramide (IP-C) and mannoysl inositolphosphate ceramide (MIP-C) (Matmati, et al., 2008). This radioassay employs ^{14}C labeled sphingomyelin, which is not present in *S. cerevisiae.* IPC, MIPC and $M(IP)_2C$ are the major yeast sphingolipids. Due to rather pronounced promiscuity of Isc1p, sphingomyelin is nevertheless cleaved into a less polar ceramide backbone and the hydrophilic headgroup phosphocholine, which is bearing three ^{14}C labeled methyl groups. After repeated washing and phase separation steps, the radioactive isotopes are either present in the aqueous phase as phosphocholine or in the organic phase as the unprocessed substrate sphingomyelin (Figure 23). Radioactivity in the water phase is detected using Ultima Gold liquid scintillation counting cocktail and disintegrations per minute (dpm) can be calculated from the counts per minute (cpm) via count yield calibration samples. Due to the nature of the numerical output from the liquid scintillation counting machine, this assay qualifies exquisitely for quantification Isc1p activity in vitro, in contrast to the NBD-SM assay which yields basically qualitative data.

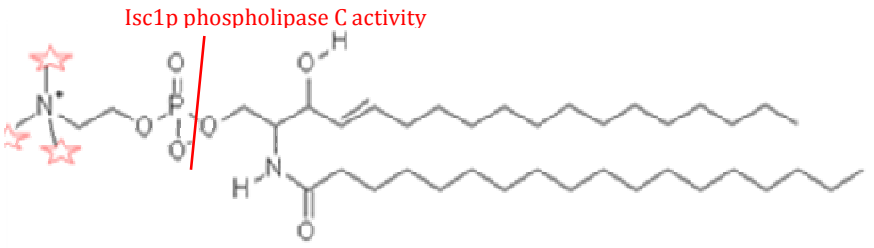


Figure 23: Schematic depiction of the radioactively labeled sphingomyelin and the site of Isc1p enzymatic activity. After cleavage of the phosphoester bond, the highly hydrophilic and radioactively labeled phosphocholine moiety is being found in the aqueous phase, whereas the uncleaved compound prefers the organic phase.

The phospholipase activity assay was modeled after an assay described in literature (Vaena de Avalos, et al., 2004). *S. cerevisiae* strains were grown in 100 ml liquid SD-Ura media and expression from the pYES2 plasmid was induced by utilizing galactose as the carbon source in the main culture and removing residual glucose containing medium from the overnight cultures, as described above. For the radioassay, the cells were disrupted and proteases were inhibited by addition of PMSF using the following protocol. The cells were pelleted by centrifugation at 3800 rpm for 5 min at room temperature and the supernatants were removed. The pellets were resuspended in 0.5 volumes (v/w) of buffer A, 0.1 M Tris, H_2SO_4, pH 9.4. Per 1 ml of buffer A, 1.54 g dithiothreitol was added and the mix was incubated for 10 min at 30°C. Buffer A was added to 50 ml and the cells were pelleted again at 3800 rpm for 5 min. The cell pellet was resuspended in 0.15 volumes (v/w) of buffer B, 20 mM KPi, pH 7.4. Upon addition of 2 mg zymolyase 20T per gram pellet weight, the mix was incubated for 60 min at 30°C under moderate shaking. Filling with buffer B to 50 ml and cell pelleting as before was followed by resuspension in 1 ml of 10 mM Tris/HCl pH 7.4 per g pellet. After addition of PMSF to 1 mM, the cells were disrupted with a Dounce homogenizer and the homogenates stored at -20 °C.

The total protein concentration of the samples was determined to use the same amount of protein in all radioassays. Twenty µl of dilute sample, amounting to 10- 300 µg total protein, were mixed with 80 µl reaction buffer to yield the following final conditions per 100 µl sample: 100 mM Tris, pH 7.5, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0,1% Triton X-100, 10 nmol phosphatidylserine, 10 nmol unlabeled sphingomyelin and 100,000 dpm [choline-methyl-^{14}C]-sphingomyelin. The reaction buffer was subjected to sonication prior to mixing with the sample in order to establish vesicles.

In brief, the 100 µl of reaction mix were incubated at 30°C for 30 min sharp and the reactions were stopped by addition of 1.5 ml chloroform:methanol (2:1 v/v) and the phase separation occurred upon addition of 0.2 ml ddH_2O and centrifugation for 1 min at 1000 rpm. Four hundred µl of the upper, aqueous, phase were transferred into 8 ml Ultima Gold liquid scintillation counting cocktail and the samples were assayed in the scintillation counting machine after 1 h.

In addition to the assay series with phosphatidylserine, that exhibits an activating effect on Isc1p, assays also were performed with phosphatidylcholine instead. Furthermore, several assays were performed with varying amounts of labeled and unlabeled sphingomyelin. Varying amounts of Triton X-100 and some assays in absence of any additional phosphatidylserine or phosphatidylcholine were performed in order to leave the membrane composition in the homogenates unchanged to mirror more closely the biological conditions.

NBD-SM ASSAY

In contrast to the radio assay, this activity assay was executed in live *S. cerevisiae* cells. A substrate that features a sphingoid base and headgroup just like sphingomyelin, but has a fluorophore labeled fatty acid, was diluted in DMSO and was then incubated for 30 min at 24°C with *S. cerevisiae* cells. NBD-C_6-SM (C_6-((*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoyl sphingosyl phosphocholine) fluorescence was detected in the G:Box using excitation by long wave UV and Sybr Gold filter. Again, like the radio assay, this assay is based upon the different separation coefficient values of the labeled sphingomyelin-analog and the labeled ceramide without the polar phosphocholine headgroup. Whereas the uncleaved sphingomyelin-analog does not migrate in the liquid phase $CHCl_3$:MeOH:H_2O 65:25:4 on a TLC plate (silica gel 60), the ceramide does migrate. Once the head group is cleaved off, the ceramide is susceptible to further modifications in live *S. cerevisiae* cells, which is why there was not one single band of (C_6-((*N*-(7-

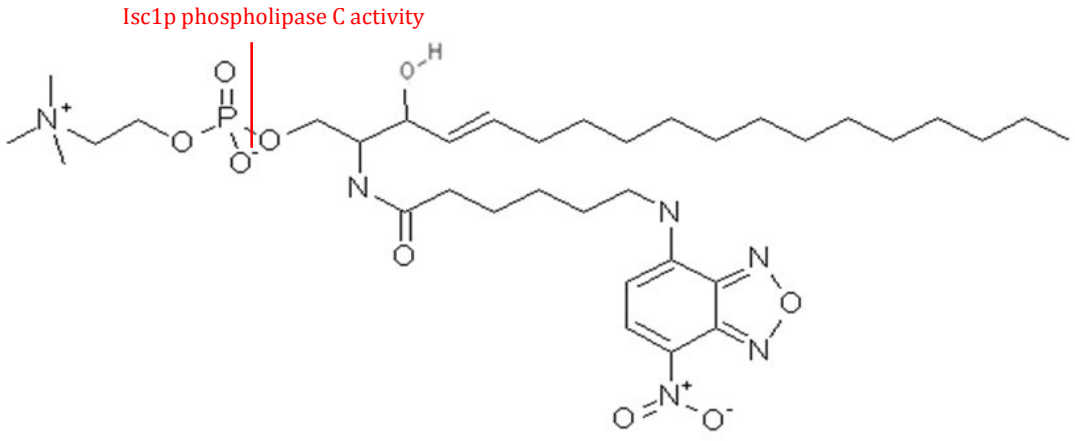


Figure 24: Schematic structure of NBD-C6-sphingomyelin. The fatty acid part of the compound features the fluorophore NBD attached to a C6 fatty acid. NBD gets excited by UV light and then emits visible light. The G:Box was used to image the migration of NBD labeled sphingomyelin and NBD labeled ceramide after phosphoester cleavage.

nitrobenz -2-oxa-1,3-diazol-4-yl)amino) hexanoyl sphingosine, but rather an array of bands. Since the final result of this assay is an image, this assay is very well suited for obtaining qualitative data about Isc1p activity in vivo. Quantification of the results turned out to be difficult.

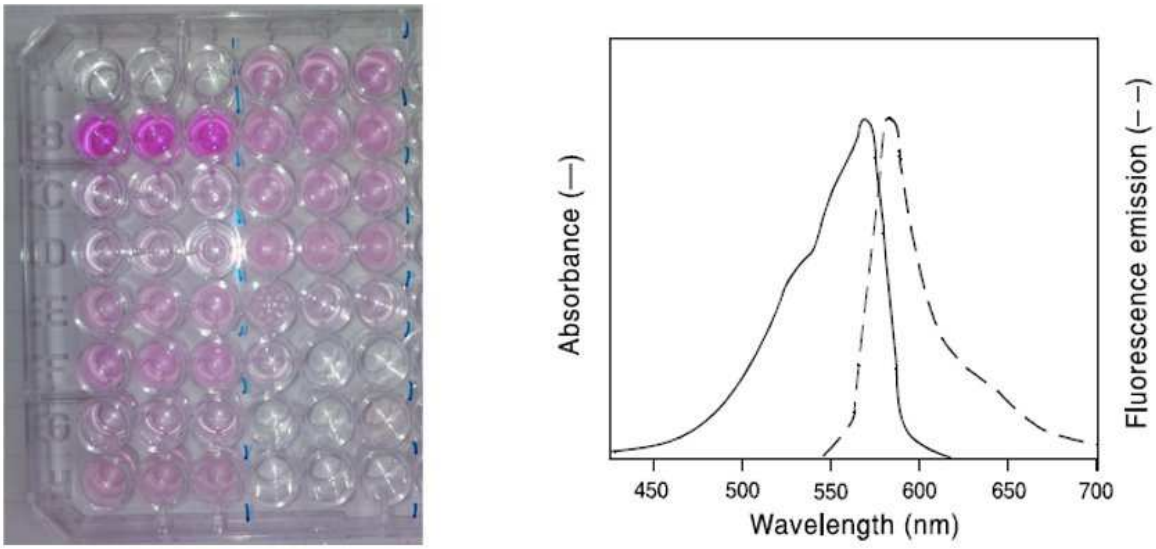
The cells were grown as described for the radio assay, but instead of homogenization the cells were diluted to 1 ml of OD_{600}=1 in SD-Ura 2% galactose medium. After addition of 0.25 µl of 10 mM NBD-C6-SM in DMSO to yield a final concentration of ~2,5 µM, the cells were incubated at 24 °C and 300 rpm for 30-60 min. Following incubation, the cells were disrupted using glass beads. The cell suspension was transferred along with 1.5 ml glass beads into glass tubes (Pyrex). Four ml $CHCl_3$:MeOH, 2:1 (v/v), were added and extraction occurred for 1-3 h at 1200 rpm. After centrifugation for 5 min at 1500 rpm, the supernatants were transferred into fresh glass tubes, and 2 ml of 0,034 % $MgCl_2$ solution was added. After 2 min of shaking and 3 min centrifugation at 2500 rpm, the upper phase was discarded. The lower phase was washed with 1.5 ml of Me:$ChCl_3$:H_2O, 48:3:47 (v/v/v), for 2 min and after centrifugation for 3 min at 2500 rpm, the upper and the interphase were discarded. Following evaporation with N_2, the extracts were dissolved in 50 µl of $CHCl_3$:MeOH, 2:1 (v/v), and 20 µl were applied onto a thin layer chromatography plate. TLCs were developed with $CHCl_3$:MeOH:H_2O, 65:25:4 (v/v/v), and the results were imaged with G:Box using EPI long wave UV excitation and SYBR Gold filter settings.

AMPLEX RED ASSAY

A third way of obtaining Isc1p activity data was tested during this thesis work. The Amplex Red Sphingomyelinase Assay by Invitrogen is an enzyme coupled assay that utilizes multiple enzyme cascades to monitor sphingomyelinase indirectly. When sphingomyelinase liberates ceramide and phosphocholine, the latter is substrate for alkaline phosphatase, generating choline and inorganic phosphate. Choline oxidase, also included in the kit, oxidizes choline to yield betaine and H_2O_2. The kit also includes horseradish peroxidase and the chromogenic compound Amplex red reagent, which reacts in presence of H_2O_2 to yield resorufin, a fluorophore. The absorption and emission maxima of resorufin are 571 and 585 nm, respectively. The generation of resorufin

can be also be seen without technical assistance by color change from transparent to pink. The assay can be performed in mircotiter plates.

The samples employed for the amplex red assay were *S. cerevisiae* homogenates from the same fermentations that also yielded cells for the radio assay and the NBD-sphingomyelin assay.



a) b)

Figure 25: a) Photograph of a 96 well microtiter plate with several amplex red sphingomyelinase assay samples. b) Excitation and emission maxima of resorufin, which is generated in the amplex red assay and can be monitored spectrophotometrically. In practice, excitation at 560 nm and emission at 590 nm gave the best results.

RESULTS

EXPRESSION OF ISC1P IN *E. COLI*

Expression of various Isc1p constructs in *E. coli* was checked by SDS-PAGE and subsequent Western blotting. The primary antibodies were mouse anti-FLAG-tag epitope M2 and the secondary antibodies were goat-anti mouse IgG with horseradish peroxidase conjugated. Detection of the bands was performed with Pierce SuperSignal West Pico Chemiluminescent substrate for HRP and Pierce 1-Step TMB blotting solution, respectively. The various constructs were expressed in the strains BL21 star and Origami B. The cells were prepared and homogenized according to the protocol described above and the pellet as well as the supernatant fractions of each sample were analyzed.

Table 23 *E. coli* samples for SDS-PAGE, Western blot and immunodetection

Sample	Strain	Plasmid insert	Expected protein size [kDa]
1	OrigamiB (DE3)	pMS470_ISC1	54
2	BL21 star	pMS470_ISC1	54
3	OrigamiB (DE3)	pMS470_flag_ISC1	56
4	BL21 star	pMS470_flag_ISC1	56
5	OrigamiB (DE3)	pMS470_flag_ISC1_370stop	43
6	BL21 star	pMS470_flag_ISC1_370stop	43
7	OrigamiB (DE3)	pMS470_flag_ISC1_417stop	48
8	BL21 star	pMS470_flag_ISC1_417stop	48
9	OrigamiB (DE3)	pMS470_linker	-
10	BL21 star	pMS470_linker	-
11	OrigamiB (DE3)	pCm_dsbC_ISC1	54
12	BL21 star	pCm_dsbC_ISC1	54
13	OrigamiB (DE3)	pCm_dsbC_flag_ISC1	56
14	BL21 star	pCm_dsbC_flag_ISC1	56
15	OrigamiB (DE3)	pCm_dsbC_flag_ISC1_370stop	43
16	BL21 star	pCm_dsbC_flag_ISC1_370stop	43
17	OrigamiB (DE3)	pCm_dsbC_flag_ISC1_417stop	48
18	BL21 star	pCm_dsbC_flag_ISC1_417stop	48
19	OrigamiB (DE3)	pCm_dsbC_linker	-
20	BL21 star	pCm_dsbC_linker	-

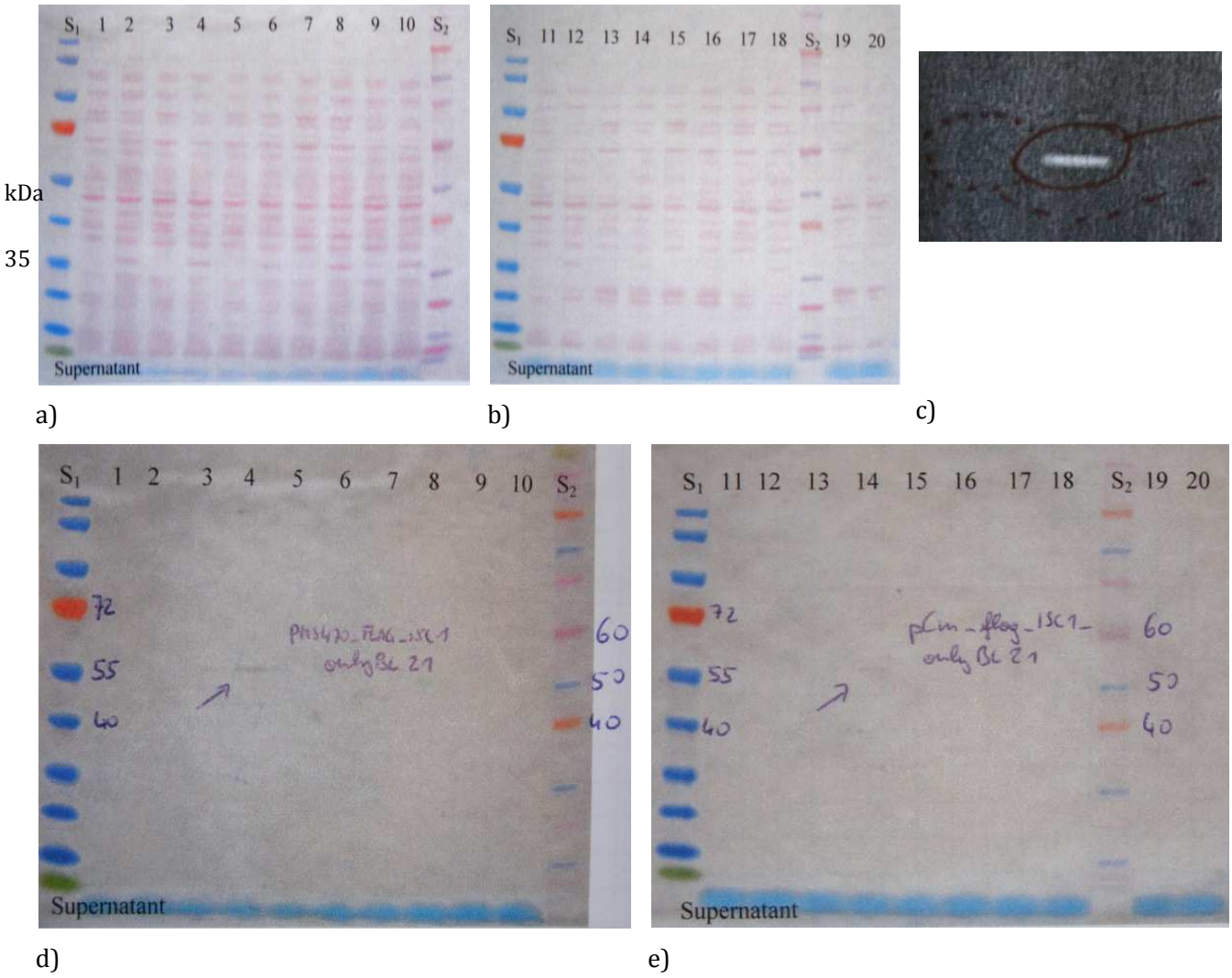
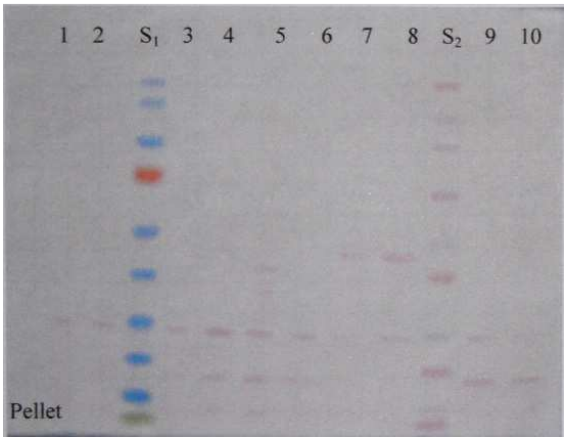
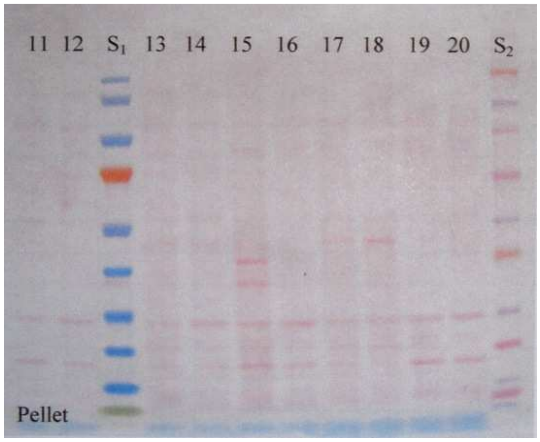


Figure 26: a) and b) Ponceau S staining after blotting the supernatant samples onto the nitrocellulose membranes. The systematic variation of bands at 35 kDa is due to the different strains. Each sample included 5 µg total proteins. c) The detection of horseradish peroxidase with SuperSignal West Pico Chemiluminescent substrate resulted in the appearance of just one single band on the two membranes. Only the flag_Isc1p construct expressed in BL21 from plasmid backbone pMS470 exhibited a band, at the correct size of 55 kDa (Table 23). The same plasmid transformed into Origami B did not yield a visible band. d) Detection of horseradish peroxidase with Pierce 1-Step TMB blotting solution. Sample 4 again is pMS470_flag_ISC1 in BL21 star. e) In contrast to the luminescent substrate, with the solution that results in insoluble precipitate, there was a very faint band on the lane of sample 14, BL21 star pCm_flag_ISC1. It seemed that only the flag_ISC1 construct in BL21 star was present in the supernatant fractions. Interestingly, this is the construct that features both transmembrane helices. Obviously, the Isc1p without FLAG-tag cannot be detected without suitable antibodies, it may have been present.



a)



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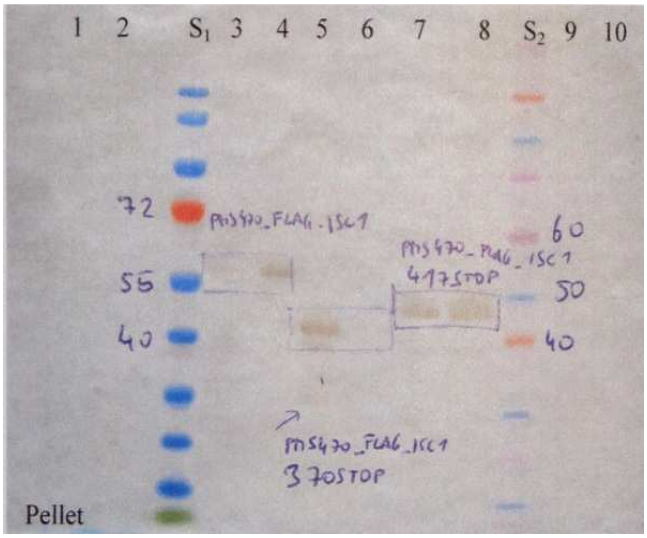
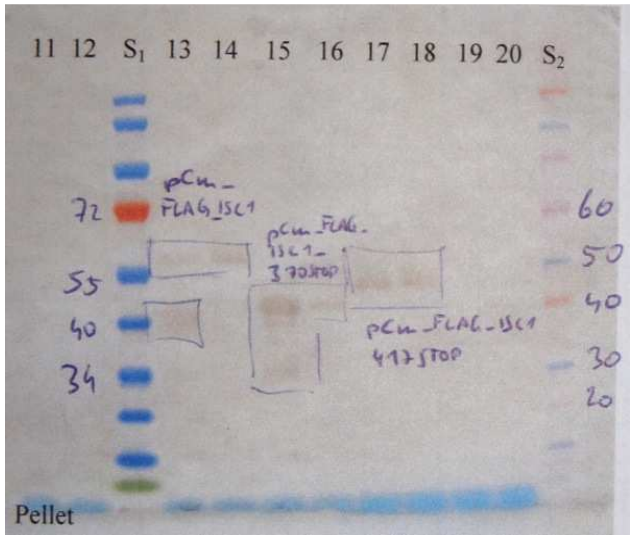
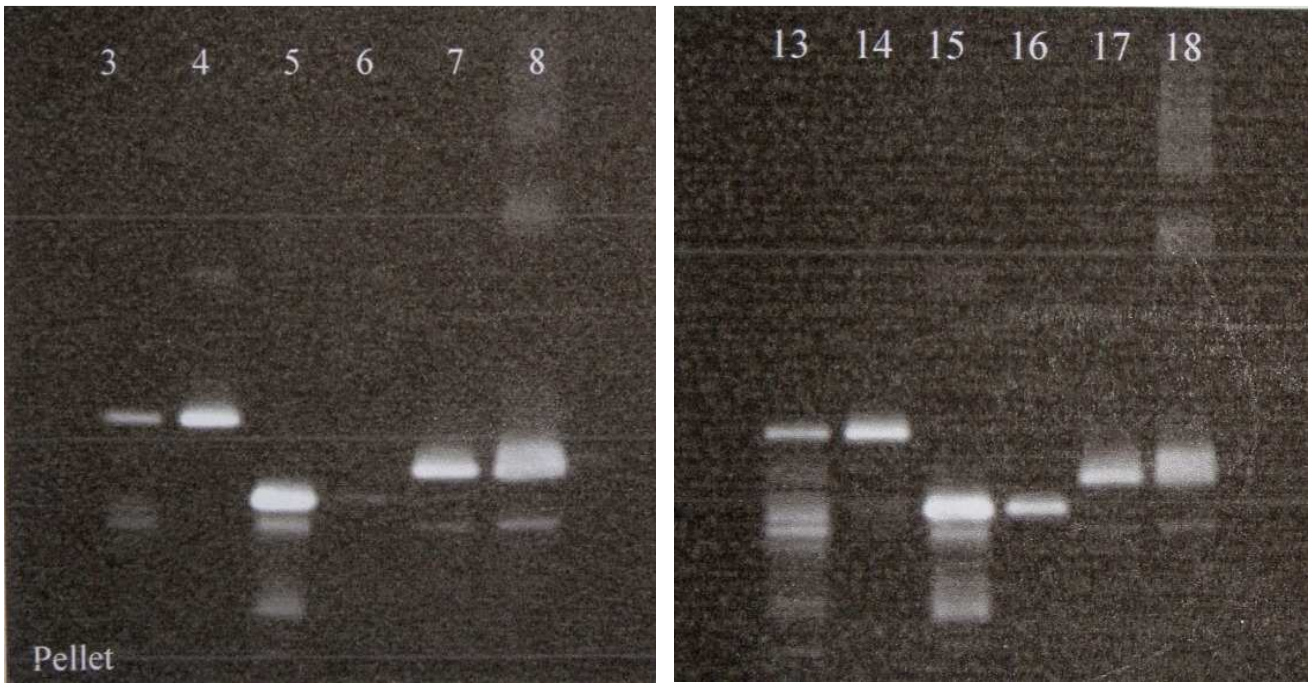


c)



d)

Figure 27: a) and b) Ponceau S staining blotting pellet samples onto the nitrocellulose membranes. Each sample comprised 5 µg total proteins. c) and d) The Isc1 protein constructs seemed to be found in the pellet fractions, which fits the expectations for an integral membrane protein. To find the constructs lacking either one or both transmembrane helices in the pellet fraction was quite a surprise, though. The expectation was that the removal of these helices would render the protein rather soluble, since the remaining catalytic domain is the water soluble part of the protein. This effect may be attributed to the formation of protein inclusion bodies. The various bands are at the expected sizes, flag_Isc1p at 55 kDa, flag_Isc1p_370stop at 43 kDa and flag_Isc1p_417stop at 48 kDa. In addition to the bands, there were also degradation products of smaller sizes. The lanes 1 + 2 and 11 + 12 feature the untagged Isc1p that cannot be detected by anti flag antibody. Lanes 9 +10 + 19 + 20 represent the empty plasmids controls.



a) b)

Figure 28: G:Box images of immunodetection of Isc1p on blots with the pellet fraction, taken to image horseradish peroxidase with chemiluminescent substrate. The lane numbering corresponds to Table 23 and the image shows in much more detail the expected bands and degradation products than in the case with the precipitation substrate for horseradish peroxidase. Interestingly, there was also unspecific smear and bands larger than the proteins, notably on lane 4, 8 and 18. On lanes 8 and 18 the constructs were loaded that feature one remaining transmembrane helix, expressed in BL21 star. The detection with chemiluminescent substrate seemed to be superior to the detection with Pierce 1-Step TMB blotting solution (compare Figure 27 c) and d)).

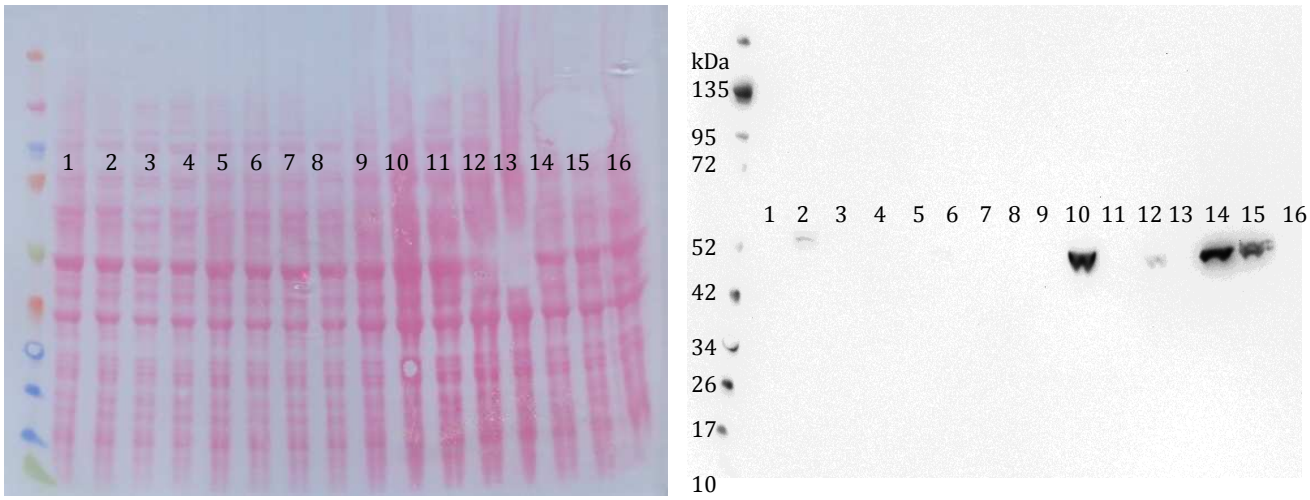
EXPRESSION OF ISC1P FROM PYES2, TAGGED AND UNTAGGED

Selected *S. cerevisiae* pYES2 transformants were subjected to further analysis. After cultivation including galactose induction, the cells were homogenized and the proteins were analyzed via SDS-PAGE and immunodetection on Western blots. Samples of the same fermentations were subjected to several sphingomyelinase activity assays in parallel, both in vitro and in vivo.

The samples for the Western blots were prepared as described above, primary antibodies were rabbit anti-Isc1p or mouse anti-FLAG-tag M2, respectively. The suitable secondary antibody-horseradish peroxidase (HRP) conjugates were goat anti-rabbit IgG or goat-anti mouse, respectively. The membranes were stripped of bound antibodies by incubation for 30 min at 50°C in 62.5 mM Tris/HCl pH 6.8, 2% SDS and 100 mM 2-mercapto-ethanol and subsequently washed with TBS and repeated blocking with TBST. It took several attempts to yield proper immunodetection results. By digitally overlaying the images taken with the G:Box without moving the membranes and adjusting the transparency, the bands can conveniently be identified.

Table 24: Samples for SDS-PAGE and Western blot analysis

	Strain		Plasmid	Hours after induction	Protein size [kDa]
1	PH2	WT	pYES2_empty	0	(54)
2	PH2	WT	pYES2_ISC1	0	54
3	PH11	*erg3 erg6*	pYES2_empty	0	(54)
4	PH11	*erg3 erg6*	pYES2_ISC1	0	54
5	PH18	*isc1*	pYES2_empty	0	-
6	PH18	*isc1*	pYES2_ISC1	0	54
7	PH18	*isc1*	pYES2_flag_ISC1	0	56
8	PH18	*isc1*	pYES2_GFP_ISC1	0	81
9	PH2	WT	pYES2_empty	17	(54)
10	PH2	WT	pYES2_ISC1	17	54
11	PH11	*erg3 erg6*	pYES2_empty	17	(54)
12	PH11	*erg3 erg6*	pYES2_ISC1	17	54
13	PH18	*isc1*	pYES2_empty	17	-
14	PH18	*isc1*	pYES2_ISC1	17	54
15	PH18	*isc1*	pYES2_flag_ISC1	17	56
16	PH18	*isc1*	pYES2_GFP_ISC1	17	81



a) b)

Figure 29: a) Ponceau S stain of Western blot (samples listed in Table 24). Protein was expressed in WT strain PH2, *erg3 erg6* strain PH11 and *isc1* strain PH18, all carrying different plasmids. b) Immunodetection of Isc1p with rabbit anti-Isc1p primary antibodies and goat anti-rabbit – HRP secondary antibodies using chemiluminescent substrate for HRP. Remarkably, all the marker proteins in the Spectra Broad range protein ladder gave a signal. It is noteworthy that on lane 2 a 54 kDa band of Isc1p expressed from pYES2 plasmid, without induction is visible. For comparison, the sample on lane 1 is the same strain with an empty plasmid. After 17 h of induction much more Isc1p was found in the cells than at the start of induction time, which is no surprise but shows that induction worked well. It seems that an air bubble was present at lane 13 at about 55 kDa during blotting. It is possible that on lane 12 the interesting *erg3 erg6* sample actually would have yielded a greater signal, were it not for that air bubble in the direct vicinity.

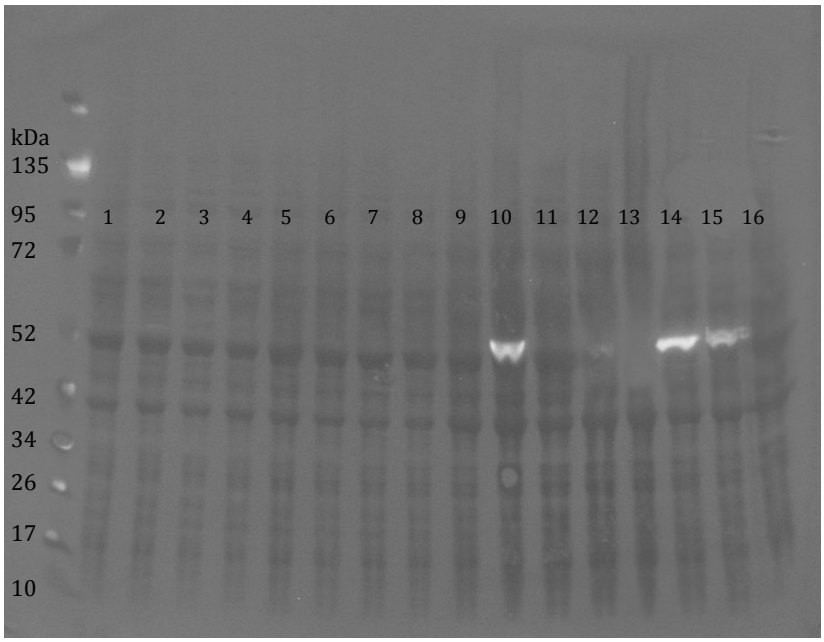
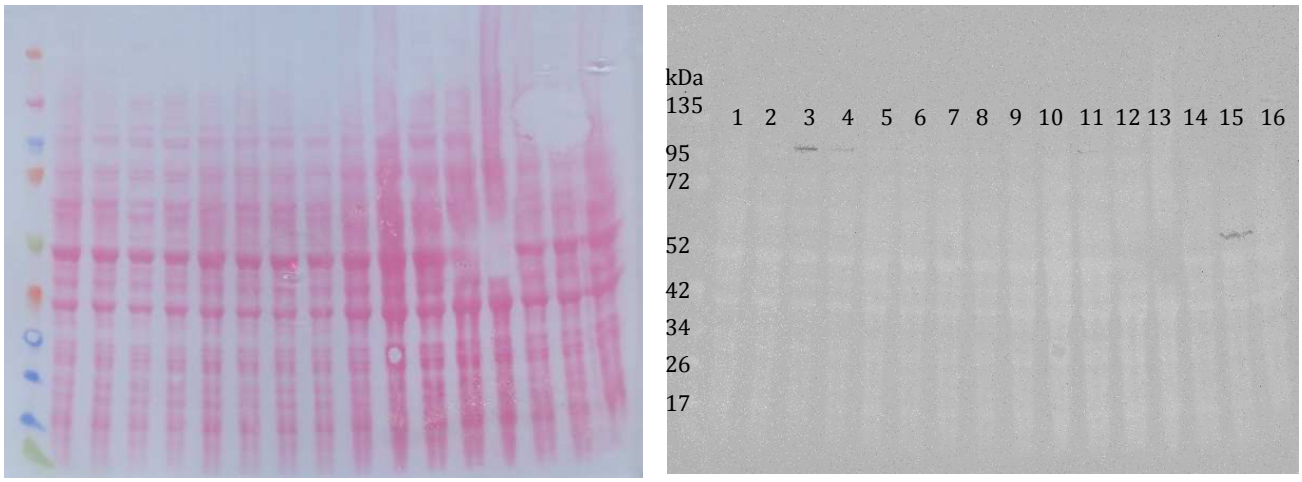
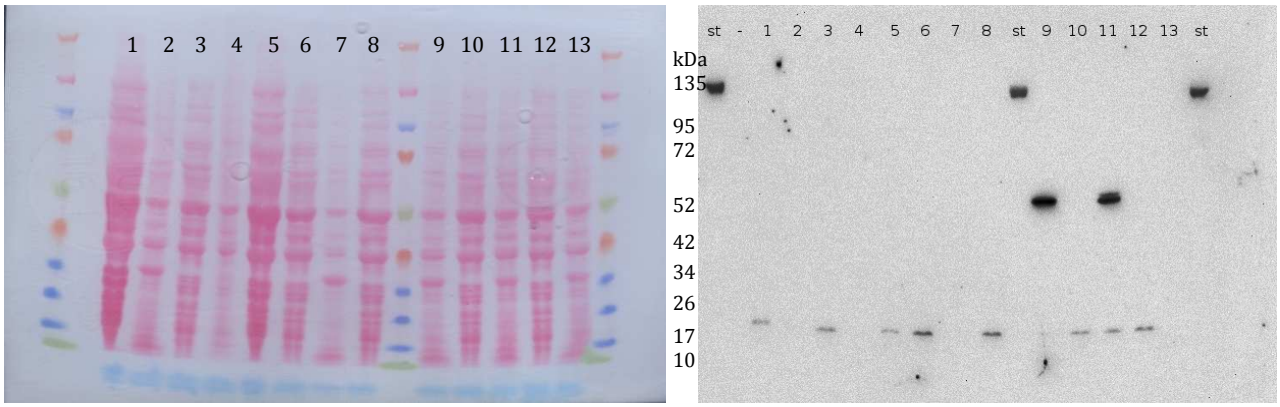


Figure 30: Overlay of the images of Ponceau S stain and chemiluminescent picture from Figure 30 (samples listed in Table 24). Here it is easy to identify the band on lane 10 as 54 kDa Isc1p, just like the faint band on lane 12. The size of the bands at lanes 14 and 15 are not that easy to tell due to a rather pronounced smiley effect on the border of the gel, but it seems that these are the 56 kDa flag_Isc1p bands. Very striking is the absence of a GFP_Isc1p band on lane 16. Especially so, because the same samples were subjected to sphingomyelinase assays in vivo and in vitro and the GFP_Isc1p sample exhibited substantial activity.



a) b)

Figure 31: a) Same blot image as in Figure 30. The membrane had been stripped of antibodies and was again incubated with primary and secondary antibodies. This time primary antibodies were mouse anti-FLAG-tag M2 and the secondary antibodies were goat-anti mouse IgG-HRP conjugates. b) Image showing photograph with illumination and photograph with chemiluminescence in one. The dark bands are the chemiluminescent bands. The 100 kDa bands appear to be specific to use of anti flag antibodies on *S. cerevisiae* lysates and are visible in some lanes, although not all. The samples on lanes 3 and 4 are from *erg3 erg6* double knockout, on lane 11 as well.



a) b)

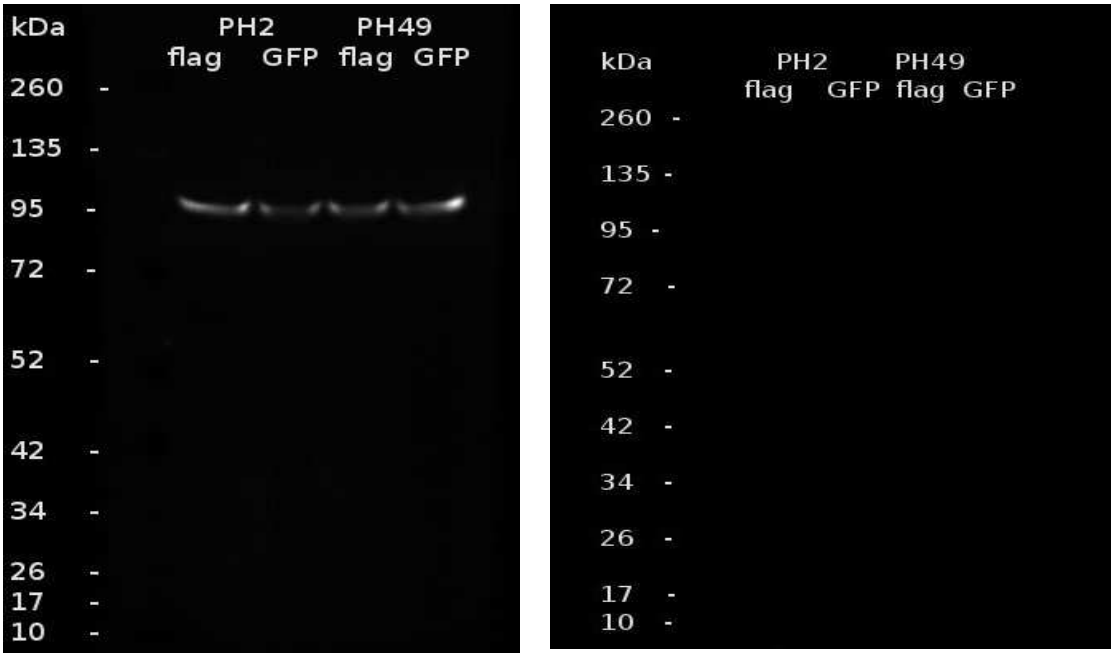
Figure 32: a) Ponceau S staining of samples from fermentation with WT and *isc1* knockout strains carrying various plasmids, the samples are listed in table 25. b) Western blot. On lane 9 there is a 54 kDa band from *isc1* knockout strain expressing Isc1p from the plasmid, on lane 11 is a sample from the *isc1* knockout strain, also exhibiting plasmid-borne expression of N-terminally FLAG-tagged Isc1p. The 56 kDa FLAG-tagged band appears to be a little higher than the 54 kDa Isc1p band. The primary antibody rabbit anti-FLAG-tag M2 and goat anti-rabbit-HRP combination also detected the 135 kDa band of Spectra broad range protein ladder under the given conditions, as well as a seemingly random ~ 20 kDa band in most samples.

Table 25: Samples for Ponceau S stain and Western blot

	Strain	Plasmid insert	Sampling
1	PH2	pYES2_empty	0h
2	PH2	pYES2_empty	17h
3	PH18	-	0h
4	PH18	-	17h
5	PH18	pYES2_empty	0h
6	PH18	pYES2_empty	5h
7	PH18	pYES2_empty	17h
8	PH18	pYES2_ISC1	5h
9	PH18	pYES2_ISC1	17h
10	PH18	pYES2_flag_ISC1	5h
11	PH18	pYES2_flag_ISC1	17h
12	PH18	pYES2_GFP_ISC1	5h
13	PH18	pYES2_GFP_ISC1	17h

WESTERN BLOTS OF S. CEREVISIAE WITH INSERTION CASSETTES

It was not clear, whether or not *S. cerevisiae* expresses Isc1p with the intrinsic promoter to a degree that is detectable by western blotting and immunodetection. The WT cells PH2 and PH49, that were transformed with either the GFP- or the FLAG-tag cassette, were fermented, homogenized and analyzed by SDS-PAGE and Western-immuno-blotting. The membranes were incubated with the primary and secondary antibodies that were used to identify Isc1p and FLAG-tagged Isc1p in overexpression experiments using pYES2 plasmid.



a) b)

Figure 33: In order to make the absence of any desired band clear, the actual images are presented here, not the color inverted ones. a) The blot presented in the left panel was incubated first with mouse anti flag M2 antibodies and then with goat anti mouse IgG-HRP. Like before, this antibody combination leads to bands of about 100 kDa size in all lanes. PH2 is a haploid WT *S. cerevisiae* strain and PH49 is a diploid WT strain. These strains have been transformed with integration cassettes, designed for homologous recombination into the *ISC1* locus on chromosome V. These strains are now expressing N-terminally FLAG-tagged and N-terminally GFP tagged Isc1p with the intrinsic promoter and it seems that the levels at which Isc1p is normally present in cells does not suffice for detection with antibodies on Western blots. b) The same samples after incubation with mouse anti Isc1p and goat anti mouse–HRP antibodies. Detection reaction was performed with Pierce SuperSignal West Pico Chemiluminescent substrate for HRP and imaged using the G:Box bioimaging device.

ACTIVITY ASSAYS

NBD-SPHINGOMYELIN ASSAY

This in vivo sphingomyelinase assay was performed with several different *S. cerevisiae* strains including WT (PH2), *erg3 erg6* double knockout (PH11) and *isc1* knockout (PH18). Some of these strains also carried pYES2 plasmids with various inserts. Cells were assayed both induced and not induced by galactose addition.

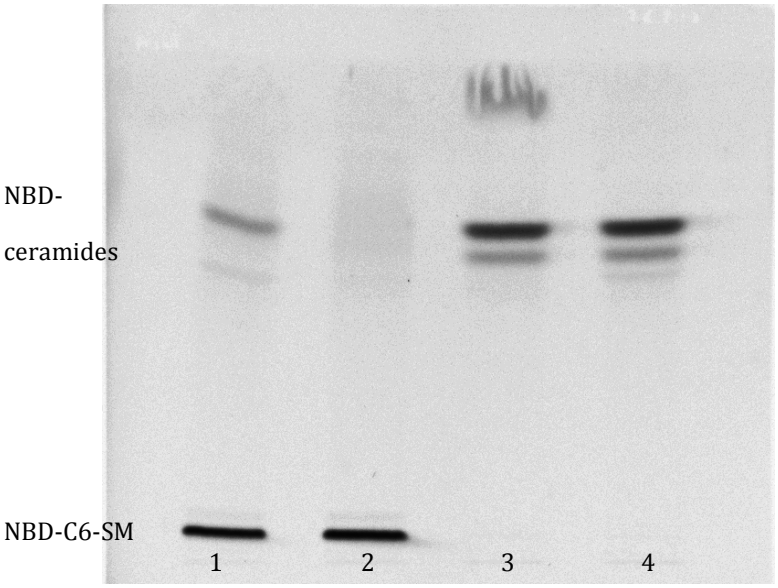


Figure 34: This image shows a very interesting case of total conversion of the labeled NBD-C6-sphingomyelin added to the fermentation samples. The samples were handled as described above. In brief, to 1 ml of cells NBD-C6-SM was added to a final concentration of 2.5 µM. After incubation for 30 min at 24°C, the lipids were extracted and analyzed via thin layer chromatography. The unprocessed substrate is less hydrophobic than the labeled ceramide that gets generated by Isc1p activity. Since the charged headgroup phosphorylcholine is particularly hydrophilic, sphingomyelin does migrate significantly less with the mobile phase than the hydrophobic ceramides. This difference results in a migration pattern where NBD-C6-SM can be found right next to the starting points and the ceramides are about two third of the way between the start and the mobile phase front. In this particular case, sample 1 was a haploid WT strain, sample 2, 3 and 4 were *isc1* knockout strain PH18. All cells were cultivated under the same conditions and the cells assayed in samples 3 and 4 performed plasmid borne expression of functional Isc1p and flag_ISC1p in the knockout background, which converted all available NBD-C6-SM. The WT in sample 1 converted little labeled sphingomyelin and the *isc1* knockout in sample 2 generated no significant amount of NBD-ceramide. It seems that the NBD-ceramides are subject to further turnover which results in more than one single band.

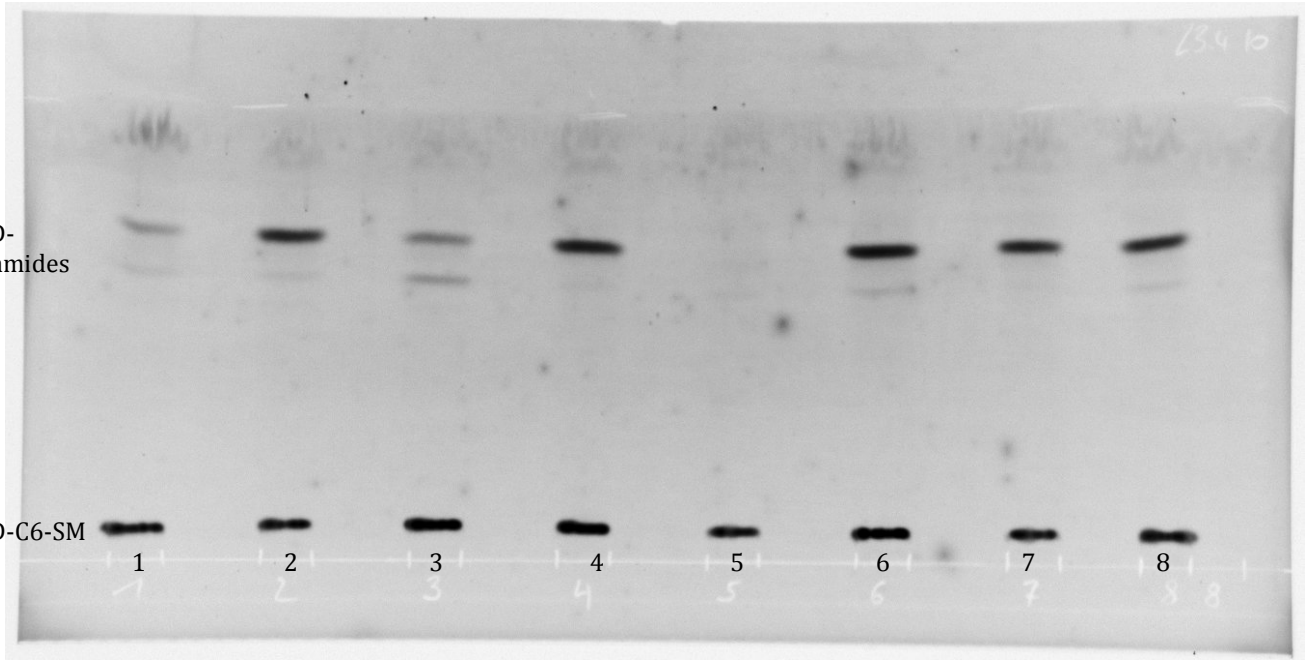


Figure 35: Isc1p activity in various *S. cerevisiae* strains. The assayed strains stem from a fermentation with galactose induction of P_{GAL} on the pYES2 plasmid in order to express Isc1p sphingomyelinase from the plasmids. The fermentation also included wild type and *isc1* knockout strains without plasmids and the samples were prepared 17 h after induction and are listed in table 24. Sample 1 is wild type and sample 2 is the wild type strain overexpressing Isc1p from a plasmid. Note the difference in turnover, the wild type strain PH2 also possesses Isc1p, but turnover in the sample with plasmid-borne overexpression is much greater. Sample 3 represents an *erg3 erg6* double knockout strain with severely different membrane sterol composition than the wild type, sample 4 represents this strain overexpressing Isc1p from the plasmid. Sample 5 obviously lacks sphingomyelinase activity, as it represents the Isc1p knockout strain. Samples 6, 7 and 8 also are *isc1* knockouts, but these strains express Isc1p, flag_Isc1p and GFP_Isc1p, respectively, from the plasmid pYES2. There is a little difference in sphingomyelinase activity. One more interesting finding is that the sample 8 has been found to not contain GFP_Isc1p by immunodetection on western blots (Figure 30). The problem seems to be in the immunodetection, since verification colony PCRs and this assay are absolutely positive about presence of the coding sequence and sphingomyelinase activity in the sample, respectively. It would have been necessary to apply the exact same amount of fluorescence per lane in order to detect a difference between the wild type and the ergosterol biosynthesis knockout strains overexpressing Isc1p, lanes 2 and 4, respectively. It was not possible to detect increased Isc1p activity in *erg3 erg6* strain, which would have been consistent with available data, compare figure 4 (Guan, 2009). This NBD-SM assay is of a semi-quantitative nature, it is difficult to identify moderate activity changes.

RADIOASSAYS WITH ^{14}C SPHINGOMYELIN

Several assays were performed. The more interesting assays included the comparison of addition of phosphatidylserine and phosphatidylcholine, since phosphatidylserine has an activating effect as compared to phosphatidylcholine (Sawai H, 2000). Another expectation was to obtain quantifiable data for the qualitatively determined sphingomyelinase activity differences in the various genetic backgrounds of assayed *S. cerevisiae* strains.

Table 26: Radioassay with added PS and PC vesicles, blank corrected liquid scintillation counting readout and derived data

Radioassay upon addition of phosphatidylserine or phosphatidylcholine, labeled sphingomyelin and unlabeled sphingomyelin. By sonication, artificial vesicles were generated and then mixed with the homogenates comprising the vesicles from the cellular membranes.									
		counts per minute, multiple samples			**cpm/(min*mg)**			**Average**	**Stdev**
PS	WT	1408	1680	1478	3911	4667	4106	4228	392
	erg3 erg6	603	1662	929	1675	4617	2581	2957	1507
	isc1	21	80	39	58	222	108	130	84
PC	WT	121	54	79	336	150	219	235	94
	erg3 erg6	191	49	79	531	136	219	295	208
	isc1	52	80	89	144	222	247	205	54

Table 27: Radioassay performed without addition of phosphatidylserine and unlabeled sphingomyelin. This assay was planned to yield higher readout values even for samples where no PS was added and the vesicles were representing the biologically occurring membranes.

	cpm						**cpm/(min*mg)**						**Average**	**Stdev**
WT	151	144	151	168	164	167	151	144	151	168	164	167	158	10
erg3 erg6	124	128	124	132	138	138	124	128	124	132	138	138	131	6
isc1	51	54	53	53	53	53	51	54	53	53	53	53	53	1

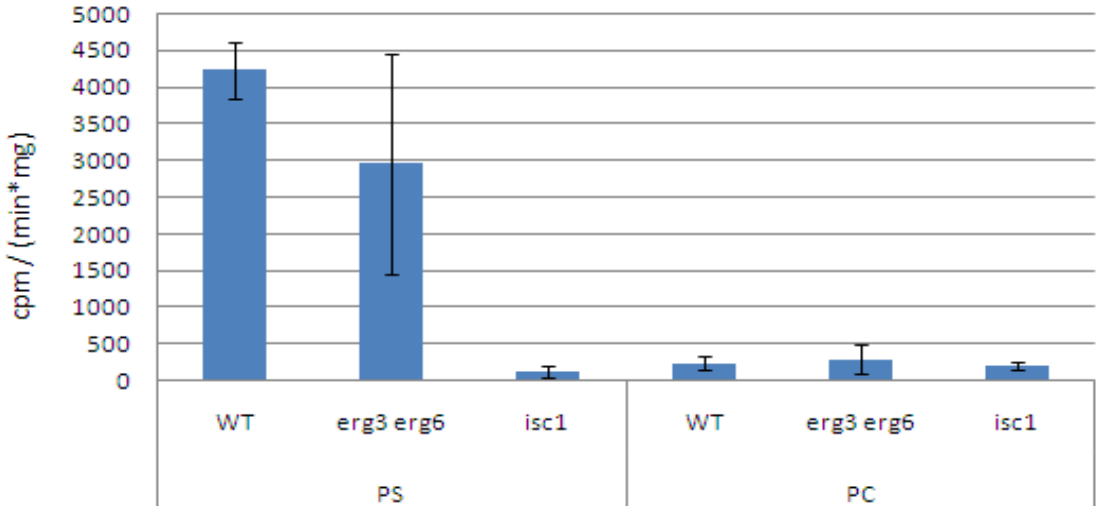


Figure 36: Graphic representation of the Isc1p activity data presented in Table 26, obtained by a radioassay with [choline-methyl-^{14}C]-sphingomyelin. The activating effects of phosphatidylserine (PS) in the membranes can be seen clearly, since activity in the wild type (PH2) samples and the *erg3 erg6* knockout (PH11) are much greater than the corresponding values from the reaction mix containing phosphatidylcholine (PC). 10 nmol PS or 10 nmol PC were included in each sample, respectively. The rather huge error in the *erg3 erg6* activity data from PS samples stems from a measuring value that is virtually twice the level of the other two, but excluding it on the basis of just two other readings seems pretentious. Sphingomyelinase activity in the *isc1* knockout strain is very low and just slightly above the blank values. The lack of activating effect by PC is grave enough to let the activity even in the WT samples drop to levels comparable to the Isc1p knockouts.

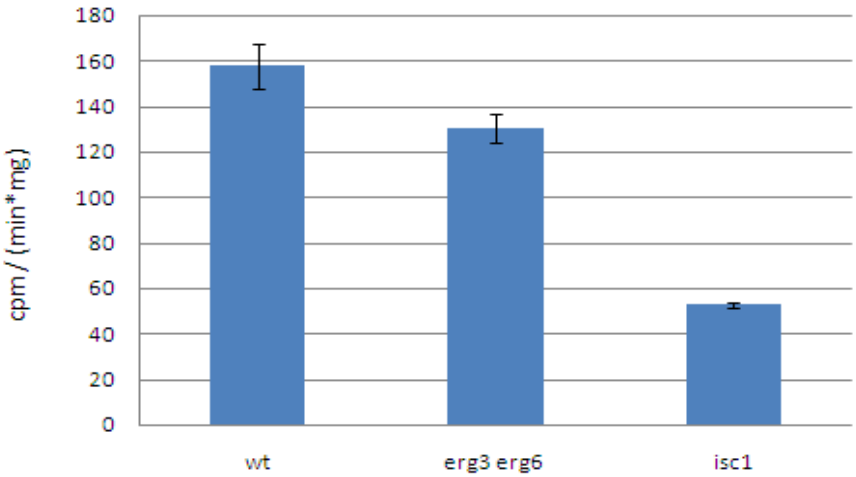


Figure 37: Graphic representation of the radioassay data from Table 27. The intended effect of obtaining higher activity even without additional phosphatidylserine, just by not adding unlabeled sphingomyelin and PS, unfortunately did not appear. The activity of the WT and *erg3 erg6* strains are similar, but the sterol biosynthesis knockout samples *erg3 erg6* were expected to be above the wild type. The activity data are similar to the ones obtained by adding PC instead of PS to the reaction mix, leaving out the unlabeled sphingomyelin did not improve the yield.

Table 28: Data from an assay that was performed with homogenates from galactose induced strains carrying pYES2 derived plasmid.

Strain	Plasmid	Counts per minute/ (min*mg protein) multiple samples per strain			Average	Stdev
WT	pYES2_empty	1672	52	1903	1209	1009
WT	pYES2_ISC1	84827	82499	78056	81794	3440
erg3 erg6	pYES2_empty	33	1644	68	582	921
erg3 erg6	pYES2_ISC1	493	515	513	507	12
isc1	pYES2_empty	15	35	725	258	404
isc1	pYES2_ISC1	90352	90635	76784	85924	7917
isc1	pYES2_flag_ISC1	78229	78146	64821	73732	7717
isc1	pYES2_GFP_ISC1	36274	23658	36074	32002	7227

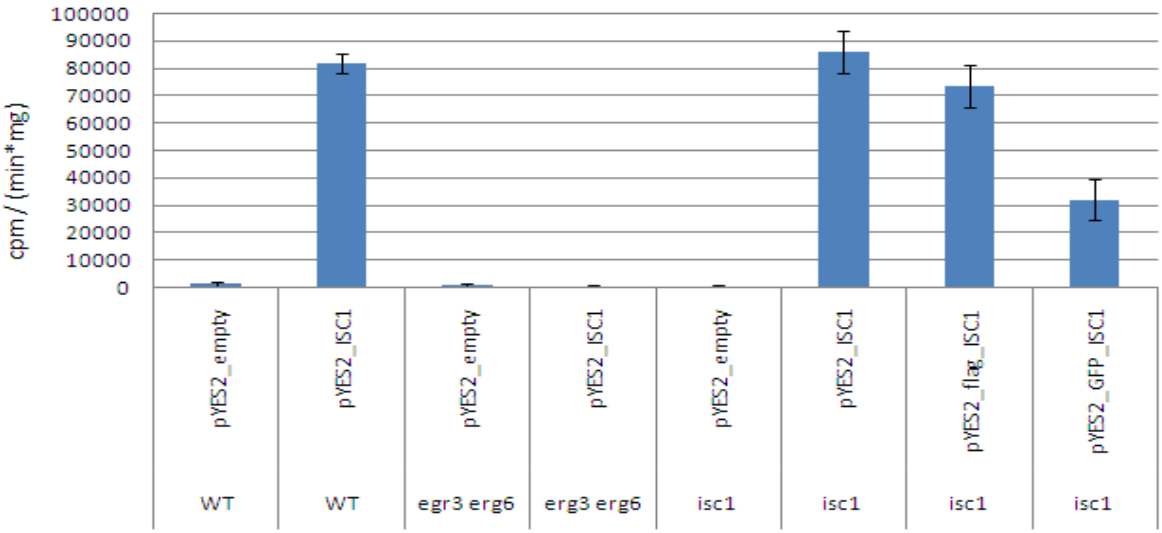


Figure 38: This plot is showing the data resulting from a radioassay with samples from *S. cerevisiae* strains overexpressing Isc1p variants from a plasmid. The expression was induced by addition of galactose, the data are listed (Table 28). The most important insights obtained by this assay series was that overexpression went very well and the strains expressing Isc1p from plasmid pYES2 exhibit much greater activity than the wild type with an empty plasmid backbone and the *isc1* knockout with an empty plasmid. This corresponds nicely to the results of the NBD-SM assay in Figure 35. The big exception were the samples from the *erg3 erg6* knockout that should also perform plasmid-borne overexpression of Isc1p. That strain is severely limited in its growth capabilities as it is and it is likely that the additional strain of overexpressing this protein did not do any good. This *erg3 erg6* double knockout strain features unusual sterols in the membranes. Perhaps the additional effect of overexpressing active sphingomyelinase put this strain over the edge, since growth was particularly poor, although the NBD-SM assay yielded proper results. Another interesting detail is that the *isc1* knockout strain overexpressing GFP_Isc1p does not exhibit activity as high as with the other protein versions, but still ~25 times the activity of the wild type, although no GFP_Isc1p was detected in Western blot experiments (Figure 30). This strain also showed elevated activity in the NDB-assay (Figure 35).

AMPLEX RED ASSAY RESULTS

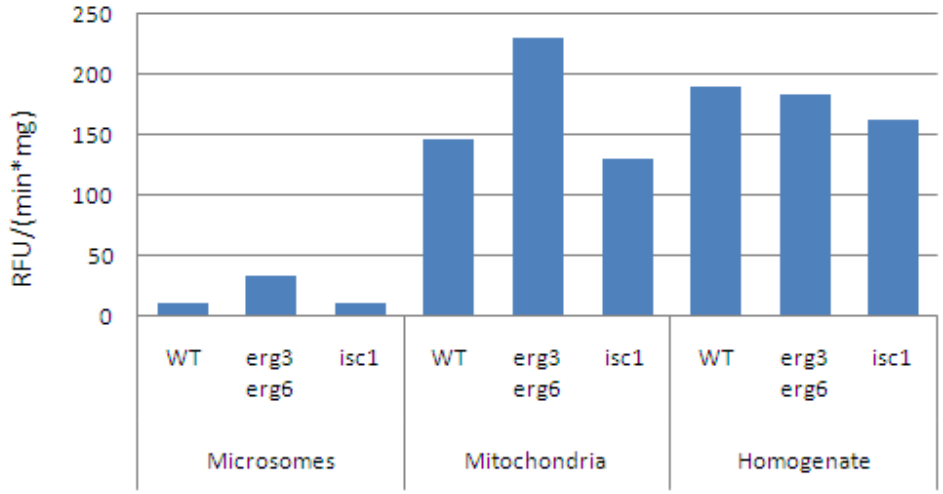


Figure 39: The homogenates of WT, *erg3 erg6* and *isc1 S. cerevisiae* strains have been further separated into microsomes and mitochondria containing fractions, these were then assayed for sphingomyelinase activity with the amplex red sphingomyelinase kit by Invitrogen. After subtraction of the blank values, the mitochondria fraction as well as the homogenate exhibited significant sphingomyelinase activity in the *isc1* knockout samples. This did not boost the confidence in this assay, especially since the NBD-assay and the radioassay suggested that there is no significant sphingomyelinase activity left after knocking out the *ISC1* gene.

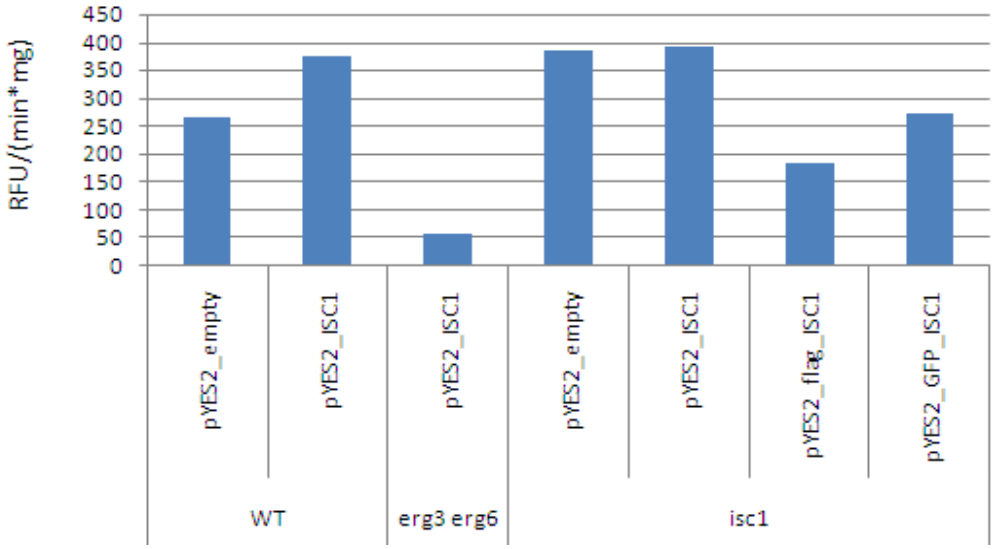


Figure 40: Despite the poor performance of the assay in the first attempts, it was also performed with samples from Isc1p overexpression that were already tested with the other assays. The *isc1* knockout strain carrying an empty plasmid was among the high achievers, yet again, which led to the idea of completely disregarding the amplex red sphingomyelinase assay results.

DISCUSSION

The planning of the thesis' work involved several major points. It was intended to represent a starting point for further investigations by (i) performing localization studies of Isc1p in live *S. cerevisiae* cells or by identifying the protein after subcellular fractionation (ii) assaying Isc1p activity in various live *S. cerevisiae* strains that feature knockouts of genes encoding enzymes that are involved in sterol biosynthesis, namely *erg3* and *erg6* in a double knockout, thereby obtaining information about sterol, sphingolipid and membrane protein interaction, (iii) establishing an in vitro Isc1p activity assay involving radioactively labeled substrate for reliable quantitation as well as an in vivo assay and (iv) performing random mutagenesis of the two predicted transmembrane domains and the whole coding sequence in order to identify the amino acids involved in the interaction with the membrane lipids.

Obviously the extent of all the planned work went beyond the scope of a single Master's thesis. To reach the stated goals, an array of *S. cerevisiae* cells have been generated that express Isc1p with N-terminal flag or GFP tags either from a plasmid or from the *ISC1* locus on chromosome V. The localization studies by either fluorescence microscopy or fractionation of the cell homogenates and immunodetection of the proteins were not pursued further than the generation of the strains expressing the tagged versions of Isc1p. Immunodetection on western blots with cell homogenates from strains expressing tagged and untagged Isc1p did not result in visible Isc1p bands, thereby suggesting that the levels of Isc1p expression under control of the intrinsic promoter is too low for detection on western blots.

Cells of the generated strains were subjected to an in vivo sphingomyelinase assay with a fluorophore labeled substrate and the cell homogenates were assayed using mainly one assay involving ^{14}C labeled sphingomyelin as substrate. A further in vitro assay, Amplex red sphingomyelinase assay, was tested, but the results seemed not to be as reliable as the radioassay. The radioassay worked flawlessly and the results are corresponding to the NBD-C6-sphingomyelin in vivo assay. The Amplex red sphingomyelinase assay did not yield proper results, as samples from the *isc1* knockout strain PH18 were among the most active samples,

under all assayed conditions. The very same samples did show little more sphingomyelinase activity than the negative controls and blank samples in the radioassay in vitro as well as with the NBD-C6-sphingomyelin assay in vivo.

It seemed that Isc1p activity is hard to determine in vitro in combination with the unusual sterols, since Isc1p is incorporated into membranes by two membrane spanning helices and the purification may result in contamination of subcellular organelles, which does not preserve the original membrane compositions of the organelles that Isc1p localizes to, be it endoplasmic reticulum or mitochondria, especially since lipids have to be added and new vesicles are being generated. Furthermore, the activity assays with Isc1p overexpression in the *erg3 erg6* strain did not yield quantifiable results. Probably the expression of active sphingomyelinase, activated by presence of unusual sterol species in the membranes, does not go well together with an *S. cerevisiae* strain that is already less viable than the wild type because of its membrane composition. As mentioned above, sphingolipids are crucial in cell cycle regulation and signal transduction. The samples with strains expressing Isc1p from its native promoter do not show elevated activity in comparison to the wild type.

The assumption that the sterol species present in the *erg3 erg6* knockout strains may lead to increased Isc1p activity could not be proved, since these samples showed activities similar to the wild type samples or even lower (Figures 37and 38). It may well be that an increase in Isc1p activity in *erg3 erg6* knockout cells can be detected, but with the samples assayed and the assays at hand, it was not possible to verify that. Establishing reliable methods of quantifying spots and bands on thin layer chromatography plates may help in assaying Isc1p activity in vivo, which means in the actual biological membranes under relevant conditions.

The fact that the amplex red sphingomyelinase assay results were completely disregarded does not hurt the conclusions, because the other assays' data correspond nicely. The NBD-C6-SM assay results are not easily quantifiable because the final result is an image of a thin layer chromatography plate. In short, the *isc1* knockout strains did not exhibit substantial sphingomyelinase activity, whereas these cells overexpressing Isc1p constructs from the *S. cerevisiae* expression plasmid pYES2 show massive sphingomyelinase activity.

The idea of omitting phosphatidylserine and unlabeled sphingomyelin when reconstituting membrane vesicles for the radioassay in order to obtain higher scintillation counting readout

values did not work. The samples with 10 nmol sphingomyelin and ~1 nmol labeled SM contained about 10 times more unlabeled SM. Statistically, only every tenth converted SM was labeled and when the unlabeled sphingolipid is being omitted, this should result in much higher counts/(min*mg protein) values, which strangely it did not. This may be due to the lack of activating phosphatidylserine in these particular samples (see Figure 38). But adding PS would alter the membrane composition more than desired.

The expression of Isc1p and its N-terminally FLAG-tagged and truncated versions in *E. coli* expression strains did result in most of the proteins being found in the pellet fraction after cell homogenization. This was particularly remarkable, since the truncated versions were designed specifically to yield a more soluble protein. Only one protein, namely FLAG-tagged Isc1p with both transmembrane helices still intact, was found in the supernatant after cell homogenization to a small extent. It is plausible that most of these overexpressed proteins ended up in protein inclusion bodies and hence are being found in the pellet fraction. The presence of the protein featuring both transmembrane helices in the supernatant still is a very interesting detail.

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