HUMBOLDT-UNIVERSITÄT ZU BERLIN

Mathematisch-Naturwissenschaftliche Fakultät I

Institut für Biologie

Arbeitsgruppe Theoretische Biophysik

Masterarbeit

zum Erwerb des akademischen Grades

Master of Science

Modellierung des Whi2-abhängigen Regulationsnetzwerkes von Mitophagie und Autophagie in *Saccharomyces cerevisiae*

Modelling the Whi2-dependent regulatory network of mitophagy and autophagy in *Saccharomyces cerevisiae*

vorgelegt von

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geb. am 20.05.1986 in München

Berlin, 1. Juli 2012

Zusammenfassung

Whi2 ist ein Protein der Bäckerhefe *Saccharomyces cerevisiae*, das spezifisch mit Mitophagie in Verbindung gebracht wurde, was sich nach derzeitigem Wissensstand nur schwer erklären lässt. In der vorliegenden Arbeit werden wir, ausgehend von Whi2 und der Mitophagie, Schritt für Schritt untersuchen, welche Faktoren damit jeweils in Verbindung stehen, und werden den Versuch unternehmen, Protein-Interaktionen und experimentelle Ergebnisse in einem größeren funktionellen Zusammenhang zu deuten. Wir präsentieren ein umfassendes Boole'sches Netzwerk, das mitochondrial lokalisierten Metabolismus, Stress-Signalwege, sowie Autophagie, speziell die Mitophagie, in einem Modell zusammenführt.

Abstract

Whi2 is a protein in baker's yeast *Saccharomyces cerevisiae*, that was found, to be linked specifically to mitophagy, which from current knowledge about Whi2 and stress response is difficult to explain. In the present work, we will, coming from Whi2 and mitophagy, step-by-step inspect the neighbourhoods of associated factors and try, to explain protein interactions as well as experimental findings in a larger functional context. We present a comprehensive Boolean network, bringing together mitochondrially localized metabolism, stress signalling as well as autophagy, specifically mitophagy, in one model.

Contents

1 Introduction

The initial discovery, that lead to the setup of the present work, was an accidental finding, encountered in a screening for mitochondrial network fission mutants in baker's yeast *Saccharomyces cerevisiae*. The mitochondrial network is a flexible, dynamic association of mitochondria, which spreads across the eucaryotic cell. Mitochondria habitually integrate (fusion) and separate (fission) from the mitochondrial network.

Mendl, Müller and Reichert originally discovered, that the mitochondrial network fission mutant Δfis1 showed diminished mitophagy. Mitophagy is the specific degradation of mitochondria by autophagy, a process in which the cell actively targets intracellular components to the vacuole for degradation (see also chapters 3.7 and 3.8). Their discovery is consistent with the assumption, that fission of mitochondria from the network is required for their specific autophagy. However, other known fission factors - Dnm1, Mdv1 and Caf4 - all turned out, not to be related to mitophagy, favoring the opposite of the previous assumption. After further analysis, Mendl discovered, that a second, unintentional mutation was present in the initially studied Δfis1 fission mutant strain: Δwhi2 [1].

Indeed, the Fis1 Δwhi2 strain reproduces the observed diminished mitophagy, linking Whi2 specifically to mitophagy. Little is known about Whi2, so this finding is difficult to explain [2].

In the present work, we will, coming from Whi2 and mitophagy, step-by-step inspect the neighbourhoods of associated factors. We will try, to explain protein interactions as well as experimental findings in a larger functional context: After having a closer look at parts of yeast growth and metabolism pathways, we will discuss, why mitophagy is important and how it may be regulated. Several stress response pathways will be inspected, to demonstrate, which function Whi2 could play in the presented context.

1.1 Yeast's lifecycle

The growth of yeast in liquid medium, as the growth of many single-cell'd organisms, is characterized by distinctable phases. At first, when cells are placed into a new medium, lag phase occurs, marked by slight to no change in cell density, while cells alter gene expression in order to adapt to their new environment: They begin collecting and synthesizing important compounds, among which are minerals, such as zinc, phosphor, sulfur, vitamins, carbon sources (glucose, glycerol, lactate, ethanol), and nitrogen sources (ammonia, urea and amino acids).

After this delay, which ranges between 3 and 15 hours, depending on the used medium, log phase begins ("log", since the exponential growth in cell density is usually depicted using a logarithmic scale): Cell cycle becomes de-arrested and cells proliferate. When nutrients start to become limited, cells interrupt their cell cycle and prepare for starvation.

The yeast cell cycle is characterized by the iterating, subsequent phases "synthesis" (S) and "mitosis"(M), interrupted by gap phases G_1 and G_2. During synthesis phase, DNA is replicated, while in mitosis phase, the cell divides in two. In the gap phases, the cells are growing. Cell size homeostasis during proliferation is ensured at the G_1/S cell cycle phase boundary ("checkpoint"), which prevents cell division until a critical cell size is attained [3]. When cells encounter starvation, they interrupt the cell cycle and arrest in the long-living gap state G_0 [4], which is characterized by increased resistance to a variety of stresses, such as heat shock [5]. It also goes along with an accumulation of storage carbohydrates (glycogen and trehalose) [6].

Having this in mind, we will now introduce the yeast protein Whi2.

1.2 Whi2 and it's suggested partner proteins

Whi2 is a 55 kDa protein of rather uncertain function in bakers' yeast *Saccharomyces cerevisiae*. It's structure has not been resolved and it's amino acid sequence shows no obvious homologies with other yeast proteins [7]. While containing neither transmembrane regions, nor signal peptides, two distinct POZ domains [8] have been identified. POZ domains are motifs, that me-

diate interactions with DNA or other proteins and have been encountered conferring protein multimerization [9]. However, no Whi2-multimerization has (yet) been reported.

With respect to yeast's cell cycle, mutant Δwhi2 cells fail to arrest in G_0 and continue proliferating after diauxic shift from fermentable to non-fermentable carbon source (glucose to ethanol) [10] [11]. This behavior is inappropriate, given the decreased energy yield and expected depletion of nutrients. The cells arrest randomly in the cell cycle, presumably due to energy exhaustion, and reach a cell density three times higher then the wild-type, while cells have only half of the respective volume. This phenotype links Whi2 to starvation-promoted cell cycle arrest. The corresponding authors pointed out, that the Δwhi2 mutant phenotype was exclusively visible during fully oxidative growth on ethanol, neither during aerobic nor anaerobic growth on glucose.

We searched for possible protein interactions with Whi2, and found Psr1 and Psr2, which were identified in 2000 as two functionally homologous protein phosphatases in yeast, required for adaption to sodium osmostress via transcriptional activation of ATP-driven sodium pump Ena1 [12]. They attracted attention, since in this function, they are independent of the Hog and Calcineurin pathways, that were previously linked to osmostress signalling in yeast.

Besides these phosphatases, in 1993 two homologous Cys_2His_2 zinc finger proteins, Msn2 and Msn4, that have striking similarity with the transcription factor Gis1, have been reported [13]. The authors stated, that deletion of Msn2 alone caused no phenotype, while simultaneous deletion with Msn4 caused defects, related to carbon utilization.

Meanwhile it is the established opinion, that Msn2/4 are transcriptional activators, localized to the cytoplasm under stress-free conditions, translocated to the nucleus upon a variety of stresses, where they specifically bind stress-response elements (STREs) [14]. Translocation is regulated via phosphorylation and depends on a nuclear import/export region inside Msn2/4's amino acid sequence [15].

The interaction of Whi2, Psr1/2 and Msn2/4 to activate STREs will be

discussed in chapter 3.6.

1.3 Reactive oxygen species

Mitochondria, among other important biosynthetic and catabolic functions like beta-oxidation, harbour the Krebs cycle (OAC) and the respiratory chain (RC). The latter is subject to intense research, since it leakes reactive oxygen species (ROS) during the processes, conferring oxidative phosphorylation (oxygen usage alongside ATP production).

ROS have a high potential, to cause mitochondrial and cellular damage - to lipids, proteins and -most importantly- DNA, especially the mitochondrial one (mtDNA), due to the latter's proximity to the sites of ROS generation.

As we will discuss, a lot of proteins in yeast exist only, to avoid the production of ROS or to counteract their effects. Among these, also Whi2 plays a role (chapter 3.6).

1.3.1 ROS production is subject to mitochondrial evolutionary processes

Since mitochondria multiply inside the eucaryotic cell during the latter's life cycle, their evolution is more efficient than their host's, and such is the evolution of mitochondrial metabolism-related genes.

The production of ROS leads to additional mutations in mtDNA, primarily affecting the mitochondrion producing them. On the one hand, these mutations facilitate mitochondrial gene diversity and thus have the potential to accelerate adaptations towards ROS tolerance and/or reduction of ROS production, since the diversity enhances mitochondrial evolution. On the other hand, especially high concentrations of ROS can damage the mtDNA to an unrecoverable extent, leading to loss of mtDNA. Understandably, cells containing and relying on such "ghost" mitochondria, exhibit severe growth defects [16], that in turn negatively selects other mitochondria of the same host.

1.3.2 ROS have a major impact on yeast's lifespan

The importance of ROS minimization is underlined by the finding, that lifespan extension of yeast in Δsch9, Δcyr1 and Δras2 mutants is reported to be conferred by enhanced expression of the mitochondrial superoxide dismutase Sod2 [17]. Additionally, co-overexpression of superoxide dismutases Sod1 and Sod2 is capable of extending yeast lifespan up to 30%, which is attributed to increased protection of the ROS-susceptible mitochondrial aconitase [17]. Other sources even report a 10-fold extension of lifespan in the Δras2 Δsch9 double mutant, when additionally combined with calorie restriction [18].

1.3.3 Yeast trades lifespan for energy

Given these findings, the question arises, why wild-type yeast is not increasing it's lifespan, e.g. also, by deleting the mentioned genes. A possible answear could be, that yeast "prefers quality for quantity": As we will discuss in the present work, the described mutants at least partially gain their increased lifespan by abstinence from mitochondrial energy metabolism. This abstinence abolishes the production of ROS. While glycolysis may generate sufficient energy for survival in culture, selection of individuals during evolution, could, however, have "judged", that this sparse metabolism is disadvantegous, outside the "wealthy" conditions of culturing. Especially when cells face interindividual or interspecies competition, an increased energy metabolism may be required for survivial and fitness, which must therefore be traded for lifespan.

1.3.4 It is unclear how ROS concentration is sensored

Concerning possible sensory mechanisms, Peroxiredoxins [19], ascorbate (vitamin C), glutathione, glutaredoxins, thioredoxins, ferredoxin, NADPH [20] as well as the mitochondrial aconitase itself [21] have been proposed, to provide feedback about intracellular ROS levels. However, ROS leakage may also be indirectly measured via the simultaneously diminished mitochondrial inner membrane potential ($\Delta\Psi$). Since we believe, that especially the latter could most easily be directly coupled to a possible energy-/ATP-driven

mitochondria-selecting mechanism, we will focus on this idea in the context of this work.

1.4 Mitophagy

Mitophagy is a process, that serves cellular homeostatis: Mitochondria and so-called pre-autophagosomal sites (PAS) co-localize. PAS are cellular complexes of autophagy-related proteins together with portions of membrane. Upon activation, these complexes can grow and surround different targets, until these are enclosed by membrane. The engulfment of targets by one or several PAS forms completed autophagosomes, which are transported to and into the vacuole, where they are degraded, together with their targeted content [22]. In the context of this work, we would like to refer to mitophagosomes, when refering to autophagosomes, which contain mitochondria.

In contrast to degradation of mitochondria via a mechanism of non-specific autophagy (bulk autophagy), where all kinds of cellular components become randomly surrounded by membrane, mitophagy targets specifically mitochondria [2].

The functional mechanism, underlying mitophagy is poorly understood. It is known, that certain environmental conditions can increase the probability, to encounter mitochondria in the vacuole. Among these are starvation for nitrogen and culturing cells to the stationary phase. Two proteins have been reported, to be specifically linked to mitophagy in yeast: Atg32 (see [23]) and Atg33 (see [24]).

Since the mechanism of mitophagy is not understood, it can only be speculated about it's purpose. However, it is a convincing assumption, that cells need to control their mitochondria for quality, especially with respect to the amount of ROS, the latter produce, which correlates also with the efficiency of the mitochondrial energy output.

This connection between mitophagy and quality control is of special interest, because quality control of mitochondria plays an important role also in humans, since mitochondrial dysfunction is a hallmark of cancer cells [25]. Additionally, the reduction of ROS production is often mentioned in the

context of lifespan extension (see also chapter 1.3.2).

In the present work, we will discuss purpose and mechanisms of mitophagy.

2 Methods

In order to analyze the interactions of Whi2 and the proteins involved in autophagy, repectively mitophagy, we decided to make use of Boolean networks. We will model interactions, we gain from extensive literature research and own ideas, using the software tools BooleanNet and biographer.

2.1 Boolean networks

A Boolean network is a model, which consists of logic rules, correlating the state of the model's components ("nodes") to each other. Nodes can stand for abstract states, possible phenotypes as well as proteins or genes. All nodes are assigned a binary state, true or false. Depending on the purpose of the network, the binary state of a node can have manifold meanings, e.g.:

- on vs. off
- present vs. absent
- active vs. inactive
- phosphorylation vs. dephosphorylation
- receptor firing vs. receptor silent
- transcription vs. no transcription
- complexation vs. dissociation

The state of each node is definded in relation to the state of other nodes of the same network via *update rules*. Listing 1 provides a simple example of a Boolean logic based update rule. For the simulation of network dynamics over time, Boolean networks are continuously iterated. During every iteration the state of every node is updated, according to it's update rule.

Listing 1: Example Boolean update rule

```
NodeA = (NodeB or NodeC) and (not NodeD)
```

A Boolean model is completely defined by it's update rules. A list of the involved nodes and a list of the edges, representing the connections of nodes to each other can automatically be derived. Thereby, Boolean networks can simulate the dynamics of a protein network *in silico*, representing an especially simple approach, to analyze the dynamics of a discrete, sequential system.

Still, the modeller must choose the initial state of it's network, which may in the biological context have significant impact on the outcome. In the present work we used an *all-true* initialization in all scenarios, meaning all nodes are turned on in the beginning of simulation, which turned out to produce good results. Boolean networks could also be initialized with a random seed, which may be interesting in perturbation-associated scenarios.

Boolean networks can be simulated synchronously, where the future state of all nodes is calculated from the present state of all nodes, as well as asynchronously, where nodes are updated in random order without separating of the future state from the present. While the second approach may to a certain degree have the advantage of painting a more realistic picture of the situation in the cell, we chose the synchronous approach, since we expect asynchronous simulation to at least partially disrupt the well-defined sequence of state-transitions over time, which the modeller might intend with his or her rules.

2.2 BooleanNet

BooleanNet is a Python library, that allows for simulation and analysis of Boolean networks [26]. It was initially presented by Istvan Albert in 2008 [27]. It is advantageous for handling Boolean networks, since it is very easy to use. Also the implementation of update rules is quickly possible: They can be provided in a human-readable syntax, using the operators = for assignment of a logical term to a node state, the terms **True**, respectively **False** for static definitions, brackets to enclose terms belonging together, and, of course, the logical operators **and**, **or** and **not** between the nodes, on which's state the logical term is based. In listing 1 an examplary update rule was presented.

2.3 biographer

In the context of this work and Google's Summer(s) of Code 2011 as well as 2012, a simulator has been implemented, that is capable of automatically visualizing networks and allowing for interactive simulation [28]. It is written in the programming language JavaScript and thus runs in the user's web browser, independent of the used operating system.

To depict our inferred interactions, we derived a simplified graphical representations from the SBGN standard [29], depicted in Fig. 1.

Our software is capable of representing the dynamics of a network over time by continuous iteration. The user can switch node state by mouse click and monitor subsequent state changes throughout the network. Nodes in the state **False** are not highlighted, while nodes in the state **True** are highlighted green. Unfortunately, we are unable to present temporal dynamics in the context of this (static) work. We will therefore present only pictures of networks, not depicting node activity, and refer to the presented logical model listings, for reproduction of results.

2.4 Sources of data

We used data from scientific papers, found via the PubMed article database [30]. Additionally, we made use of bioinformatical methods and different online databases: Protein amino acid sequences as well as protein domain predictions were retrieved from the *Saccharomyces* Genome Database [7]. Possible protein interactions were found in the BioGRID repository [31]. Protein phosphorylations sites were looked up in the PhosphoGrid database [32]. Protein sequence comparison was carried out, using the EMBL-EBI EMBOSS needle software [33]. Protein interaction networks were predicted and visualized using GeneMANIA [34].

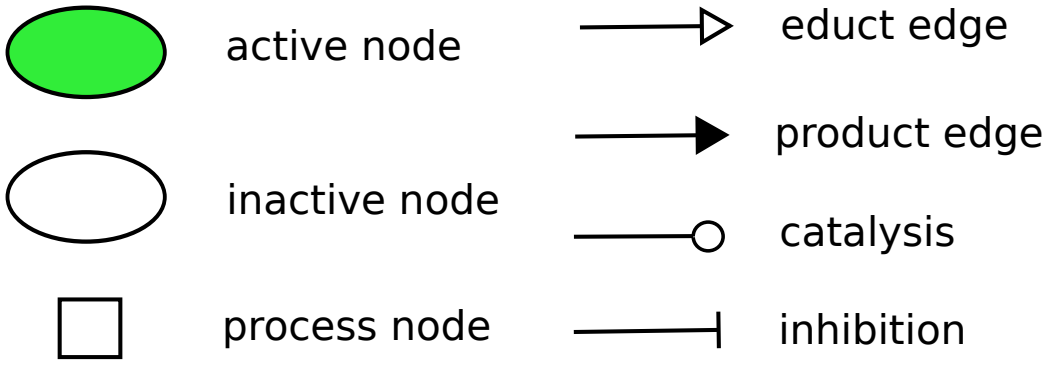


Figure 1: The graphical elements, used in the present work, and their respective meanings

3 Results

In this chapter, we will present the data, we gained from literature and databases, which we found, to be important in order to understand the processes, we describe in this work. We will explain our ideas and summarize our respective conclusions in listings of Boolean logic, which we will combine, to model stress response and autophagy in yeast.

3.1 Framework

The model, we propose here, is designed as a functional module, meaning it is closed in itself: Our module expects input signals, like glucose, glycerol, nitrogen and rapamycin (presence, respectively), and provides the uniform, integrative output signal energy.

Nitrogen, of course, does not refer to the gas, but soluble, biochemical substances, such as ammonia, aspartate or glutamate, yeast is capable of using as a source for it's nitrogen pool of glutamin/glutamate. Fig. 13 depicts yeast's nitrogen metabolism, which may be of interest in this context.

Glucose and glycerol are potential carbon and energy sources. Limitations in carbon and nitrogen, but also other compounds, like phosphor or sulfur, induce different types of starvation, respectively.

The module's output (energy) shall be a rough indicator, whether our model predicts viable or lethal changes. In our model, energy is generated by anaerob fermentation on glucose, oxidative phosphorylation on glycerol, lactate or ethanol, and by degradation of autophagosomes in the vacuole. The resulting Boolean logic is presented in listing 2 and figure 2.

Listing 2: Boolean model framework

```
fermentation*                = glucose
pyruvate*                    = glucose or glycerol or lactate or ethanol
oxidative_phosphorylation*   = pyruvate and (not glucose)
vacuolar_catabolism*         = stress
energy*                      = fermentation or oxidative_phosphorylation or vacuolar_catabolism
```

3.2 Metabolism

In this subchapter, we will model the processes, involved in catabolism of fermentable and non-fermentable carbon sources.

3.2.1 Glycolysis

Glucose represents the carbon source of choice, as long as it is present in the medium. It is metabolized to pyruvat by glycolysis, which does not require oxygen or mitochondria, while pyruvate can be converted, e.g. to ethanol, but not be further metabolized in the absence of oxygen or mitochondria. In listing 3 we present the logic, we used to model glycolysis.

Listing 3: Boolean model of glycolysis

```
#
# glycolysis
#

Glucosephosphate*                    = Glucose and Hexokinase and ATP
Fructosephosphate*                   = Glucosephosphate and Phosphoglucokinase
Fructosebisphosphate*                = Fructosephosphate and Phosphofructokinase and ATP

processF*                            = Fructosebisphosphate and Aldolase
processD*                            = Dihydroxyacetonephosphate and Triosephosphateisomerase
processG*                            = Glycerolphosphate and Glycerolphosphatedehydrogenase
                                       and FAD and NADH

NAD*                                 = processG or processAlc2
NADH*                                = processA or processI or processK
                                       or processM or processLactate
FADH2*                               = processG or processS2

# glycerol

Glycerolphosphate*                   = Glycerol and Glycerolkinase and ATP
processA*                            = Glyceraldehydephosphate and
                                       Glyceraldehydephosphatedehydrogenase and NAD
Bisphosphoglycerate*                 = processA
Glyceraldehydephosphate*             = processF or processD
Dihydroxyacetonephosphate*           = processF or processG
Phosphoglycerate*                    = Bisphosphoglycerate and Phosphoglyceratkinase

processB*                            = Phosphoglycerate and Enolase
Phosphoenolpyruvate*                 = processB

processE*                            = Phosphoenolpyruvate and Pyruvatkinase
Pyruvate*                            = processE or processLactate
ATP*                                 = processB or processE or processCV
processP*                            = Pyruvate and Pyruvatedecarboxylase

#
# alcoholic fermentation
# modelled as production of ethanol
#

# oxygen repression of alcoholic fermentation
processAlc1*                         = Pyruvate and Pyruvatedecarboxylase and (not O2)
```

```
Acetaldehyde*                          = processAlc1
processAlc2*                           = Acetaldehyde and Alcoholdehydrogenase and NADH
Ethanol*                               = processAlc2

#
# lactic acid fermentation
# modelled as uptake of lactate
#

processLactate*                        = Lactat and Lactatdehydrogenase and NAD
```

3.2.2 Oxidation of carbon: The organic acid cycle

Glycerol, ethanol, lactate and pyruvate represent alternate carbon sources, being metabolized, after glucose is depleted in the medium. Additionally to other pathways (like protein catabolism), they, via pyruvate, affere into a very general, conserved and, among eucaryotes, "omnipresent" pathway, commonly referred to as the Krebs cycle, named after it's discoverer [35]. We constructed a Boolean model in listing 4, reflecting the processes, that are taking place.

Listing 4: Boolean model of the organic acid cycle

```
AcetylCoA*                             = Pyruvate and Pyruvatdehydrogenase and CoA
Citrate*                               = Oxaloacetate and AcetylCoA and Citratesynthase
Aconitate*                             = Citrate and Aconitase
Isocitrate*                            = Aconitate and Aconitase

processI*                              = Isocitrate and Isocitratedehydrogenase and NAD
Ketoglutarate*                         = processI
CO2*                                   = processI or processK or processAlc1

processK*                              = Ketoglutarate and Ketoglutaratedehydrogenase and CoA
SuccinylCoA*                           = processK

processS*                              = SuccinylCoA and SuccinylCoAsynthetase and GDP
Succinate*                             = processS
GTP*                                   = processS
CoA*                                   = processS

processS2*                             = Succinate and Succinyldehydrogenase and FAD
Fumarate*                              = processS2

Malate*                                = Fumarate and Fumarase

processM*                              = Malate and Malatedehydrogenase and NAD
Oxaloacetate*                          = processM or processP
```

Most compounds in the Krebs cycle are organic acids, which is also often referred to as citric acid cycle. In the context of this work, we would like to not use the latter term, since citric acid is only one of the involved acids. Also the term tricarboxylic acid cycle is sometimes used, although not all involved

compounds actually contain three carboxylic acid groups: oxaloacetic, α-ketoglutaric, succinic, fumaric and malic acids only contain two. Therefore we will refer to the more general term "organic acid cycle" (OAC) in this work.

Every time, the OAC "steps forward", besides to reduction units $NADH$ and $FADH_2$, one molecule of CO_2 is released. The latter lowers the pH, as long as it stays dissolved as $H_2CO_3/HCO_3^-/CO_3^{2-}$ and does not evaporate (gaseous environment) or thin out (liquid environment).

3.2.3 Reduction of oxygen: The respiratory chain

Membrane complexes I to V inside the mitochondrial inner membrane(s) use the intermediary products of the OAC, to generate a proton gradient between the matrix and the intermembrane space, the membrane potential $\Delta\Psi$. During this process, electrons from reduction units ($NADH$ and $FADH_2$) are used to reduce ubiquitin (Ubi) to ubiquinol ($UbiH_2$). From there, electrons are further transported onto cytochrom C and subsequently onto O_2, to finally form H_2O. We want to outline, that this process formally assimilates two protons from the medium, thereby raising the overall pH. ATP-Synthetase in parallel, independently from the respiratory chain complexes, uses $\Delta\Psi$ for ATP synthesis. In listing 5 we present our Boolean model for the respiratory chain processes. Figure 3.

Listing 5: Boolean model of the respiratory chain

```
processCI*                          = NADH and Complex_I and Ubi

processCII*                         = FADH2 and Complex_II and Ubi
UbiH2*                              = processCI or processCII
processCIII*                        = cytochromeC and UbiH2
cytochromeC_minus*                  = processCIII

processCIV*                         = cytochromeC_minus and O2 and UbiH2
cytochromeC*                        = processCIV
H2O*                                = processCIV
Ubi*                                = processCIV

proton_gradient*                    = processCI or processCIII or processCIV

processCV*                          = ADP and ATPsynthase and proton_gradient
```

Figure 2: Graphical representation of the framework in listing 2

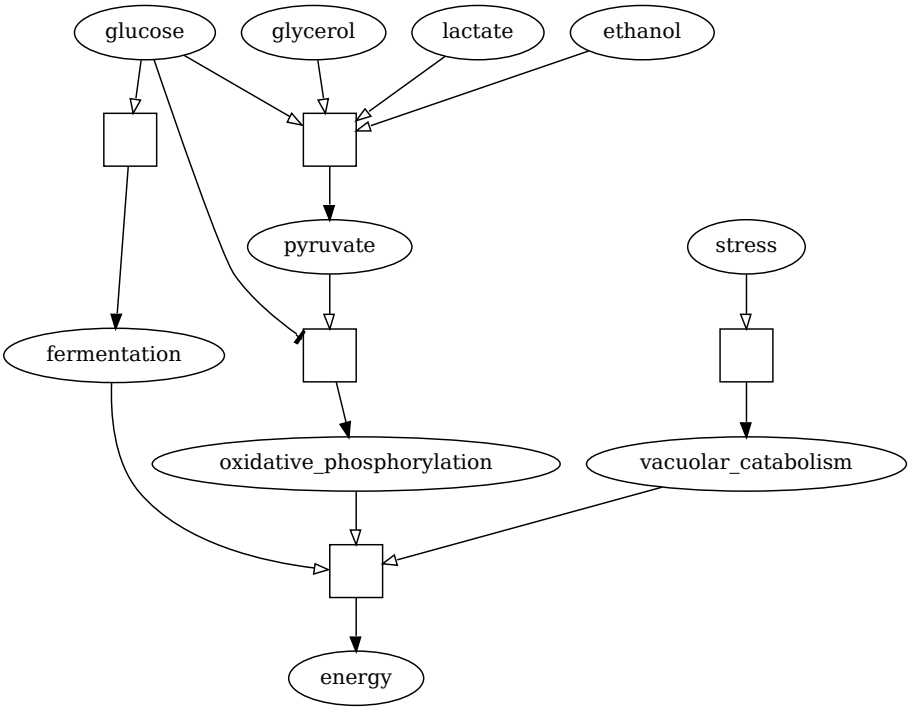
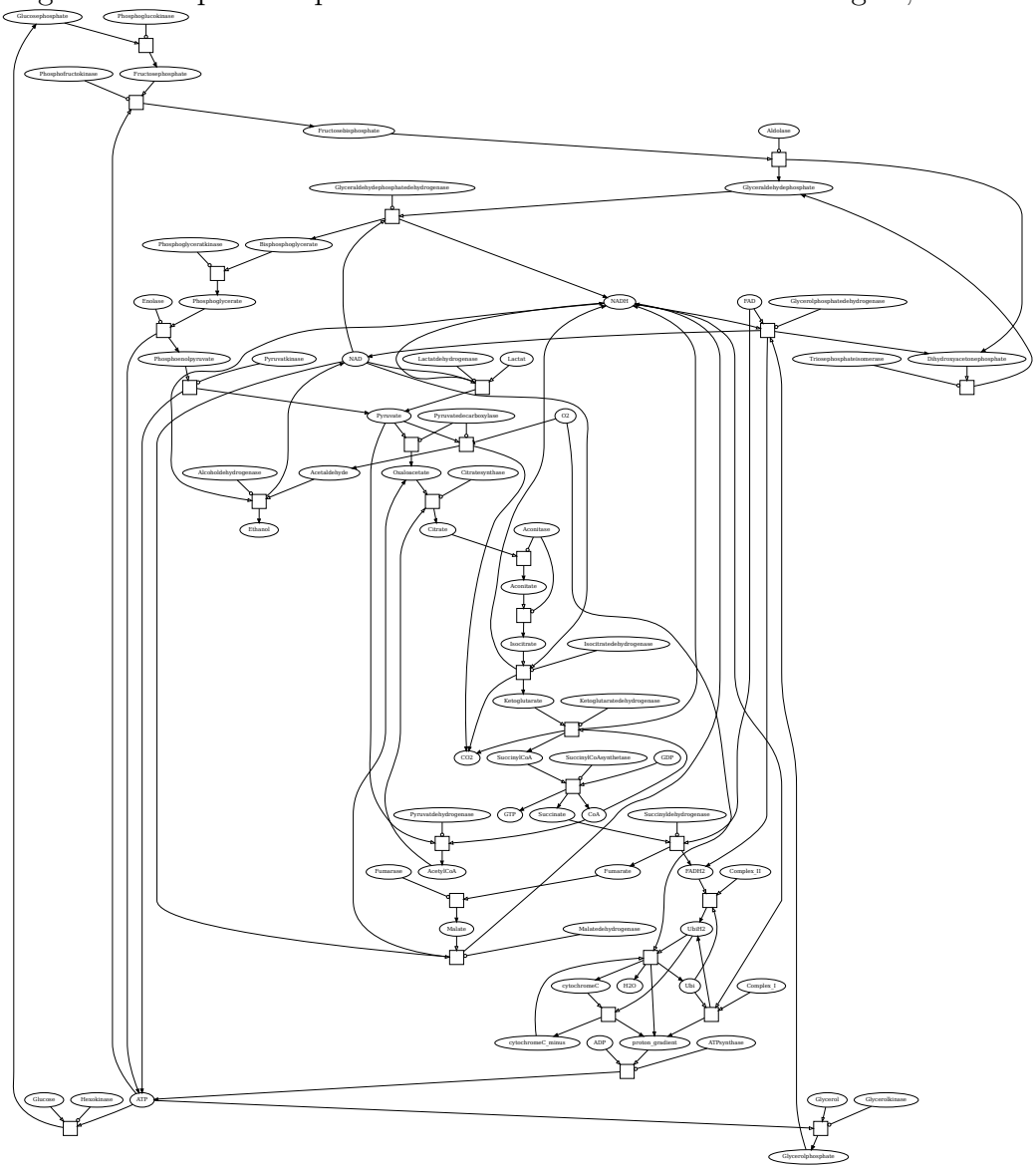


Figure 3: Graphical representation of the metabolism in listings 3, 4 and 5



3.3 Ras, Cyr1 and the PKA

The protein kinase A (PKA) is a downstream effector kinase of a pathway, conserved among eucaryotes. PKA phosphorylates a variety of proteins in response to (at least) two upstream signals and is also linked to stress response. PKA kinase activity is modulated, especially by Ras1/2 and by Gpr1. In this chapter, we will discuss the associated mechanisms of function.

3.3.1 PKA regulates autophagy via Psr-Whi2-Msn

As we will conclude in chapter 3.6, in which we discuss the Psr-Whi2-Msn pathway, Msn2/4 are stress response transcription factors, activated by dephosphorylation. PKA kinase activity inhibits their activity [36]. As presented in chapter 3.7, increased autophagy is one of the phenotypes, promoted by activation of Msn2/4, so autophagy can be modulated by PKA activity.

3.3.2 Gpr1 announces glucose presence

Glucose is detected by glucose receptor Gpr1, which is a G-protein coupled receptor, in the plasma membrane. Gpr1 interacts (at least) with Gpa2 [37], which in turn activates the adenylate cyclase Cyr1 [38].

Addition of glucose to cells, grown on a non-fermentable carbon source or to stationary phase, triggers a short-termed, transient rise in the intracellular level of cyclic adenosine monophosphate (cAMP), mediated by Gpa2 [37]. cAMP is the messenger, which directly conferres PKA activation. Listing 6 summarizes the derived Boolean logic.

The previous finding is especially interesting, since both states, non-fermentable carbon metabolism and stationary phase, are critical and both are linked to mitochondria:

The presence of glucose is ”interesting” for cells, grown on non-fermentable carbon source, since glycolysis is inactive, OAC and RC are upregulated, cells rely on their mitochondria for energy production. They have to adapt by enabling glycolysis and announcing an upcoming increase in pyruvate concentration to their mitochondria. The Gpr1 transmitted signal

may even ”allow” the cell, to increase stringency with respect to mitochondrial quality control, since mitochondria are expected to become non-essential and hence inefficiencies or defects no longer need to be tolerated. Also, the cell, of course, wants to metabolize the expected pyruvate in an energy-efficient manner, requiring efficient mitochondria, while the degraded, ”rejected” mitochondria may quickly be recovered by proliferation of the more efficient mitochondria on basis of the new carbon and energy source, which, simultaneously, promotes evolution of mitochondria towards higher efficiency.

Also in stationary phase, glucose presence is ”interesting”, since mitochondria are inactive and their density is reduced to a minimum. Cells have to adapt by reviving mitochondria from quiescence and promoting their multiplication. So, Gpr1 activity is expected to have a mitochondrial metabolism-stimulating downstream signal. With respect to the fact, that stationary phase can last indefinitely long, the cell's mitochondrial pool quality may have faded. Gpr1 may therefore, similar to the previous hypothesis, also in stationary phase promote an increase in mitochondrial quality control.

Listing 6: Glucose sensory model

```
#
# G-Protein coupled receptor in the plasma membrane
#
Gpr1*                                   = glucose

#
# alpha-subunit of heterotrimeric G-protein
# downstream of Gpr1
#
Gpa2*                                   = Gpr1

Cyr1*                                   = Gpa2
```

Inspecting listing 6, the mechanism of PKA activation appears unexpectedly complicated: Gpr1 is not directly activating it's final downstream target PKA, but via intermediates Gpa2, Cyr1 and cAMP. As further discussed in subchapter 3.3.3, this may be explainable from Cyr1 responding to (at least) two sensors: Gpr1 and Ras [39]. Notably, Gpr1 activity is not sufficient to suppress PKA-Msn2/4 mediated stress response (see subchapter 3.3.3), which suggests, that Ras may promote the more ”important” signal. We will confirm this assumption below.

3.3.3 Ras is a GTP-binding protein and associated to stress response

Ras1 and Ras2 ("Ras") are two highly related GTP-binding proteins and homologs of the mammalian oncogene Ras [40]. Ras plays a role in a MAPK and the PKA pathway [41]. In the context of this work, we will focus on the latter function.

Deletion of Ras or Cdc25 induces (drastic) stress response via Msn2/4 [42]. Cdc25 is a guanine nucleotide exchange factor for Ras, stimulating the exchange of GDP for GTP, thus assisting in efficient activation of Ras. It is usually interpreted, that ${}^{GTP}Ras$ is required for efficient activation of Cyr1: The levels of cAMP are significantly decreased in the mutants, leading to inactivation of PKA, which, in turn, initiates the Psr-Whi2-Msn pathway (chapter 3.6). However, we will provide a different explanation for the mutants' phenotypesbelow, suggesting an inhibitory control of ${}^{GTP}Ras$ over Cyr1.

3.3.4 Post-translationally modified Ras was encountered on the surface of mitochondria

Ras, like many Ras homologs, becomes post-translationally modified: Shr5 [43], Erf2 and Erf4 [44] constitute an acyltransferase, palmitoylating Ras. Ras also gets farnesylated, which requires at least Ram1 and Ram2 [45]. While palmitoylation is required for membrane association, but not for interaction with Cyr1, farnesylation increases Cyr1 affinity at least 100-fold [46]. We interpret, that the resulting Ras^{farn}_{palm} is capable of recruiting Cyr1, thereby gaining dominant control over cAMP production and thus the PKA. This idea is consistent with the dominant activity of Ras compared to Gpr1, suggested above, and especially interesting, since Ras was found localized to the mitochondria [47], (at least) in a scenario, where we expect stress response. Contradicting Leadsham et al., we speculate, that Ras was not by accident, but on purpose localized to the mitochondria (see below).

In our model, the function of Gpr1-Gpa2 signalling is, to deliver a "glucose presence" signal from the plasma membrane to Cyr1, especially acting

at the mitochondria: There, the membrane-bound Ras^{farn}_{palm}-Cyr1 complex is localized. Cyr1 becomes activated, much less, than by Ras activation, but still, in turn producing cAMP. cAMP downregulates stress response via stimulation of PKA. The reaction, producing cAMP is consuming ATP, so it is at least to some degree proportional to the individual mitochondrion's ATP output. ATP is degraded especially in the environment, close of the mitochondria, which might influence other ATP-dependent processes at the mitochondrial membrane. Since Gpr1-Gpa2-mediated Cyr1 activation is inefficient (see above), the consumption of ATP is low. This is reasonable, since the mitochondrial ATP output is required as energy source and must hence not be degraded without fulfilling a purpose.

3.3.5 No direct link could be established between Ras and Whi2

It has been reported, that Ras degradation requires Whi2 [47]. From literature and database searches, we were unable to infer an interaction between Ras and Whi2, so we could not model it.

Rather, since we will link Whi2 to stress response in chapter 3.6, we conclude, that no direct interaction, but a stress response is responsible for Ras degradation. Adding the finding, that palmitoylation binds Ras to the mitochondrial membrane, mitophagy itself may be responsible for degradation of activated Ras. This also makes sense with respect to the next subchapter.

3.3.6 Ras activity is linked to mitochondrial efficiency

Ras has been shown to respond primarily to acidification [48]. In chapter 3.2 we explained, that the OAC lowers the pH, while RC raises it. A dropdown of pH near the mitochondria indicates, that the OAC in the mitochondrion metabolizes more efficient than the RC. This way, Ras becomes activated, when mitochondrial respiration efficiency drops.

This hypothesis goes together well with the fact, that the Ras substrate GTP also is a product of the OAC: GTP, a signalling molecule, rather than ATP, the chemically homologous energy unit. This way, Ras activity is stimulated by the OCA in a two-fold manner, while RC inhibits it, with protons

being the second messenger.

In listing 7 we have summarized the inferred interactions, concerning Ras. mtATP refers to the mitochondrial ATP output, that is constutively consumed by Cyr1, independent of activation, while activation of Ras dominantly inhibits cAMP production. As one representative of the acids, involved in OCA, we introduced CO_2, since it is produced but not consumed. CO_2 has the chance (and is supposed) to leave the cell and thereby re-raise the cellular pH (buffer capacity).

Listing 7: Ras sensory model

```
Ras_GTP_palm_farn*                    = Ras_palm_farn and GTP and CO2
Cyr1*                                 = (mtATP or Gpa2) and (not Ras_GTP_palm_farn)
```

3.3.7 Cyr1 may be linked to mitophagy

From our model, the Δras2 and Δcyr1 phenotype of extended lifespan can be explained. The absence of Ras, leads to improper localization of Cyr1, implying reduced cAMP levels and abolished PKA signalling. Similarly in the Δcyr1 mutant. Improper downregulation of PKA, would induce stress response via Msn2/4 (see also chapter 3.8) and thus indirectly upregulate (mitochondrial) quality control / mitophagy.

Additionally, we would like to mention, that besides it's adenylate cyclase and Ras-binding domain, Cyr1 contains a protein phosphatase domain (localized approx. between aa 1300-1600 in Fig. 4). We could not find a reference in literature, addressing the function of this domain.

Especially with respect to mitophagy, we consider this a rather interesting finding, since the mitophagy-associated factors Atg32 and Atg33, which are also localized to the mitochondrial surface, contain sites, which are target to kinase/phosphatase-mediated regulation (see chapter 3.8). Since it is probable, that Δras2-promoted lifespan extension is due to effects, that involve specifically mitochondria, we speculate, that Cyr1 responds to a local pH dropdown, sensed by Ras, by promoting mitophagy via changes in the phosphorylation state of Atg32 and/or Atg33 (directly or indirectly). This would, of course, shed a new light on the function of Ras and Cyr1, rendering them mitochondrial quality sensor and effector.

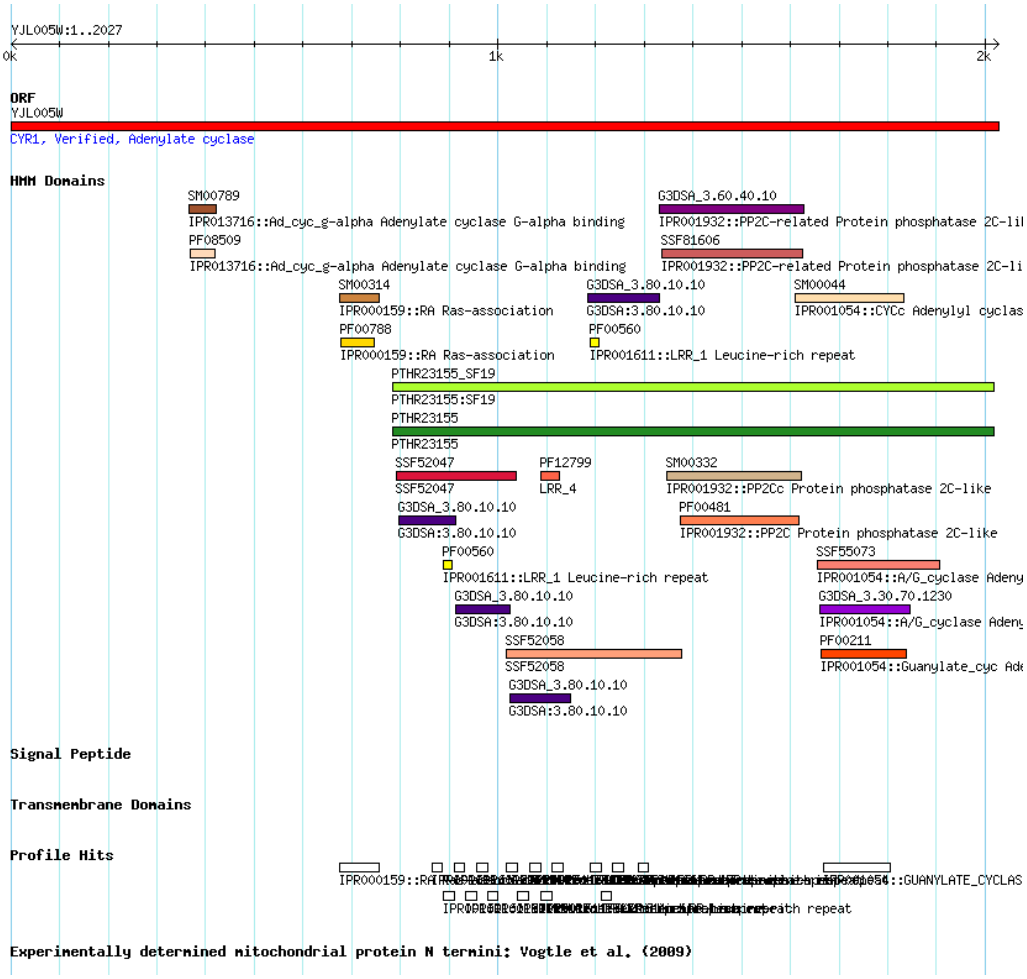


Figure 4: Protein domains of Cyr1, as predicted by SGD

This idea perfectly fits with our previous assumption, that Gpr1 acts as a stringency enhancer for mitophagy.

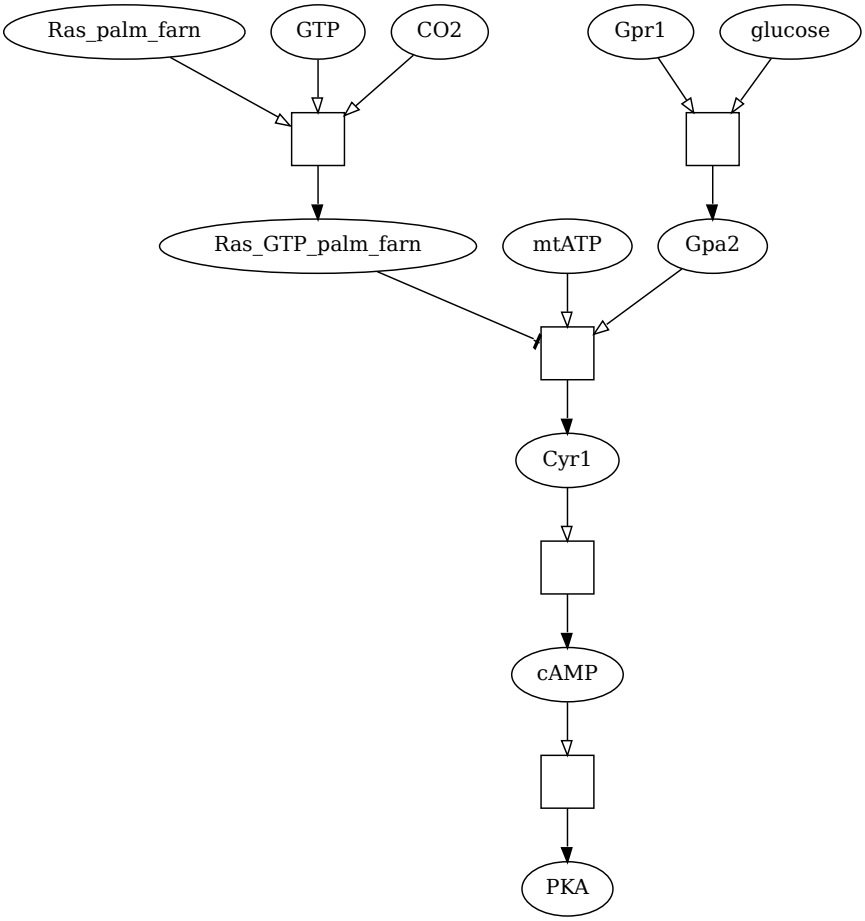
3.3.8 Conclusion

As listing 8 and figure 5 present, adenylate cyclase Cyr1 integrates upstream signals from the OAC-responsive Ras (post-translationally modified, GTP-bound ${}^{GTP}Ras_{palm}^{farn}$) and the glucose-responsive Gpa2. Cyr1 produces cAMP, which activates PKA.

Listing 8: Boolean model for the PKA pathway

```
Gpa2*                                = Gpr1 and glucose
Ras_GTP_palm_farn*                   = Ras_palm_farn and GTP and CO2
Cyr1*                                = (mtATP or Gpa2) and (not Ras_GTP_palm_farn)
cAMP*                                = Cyr1
PKA*                                 = cAMP
```

Figure 5: Graphical representation of the PKA network in listing 8



3.4 The Tor network

The Tor network is a pathway, responding to different nutritional conditions with a fine-tuned response, that relies on many interacting components.

A central role is effectuated by two (functionally differing) Tors: Tor1 and Tor2. Complexes involving Tor1 (TORC1) control ribosome biogenesis, translation, protein turnover and transcription of starvation-specific genes. Complexes involving Tor2 (TORC2) control actin cytoskeleton organization [49]. Several "prominent" kinases, originally identified in different contextes, have been linked to the Tor network: Hog1, PKA, Yak1, Sit4, Slt2 and PP2As [7]. This underlines the central role and importance of Tor's signalling.

A TORC1 has also been linked to mitophagy, which is consistent with the assumption, that mitophagy is regulated in a fine-tuned response to nutritional conditions.

In this chapter, although we know, that an appropriate examination of Tor functionalities would go beyond the frame of this work, we will shed some light on the Tor network.

3.4.1 Rapamycin

Rapamycin is an organic compound, originally isolated from *Streptomyces hygroscopicus* on Rapa Nui (easter islands) [50]. It has an antifungal effect and is used to prevent rejection of organ transplants [51].

Mechanisticly, Rapamycin binds Fpr1, thereby inactivating Tor1 complexes (but not Tor2's complexes). We are unaware, which Tor complexes are affected by rapamycin, but in experiments, rapamycin treatment induced nitrogen starvation response. Probably it inactivates several, if not all Tor1 complexes.

3.4.2 Tor induces autophagy via STREs

Tor1 was linked to autophagy: Treatment of cells with rapamycin, induced autophagy, while a high concentration of cAMP could abolish this phenotype [52]. Tor1 is also involved in Msn2/4 activation: Msn2, usually cytoplasmatic, is activated (translocated to the nucleus) during nitrogen starva-

tion [53]. We conclude, that Tor1 mediates STRE activation upon nitrogen starvation via Msn2/4 (via the Psr-Whi2-Msn pathway, see chapter 3.6). It is unclear, whether this occurs in parallel, upstream or downstream of PKA.

However, it anyway can not sufficiently explain results, specifically involving mitophagy. Also no combination with PKA activity could, at least not in any of our tested scenarios.

3.4.3 STRE activation occurs via a PP2A

Rapamycin as well as inactivation of Tap42 causes sustained Msn2 activation. We don't know at which point rapamycin acts in the Tor complexes, so we can only assume, that Tor1 activates Tap42. It might as well be an inhibitory action upon the individual complex formation. Tip41 plays a role in transducing the Tap42 signal downstream. Tap42 inhibits type 2A phosphatase complexes (PP2As). A PP2A (or several) is/are required for nuclear localization of Msn2 in response to heat shock as well as osmotic shock and in response to nitrogen starvation, but not for glucose starvation response [53].

In our model, Tor1 mediates STRE activation, by delegation of an effector branch, composed of Tap42, Tip41 and a PP2A, as presented in Lst. 9.

Listing 9: Boolean model of Tor's Tap42 effector branch

```
Tap42*                          = Tor1
PP2As_enabled*                  = not (Tap42 and Tip41)
```

3.4.4 PP2As are activated by Rrd1/2

Tor1's activation of STREs depends on Rrd1 and Rrd2: Single mutants Δrrd1 and Δrrd2 are sensitive to osmostress and don't respond to rapamycin treatment [54]. Rrd1 and Rrd2 are potential activators of PP2As. We conlude, that PP2As are controlled two-fold by the corresponding upstream Tor. Combining this with the previous pathway, the logic in Lst. 10 results.

Listing 10: Boolean model of Tor's Rrd1/2 effector branch

```
Tap42*                          = Tor1
Rrd1_2*                         = not Tor1
PP2As_enabled*                  = (not (Tap42 and Tip41)) or Rrd1_2
```

3.4.5 Also Hog1 is linked to Rrd1/2

In the previous subchapters, osmostress response seemed to be related to the Tor network.

As a matter of fact, Δrrd1 Δrrd2 double mutants are inviable on glucose, but grow in the presence of an osmotic stabilizer [54]. This suggests, that the osmotic stress signalling or effector pathway is disrupted in the absence Rrd1/2. Δrrd1 Δrrd2 could partially be rescued by deletion of Hog1 or Pbs2, which are also related to osmotic stress response.

3.4.6 Also Slt2 is linked to Rrd1/2

Compared to the Δrrd1 Δrrd2 double mutant, the three-fold mutant Δslt2 Δrrd1 Δrrd2 showed improved growth on sorbitol-containing medium [54]. However, we are currently unable to explain this finding, but mention it, since in chapter 3.8 we will present results, indicating Hog1 and Slt2 to be involved in mitophagy.

3.4.7 Tor controls manifold effector branches

PP2A stands for protein phosphatase type 2A complex. Different type 2A phosphatases exist in yeast: We are aware of Sit4, Pgp1, Pph3, Pph21 and Pph22.

Tor seems to control the specific (in)activation of type 2A phosphatases in a manner, dependent of Tap42, Tip41, Rrd1 and Rrd2 [55].

The mechanisms, underlying Tor-mediated nutritional responses, are not yet fully elucidated. In our model, different nutrients assemble functionally differing Tor complexes: Downstream effector phosphatases (PP2As) are controlled via Tap42-Tip41 and Rrd1/2 in a nutrient-dependent manner. Each nutrient, that is detected by the Tor network, is addressed by a specific Tor complex, involving a dedicated sensor and a dedicated phosphatase (or phosphatase complex). Upon sensor activation, PP2As dephosphorylate further downstream targets, like transcriptional activators, in a sensor-activity-dependent fashion [56] [57] [58].

We expect, that the targeted (de)phosphorylation sites in the downstream effectors intrinsically contain the information in their amino acid motif(s), upon which specific, binary encodeable combination of (nutritional) conditions they shall become (de)activated. This functional mechanism renders the Tor network a digital information processor.

In Lst. 11 we present a preliminary model of the Tor1 network.

Listing 11: Boolean model of Tor1 complexes, we are aware of

```
# we are aware of 5 TORC1-type branches
Tor1_Sit4*                    = Sit4_sensor and Sit4_signal and Sit4 and PP2As_enabled
Tor1_Pgp1*                    = Pgp1_sensor and Pgp1_signal and Pgp1 and PP2As_enabled
Tor1_Pgh3*                    = Pgh3_sensor and Pgh3_signal and Pgh3 and PP2As_enabled
Tor1_Pph21*                   = Pph21_sensor and Pph21_signal and Pph21 and PP2As_enabled
Tor1_Pph22*                   = Pph22_sensor and Pph22_signal and Pph22 and PP2As_enabled
```

3.4.8 Tor controls mitophagy

A Tor1 complex, which responds to rapamycin, responds to nitrogen starvation (see above). Nitrogen starvation induces mitophagy (discussed in chapter 3.8). From this we can immediately conclude, that a Tor1 complex exists, that controls specifically mitophagy and presumably via dephosphorylation of a downstream target. The dedicated sensor would most probably respond to glumatin or glutamate (see nitrogen metabolism). As described in the previous subchapter, the effector is expected to be targeted by a dedicated PP2A. Maybe also here, a detector for mitochondrial metabolism could act.

We can only speculate how nitrogen sensing could work via the Tor network, so we are unable to present a Boolean network for nitrogen starvation induced mitophagy.

3.5 Rim15 integrates different stress signals

Rim15 is a kinase, that integrates four (or more) upstream kinase signals about nutrient availability. We mention Rim15, since additionally to the Psr-Whi2-Msn pathway it is required, for appropriate STRE activation via Msn2/4. The function of Rim15 is the remodelling of chromatin, which is not sufficient, but required for stress response, and occurs downstream of Rim15 via (de)acetlyation of chromatin via Ume6, Ime1, Ime2, Rpd3 and Sin3 [49].

Rim15 is a direct downstream target of PKA: phosphorylation by PKA inhibits Rim15 [59].

Also inactivation of TORC1 (via rapamycin treatment or nitrogen starvation), leads downstream to dephosphorylation of a cytoplasmatic anchor sequence in Rim15 and subsequently to nuclear localization. TORC1's effect on Rim15 appears not directly, but via a TORC1 effector branch, but not via Sit4 and probably also not via Pph21 or Pph22 [49].

Additionally, Rim15 also responds to the phosphate-sensing Pho85 kinase, in conjunction with it's cyclin partner Pho80.

Sch9, a kinase, that senses the presence of a fermentable growth medium, also acts on Rim15.

Also starvation for sulfur was shown to influence Rim15 [60].

In addition to nutrient-dependent kinases, it has been suggested, that Rim15, maybe via a special protein domain, responds also to oxidative stress, enabling e.g. the transcription of superoxide dismutases Sod1 and Sod2 [61].

We summarize and present in Lst. 12: Among other transcriptional activation related effects, Rim15 responds to nutritional depletion, by enabling Msn2/4-mediated STRE activation (Psr-Whi2-Msn pathway, see chapter 3.6), via decondensation of chromatin from heterochromatin to euchromatin.

Listing 12: Boolean model for the role of Rim15

```
Rim15*                          = (not PKA) or (not PP2A) or (not Pho85_80) or (not Sch9)

STRE*                           = Rim15 and Psr_Whi2_Msn
```

We would like to add, that, since, as chromatin remodeller, Rim15 is curcial for any kind of starvation, it may linked tightly to Tor complexes, which sense nutritional exhaustion. Therefore we expect, that an analysis of

the interactions between Tor complexes and Rim15 and of the phosphorylation sites in Rim15's amino acid sequence, could provide insight into Tor's capabilities, especially with respect to nitrogen sensing.

3.6 The Psr-Whi2-Msn complex induces stress response

Whi2 has been introduced as a factor, which is important for induction of stress reponse. In this chapter we will derive the probable mechanism of function, by which Whi2 is capable of doing so.

3.6.1 Whi2 binds Psr1 and Psr2 to activate STREs

The binding of Whi2 to Psr1 was shown to be required for STRE activation: Upon deletion of Whi2, expression of genes, controlled by STRE promotors, was significantly reduced under various stress conditions [62].

Evidence was presented, that Whi2 binds Psr1 [62]. The authors additionally reported, that the Δwhi2 mutant shows a similar phenotype, as the double mutant Δpsr1 Δpsr2. As we already stated in the introduction (chapter 1.2), Psr1 and Psr2 fulfill redundant functions in yeast. The single mutant results are not presented, so redundancy of Psr1 and Psr2 with respect to the binding of Whi2 is not evident.

Provided the striking similarity of protein domains (presented in Fig. 6 and 7), it is likely, that also Psr2 binds Whi2 and induces STRE activation. This assumption is additionally supported by the protein sequence similarity, depicted in listing 13: Although the amino-terminal half of the alignment contains gaps and a weak similarity between Psr1 and Psr2, the carboxy-terminal half, which was identified as nuclear import sequence phosphatase (see Fig. 6 and 7) and is the functionally important region, is mostly identical.

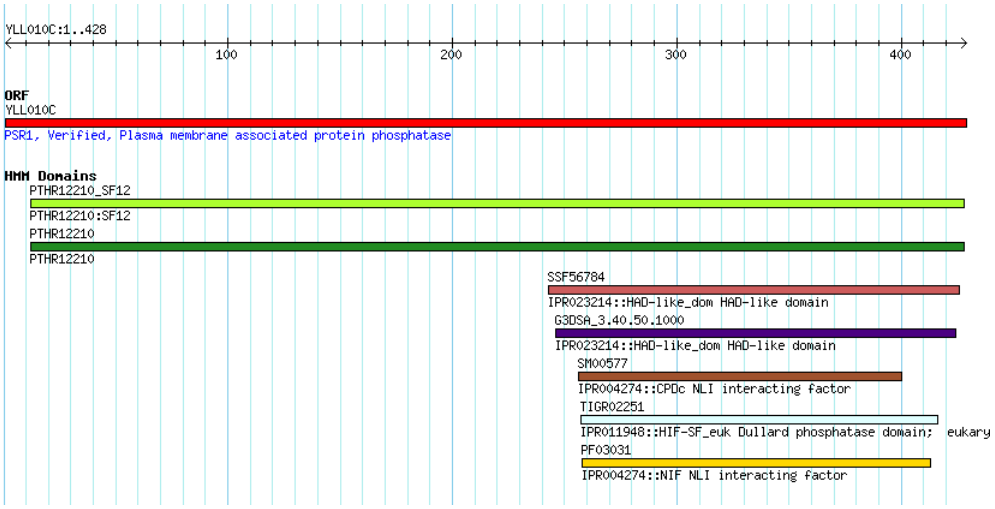


Figure 6: Psr1 protein domains, as predicted by SGD

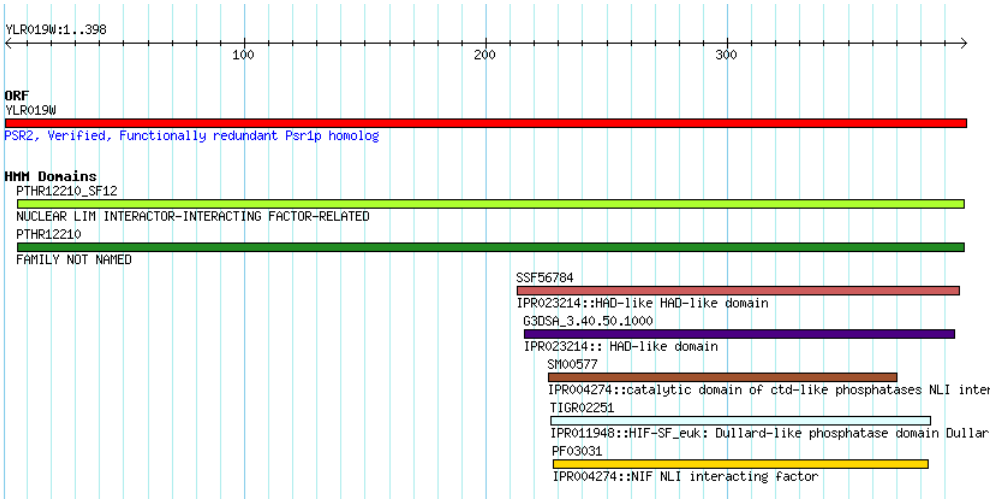


Figure 7: Psr2 protein domains, as predicted by SGD

Listing 13: Protein sequence comparison Psr1 vs. Psr2

```
#=======================================
#
# Aligned_sequences: 2
# 1: YLL010C
# 2: YLR019W
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 442
# Identity:     226/442 (51.1%)
# Similarity:   276/442 (62.4%)
# Gaps:          58/442 (13.1%)
# Score: 1025.5
#
#
#=======================================

YLL010C             1 MGFISSILCCSSETTQSNSNSAYRQQQSSSLNKNRSVKHSNTKSRTRGVH     50
                      ||||::||||||:|::::..   ||...::.|:||:.|||:.|::|:|..
YLR019W             1 MGFIANILCCSSDTSKTHRQ---RQPPETNHNRNRNRKHSSNKAQTQGRK     47

YLL010C            51 QTNSPPSKTNSAAT---FSSTERSTGKSGISTNDNEKKKPSSPTAAVTAT     97
                      |..:|.......:|   ..|:..|...:|..|........:...|.::..
YLR019W            48 QKATPNGDKMQYSTPEILLSSSDSGSNAGSKTMQENGNSGNGKLAPLSRD     97

YLL010C            98 TTNNMTKVEKRISKDDLYEEKYEVD----------EDEEIDDEDNRRSRG    137
                      .:||....||.      ||:..|.|          |:||.|||...:...
YLR019W            98 HSNNSYDEEKE------YEDYNEGDVEMTEVNNAGEEEEEDDEAKEKQDH    141

YLL010C           138 IVQEKG-DAVKDTSRQKKQQQQQQQQSQPQPQPQSQSQSQSQSQSQQRGP    186
                      :|.|.. ||.:::|...:...||......|
YLR019W           142 VVHEYNVDADRNSSINDEAPPQQGLYQVGQ--------------------    171

YLL010C           187 TVQVSSDHLIQDMNLSRVSSSSQASETSNDADDEDDEDEEYIDLTLLQQG    236
                                :|||...|:||     ..||.:.....|::.|||.||..
YLR019W           172 ----------EDMNPQYVASS-----PDNDLNLIPTTEEDFSDLTHLQPD    206

YLL010C           237 QYHAPGYNTLLPPQDESTKGKKCLILDLDETLVHSSFKYLRSADFVLSVE    286
                      |||||||:|||||:.:.:.:.|||||||||||||||||||:.|||||.||
YLR019W           207 QYHAPGYDTLLPPKLQEFQQKKCLILDLDETLVHSSFKYMHSADFVLPVE    256

YLL010C           287 IDDQVHNVYVIKRPGVEEFLERVGKLFEVVVFTASVSRYGDPLLDILDTD    336
                      ||||||||||||||||:|||.||.:|:|||||||||||.:||||.||.:
YLR019W           257 IDDQVHNVYVIKRPGVDEFLNRVSQLYEVVVFTASVSRYANPLLDTLDPN    306

YLL010C           337 KVIHHRLFREACYNYEGNYIKNLSQIGRPLSDIIILDNSPASYIFHPQHA    386
                      ..|||||||||||||||||||||||||||:.|||||||||||||||||
YLR019W           307 GTIHHRLFREACYNYEGNYIKNLSQIGRPLSETIILDNSPASYIFHPQHA    356

YLL010C           387 IPISSWFSDTHDNELLDIIPLLEDLSVKTSLDVGKILDVTI*    428
                      :||||||||||||||||||||||....||||.:|||||
YLR019W           357 VPISSWFSDTHDNELLDIIPLLEDLSSGNVLDVGSVLDVTI*    398
```

Given these facts, we feel safe to assume redundancy of Psr1 and Psr2. The logic for Whi2 binding Psr1 and Psr2 is presented in listing 14.

Listing 14: Boolean logic for Whi2's interaction with Psr1 and Pr2

```
Psr_Whi2*               = Whi2 and (Psr1 or Psr2)
```

3.6.2 Whi2 binds Msn2 and Msn4 to activate STREs

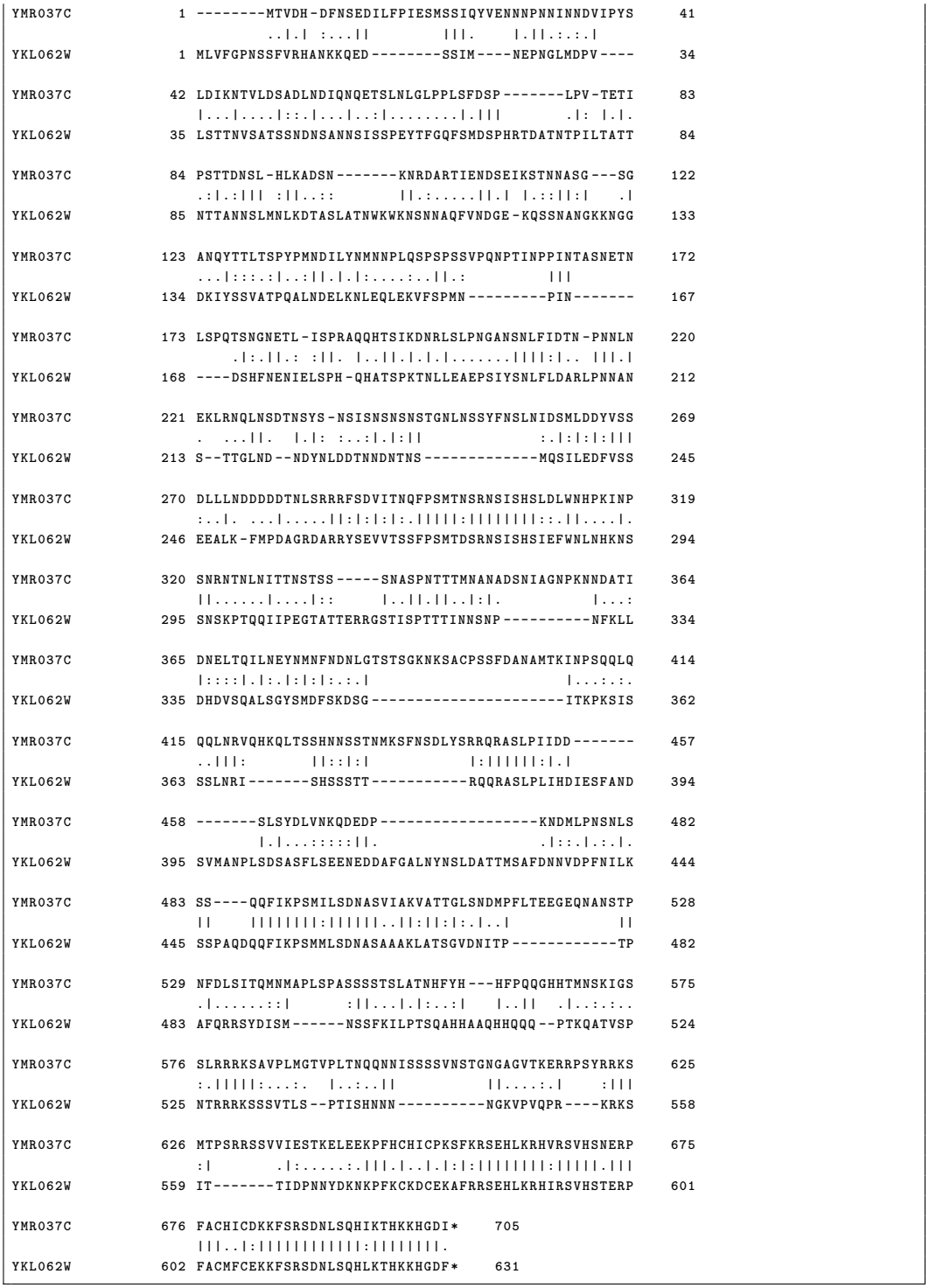
Msn2 and Msn4 are the only transcription factors, that up to our knowledge are capable of inducing STRE-promoted gene expression.

In literature, Msn2 and Msn4 are mostly used in a redundant manner, assuming functional redundancy. Evidence was presented, that Whi2 binds Msn2 [62], but we didn't find a reference, that Msn4 binds Whi2, so we cannot rule out, that Msn4 is activated in a Whi2-independent fashion, although Msn4 and Whi2 have been found co-localized [63]. In experiments, a decrease of stress response was observed upon deletion of Msn2, while deletion of Msn4 caused little to no decrease in stress response [42]. This may be an experimental artifact, since the experiments were not carried out to specifically analyze the function of Msn2 and Msn4. It may, of course,a also hint to functional differences between Msn2 and Msn4.

We aligned the protein sequences of Msn2 and Msn4, which supports their redundancy (see Lst. 15): We found 45% of the amino acids similar, but also 29% gaps.

Listing 15: Protein sequence comparison Msn2 vs. Msn4

```
#=======================================
#
# Aligned_sequences: 2
# 1: YMR037C (Msn2)
# 2: YKL062W (Msn4)
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 780
# Identity:     251/780 (32.2%)
# Similarity:   349/780 (44.7%)
# Gaps:         224/780 (28.7%)
# Score: 794.0
#
#=======================================
```



Considerring also, that redundant usage in literature may also be due to

facts, we are not aware of, we will assume Msn2/4 redundancy at least in the context of this work. The logic for the interaction with Whi2 is presented in listing 16.

Listing 16: Boolean logic for Whi2's interaction with Msn2 and Msn4

```
Whi2_Msn*              = Whi2 and (Msn2 or Msn4)
```

Additionally to Whi2, Rim15 was presented as potential Msn2/4 activator: It has been shown, that Msn2/4 activity depends on protein kinase Rim15, suggesting that Rim15 could phosphorylate Msn2/4. But, as we discuss in chapter 3.5, Rim15 integrates upstream stress signals to mediate chromatin remodelling. Therefore we believe, that Rim15 does not directly interact with Msn2/4, but rather mediates decondensation of heterochromatized DNA regions, which contain the Msn2/4-responsive genes, in a parallel pathway.

3.6.3 Psr1/2 activates Msn2/4 via Whi2

As we already stated, protein sequence analysis recognizes the amino-terminal region of both Psr1 (Fig. 6) and Psr2 (Fig. 7) as a nuclear import sequence phosphatase (NIF NLI). Combined with the above conclusions of Whi2 binding Msn2/4 as well as Psr1/2, it is reasonable to assume, that Psr1/2 binds Msn2/4 in four-fold redundant manner, mediated by Whi2: The phosphatase(s), upon activation, dephosphorylating the transcription factor(s) and thereby inducing STRE activation.

This assumption goes together well with the finding of STRE activation being reduced to half in Δwhi2 cells, since Psr1/2 may still become activated upon an upstream stress signal, but may be inefficient in dephosphorylating it's downstream target Msn2/4 in the absence of the Whi2.

The fact, that Δwhi2 and Δpsr1 Δpsr2 mutants exhibit the same phenotype of defective stress response [62], leades us to the conclusion, that Whi2 is the only mediator between Psr1/2 and Msn2/4. It, however, does not rule out, that Psr1/2 also activates other transcription factors, e.g. via other mediators.

3.6.4 Why are three proteins required for STRE activation ?

Psr1/2 was claimed to be "strictly localized to the plasmamembrane due to a conserved, amino-terminal sequence motif, suggesting a close functional relationship with plasmamembrane-bound environmental sensors" [62]. However, from protein sequence analysis we cannot confirm the presence of a membrane-associative region in neither Psr1, nor Psr2. It seems to us, that both phosphatases are globular, cytoplasmatical proteins.

In case there exists data we are unaware of, confirming the localization of Psr1/2 to the plasmamembrane, Whi2, as a cytoplasmatic protein, could act as an intermediate signal relay, transmitting Psr1/2 activity from the plasma membrane to the cytoplasm or the nuclear membrane (if we assume Msn2/4 to be waiting there for activation). We lack data to further evaluate this hypothesis.

3.6.5 PKA suppresses STRE activity via Msn2/4 and Rim15

A nuclear import as well as a nuclear export sequence was identified in Msn2, which is recognized by nuclear pore transporters Kap121 and Kap123 [64]. Similar sequences exist also in Msn4 [65]. Both, Msn2 and Msn4, carry several phosphorylated motifs (Fig. 8 and Fig. 9): Different kinases phosphorylate Msn2/4, among them the PKA, which phosphorylates the nuclear import sequence.

Taken together with the finding, that PKA also inactivates Rim15 via phosphorylation (see chapter 3.5), PKA seems to suppress STRE activation in a two-fold manner: via inhibition of transcription factors as well as via heterochromatization of genes. The concluded logic is presented in listing 17.

Listing 17: Boolean logic, modelling PKA's inhibition of stress response

```
Msn2*                   = (not PKA)
Msn4*                   = (not PKA)
Rim15*                  = (not PKA)

Psr_Whi2_Msn*           = (Psr1 or Psr2) and Whi2 and (Msn2 or Msn4)

STRE*                   = Psr_Whi2_Msn and Rim15
```

Protein Sequence

```
  1 MTVDHDFNSE DILFPIESMS SIQYVENNNP NNINNDVIPY SLDIKNTVLD SADLNDIQNQ
 61 ETSLNLGLPP LSFDSPLPVT ETIPSTTDNS LHLKADSNKN RDARTIENDS EIKSTNNASG
121 SGANQYTTLT SPYPMNDILY NMNNPLQSPS PSSVPQNPTI NPPINTASNE TNLSPQTSNG
181 NETLISPRAQ QHTSIKDNRL SLPNGANSNL FIDTNPNNLN EKLRNQLNSD TNSYSNSISN
241 SNSNSTGNLN SSYFNSLNID SMLDDYVSSD LLLNDDDDDT NLSRRRFSDV ITNQFPSMTN
301 SRNSISHSLD LWNHPKINPS NRNTNLNITT NSTSSSNASP NTTTMNANAD SNIAGNPKNN
361 DATIDNELTQ ILNEYNMNFN DNLGTSTSGK NKSACPSSFD ANAMTKINPS QQLQQQLNRV
421 QHKQLTSSHN NSSTNMKSFN SDLYSRRQRA SLPIIDDSLS YDLVNKQDED PKNDMLPNSN
481 LSSSQQFIKP SMILSDNASV IAKVATTGLS NDMPFLTEEG EQNANSTPNF DLSITQMNMA
541 PLSPASSSST SLATNHFYHH FPQQGHHTMN SKIGSSLRRR KSAVPLMGTV PLTNQQNNIS
601 SSSVNSTGNG AGVTKERRPS YRRKSMTPSR RSSVVIESTK ELEEKPFHCH ICPKSFKRSE
661 HLKRHVRSVH SNERPFACHI CDKKFSRSDN LSQHIKTHKK HGDI
```

Protein Kinase Motifs

301 - 304: Casein Kinase I (CKI, CK-1)	302 - 304: cAMP-dependant Protein Kinase (PKA, cAPK)
304 - 308: Glycogen Synthase Kinase 3 (GSK-3)	449 - 451: cAMP-dependant Protein Kinase (PKA, cAPK)
579 - 582: cAMP-dependant Protein Kinase (PKA, cAPK)	580 - 582: cAMP-dependant Protein Kinase (PKA, cAPK)
617 - 620: cAMP-dependant Protein Kinase (PKA, cAPK)	618 - 620: cAMP-dependant Protein Kinase (PKA, cAPK)
622 - 625: cAMP-dependant Protein Kinase (PKA, cAPK)	623 - 625: cAMP-dependant Protein Kinase (PKA, cAPK)
625 - 629: Glycogen Synthase Kinase 3 (GSK-3)	627 - 630: CDK1 Protein Kinase
630 - 633: cAMP-dependant Protein Kinase (PKA, cAPK)	631 - 633: cAMP-dependant Protein Kinase (PKA, cAPK)

Figure 8: Sites, that have been encountered phosphorylated in Msn2

Protein Sequence

```
  1 MLVFGPNSSF VRHANKKQED SSIMNEPNGL MDPVLSTTNV SATSSNDNSA NNSISSPEYT
 61 FGQFSMDSPH RTDATNTPIL TATTNTTANN SLMNLKDTAS LATNWKWKNS NNAQFVNDGE
121 KQSSNANGKK NGGDKIYSSV ATPQALNDEL KNLEQLEKVF SPMNPINDSH FNENIELSPH
181 QHATSPKTNL LEAEPSIYSN LFLDARLPNN ANSTTGLNDN DYNLDDTNND NTNSMQSILE
241 DFVSSEEALK FMPDAGRDAR RYSEVVTSSF PSMTDSRNSI SHSIEFWNLN HKNSSNSKPT
301 QQIIPEGTAT TERRGSTISP TTTINNSNPN FKLLDHDVSQ ALSGYSMDFS KDSGITKPKS
361 ISSSLNRISH SSSTTRQQRA SLPLIHDIES FANDSVMANP LSDSASFLSE ENEDDAFGAL
421 NYNSLDATTM SAFDNNVDPF NILKSSPAQD QQFIKPSMML SDNASAAAKL ATSGVDNITP
481 TPAFQRRSYD ISMNSSFKIL PTSQAHHAAQ HHQQQPTKQA TVSPNTRRRK SSSVTLSPTI
541 SHNNNNGKVP VQPRKRKSIT TIDPNNYDKN KPFKCKDCEK AFRRSEHLKR HIRSVHSTER
601 PFACMFCEKK FSRSDNLSQH LKTHKKHGDF
```

Protein Kinase Motifs

260 - 263: cAMP-dependant Protein Kinase (PKA, cAPK)	261 - 263: cAMP-dependant Protein Kinase (PKA, cAPK)
313 - 316: cAMP-dependant Protein Kinase (PKA, cAPK)	314 - 316: cAMP-dependant Protein Kinase (PKA, cAPK)
316 - 319: Casein Kinase I (CKI, CK-1)	486 - 488: cAMP-dependant Protein Kinase (PKA, cAPK)
488 - 492: Glycogen Synthase Kinase 3 (GSK-3)	556 - 558: cAMP-dependant Protein Kinase (PKA, cAPK)

Figure 9: Sites, that have been encountered phosphorylated in Msn4

3.6.6 Psr1/2 may autodephosphorylate

The BioGRID contains data about physical interactions of Psr1 with Psr2 [66] and vice-versa [67]. This could hint to Psr1/2 dimerization. Given Psr1/2's phosphatase activity, dimerization could induce autodephosphorylation. The latter would require Psr1/2 to contain the phosphorylation motif, they are by themselves capable to dephosphorylate. In fact, Psr1 has been encountered phosphorylated by the PKA (Fig. 10). Experimental confirmation is required to enstrengthen or falsify this hypothesis.

3.6.7 Glycogen may inhibit STRE activation

We performed further phosphorylation site analysis and looked up the phosphorylation sites of Whi2: Whi2 is phosphorylated by GSK-3 (Fig. 11).

The term GSK-3 summarizes different glycogen synthase type-3 kinases. A possible interpretation could be, that presence or anabolism of the storage carbohydrate glycogen could diminish or abolish the induction of stress response, by inhibition of efficient mediation between Psr1/2 and Msn2/4.

This idea is supported by a review of the phosphorylation patterns of Msn2 (Fig. 8) and Msn4 (Fig. 9): Both factors are also phosphorylated by a GSK-3.

We are aware of four glycogen synthase type-3 kinases in yeast: Mck1, Ygk3, Mrk1 and Rim11, and in fact, all of them have been reported to interact with Msn2, so glycogen-related processes may inhibit stress reponse. Experiments are required to test our hypothesis.

3.6.8 Autophagy can be stimulated or induced via Msn2/4

The induction of autophagy was observed, when Sch9 and PKA were simultaneously inactivated [36]. TORC1 was also shown to play a role in the control of autophagy induction. The Atg1-13-17 kinase complex was essential for induction of autophagy in all pathways.

In our model, the Atg1-13-17 kinase complex represents the initiator of autophagosome formation, inducing bulk autophagy as well as providing pre-autophagosomes for selective types of autophagy. Induction of autophagy,

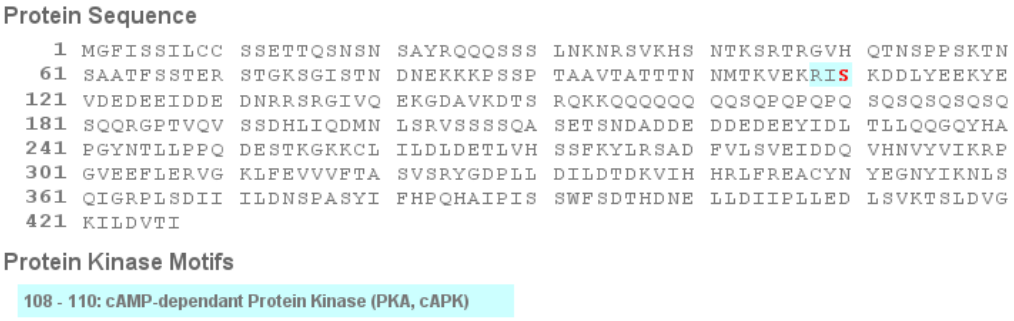


Figure 10: Sites, that have been encountered phosphorylated in Psr1

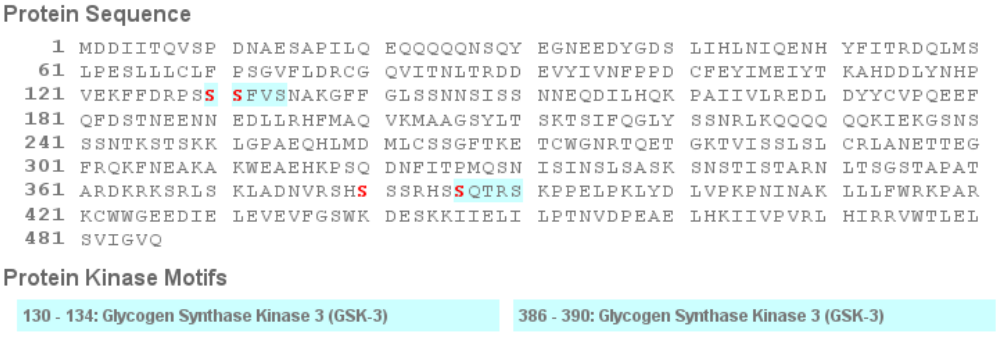


Figure 11: Sites, that have been encountered phosphorylated in Whi2

of course, depends on expression of autophagy-related genes, which may be mediated via Msn2/4.

Autophagy is presented in chapter 3.7.

3.6.9 Databases contain no data, linking Whi2 to mitophagy

Up to this point, we were unable to uncover a direct link between Whi2 and mitophagy. We tried to make use of all available information concerning Whi2, so we also took the corresponding BioGRID dataset of protein interactions into consideration [68]. The BioGRID lists 16 physical and 277 genetic interactions for Whi2 (June 2012).

None of the physical interactions are in an obvious manner related to autophagy or mitophagy. Among the many inferred genetic interactions, however, some concern proteins, we already encountered:

- overexpression of Whi2 rescues mutations in Msn2
- overexpression of Msn4 enhances the phenotype of Whi2 mutations
- simultaneous mutation of Whi2 and Pde2 causes severe fitness defects
- simultaneous mutation of Whi2 and Pho80 causes severe fitness defects
- simultaneous mutation of Whi2 and Tor1 enhances the individual mutation's phenotype
- simultaneous mutation of Whi2 and Ras2 enhances the individual mutation's phenotype
- simultaneous mutation of Whi2 and Sch9 causes severe fitness defects
- simultaneous mutation of Whi2 and Snf4 (part of Snf1 kinase complex) enhances the individual mutation's phenotype
- deletion of Whi2 rescues the lethality of Tpk3 deletion

Again, we did not find a link to mitophagy.

3.6.10 Conclusion

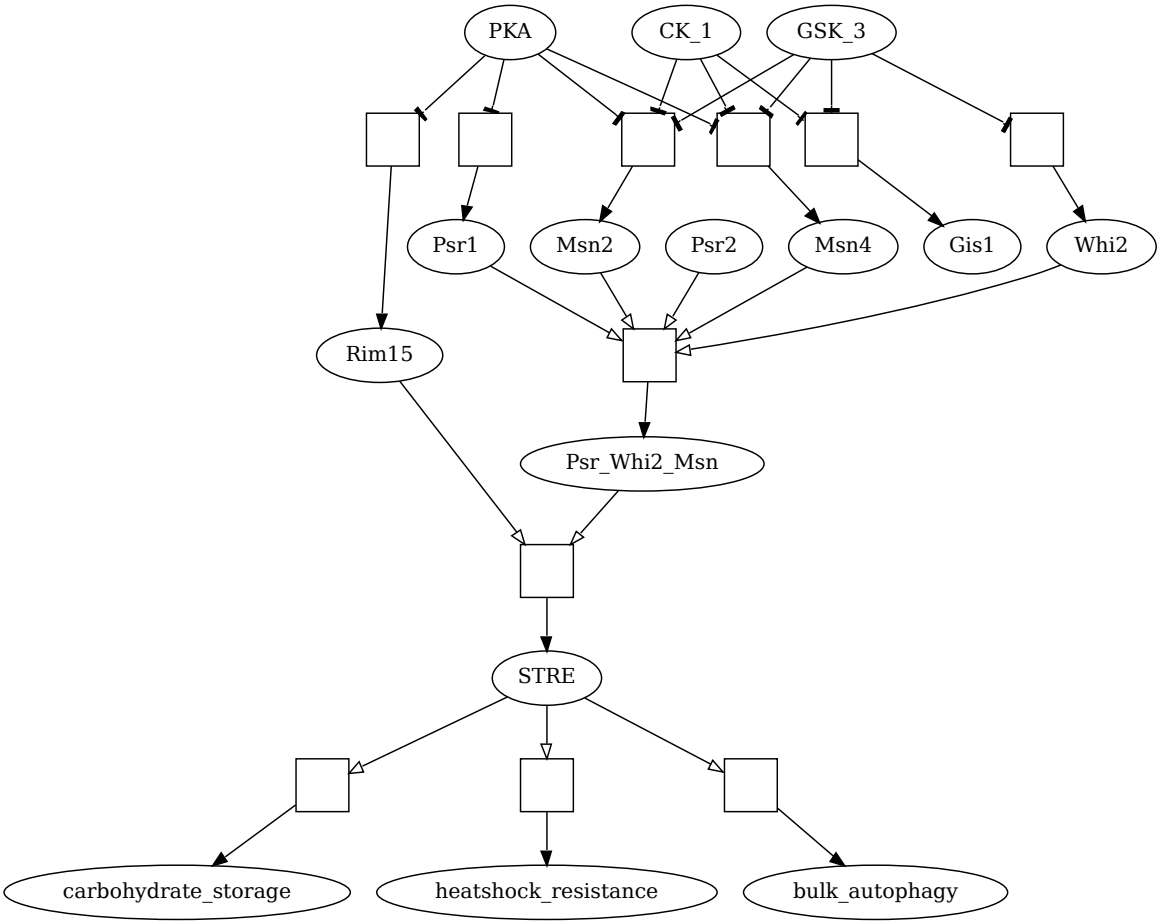
To summarize the efforts, presented in the previous subchapters, we were unable to infer a direct interaction between Whi2 and mitophagy and can thus not explain the diminished mitophagy in the Δwhi2 mutant [1]. We suggest, that regulation of mitophagy might occur via a different mechanism.

Still, we have deducted, that Psr1/2, Whi2 and Msn2/4 form either a complex or a sequential pathway to activate STREs and this way induce, repectively increase expression of autophagy-related genes. Listing 18 and figure 12 combine our results about Ras, Whi2, Psr1/2, Msn2/4, Rim15 as well as STREs.

Listing 18: Boolean model of the Psr-Whi2-Msn effector

```
Rim15*                                  = not PKA
Psr1*                                   = not PKA
Whi2*                                   = (not GSK_3)
Msn2*                                   = (not PKA) and (not GSK_3) and (not CK_1)
Msn4*                                   = (not PKA) and (not GSK_3) and (not CK_1)

# Gis1, also depicted because of the striking similarities in sequence,
# functional mechanism and upstream kinases
Gis1*                                   = (not GSK_3) and (not CK_1)


Psr_Whi2_Msn*                           = (Psr1 or Psr2) and Whi2 and (Msn2 or Msn4)

STRE*                                   = Psr_Whi2_Msn and Rim15

#
# phenotypes accounted to STRE activity
#
bulk_autophagy*                         = STRE
heatshock_resistance*                   = STRE
carbohydrate_storage*                   = STRE
```

Figure 12: Graphical representation of the Whi2 network in listing 18



3.7 Autophagy

In this chapter, we will provide a quick introduction to general autophagy, before we come to the specific mitophagy.

Autophagy is a cellular process, by which either non-specifically or specifically, certain cellular components can be marked and subsequently transported to the vacuole. Pre-autophagosomal sites are present in the cell, that, upon induction of autophagy, begin growing and, during bulk autophagy, randomly engulf cytoplasmatic components into a double membrane, forming an autophagosome. The latter is transported to the vacuole, where binding, fusion, breakdown and lysis of targets subsequentially occur via active mechanisms.

The cell uses autophagy to gain nutrients, like amino acids, but also to prevent damage by "defective" intracellular components, like protein aggregates or ROS-leaking mitochondria.

3.7.1 Induction

Induction of autophagy can occur on the level of gene transcription, but also on protein level. The latter is mediated via the autophagy initiator kinase complex, consisting of Atg1, 13 and 17 [7]. Downstream autophagy processes rely on CVT.

3.7.2 CVT

Cytoplasm-to-vacuole targeting (CVT) is a pathway, that actively imports hydrolases into the vacuole. Among subsequent peptide cleavage, these are activated and promote vacuolar degradation. Understandably, CVT is required for all types of autophagy.

Although functionally partially relying on factors, that also play a role in processes of selective autophagy, we would like to differentiate CVT from these, since CVT is, from the epistastical point of view, dominant, so, required for the others.

In listing 19 we present our model, including the autophagy-related proteins (Atgs) and the hydrolases. The details, however, are not of further

importance for stress signalling or mitophagy.

Listing 19: Boolean model of the CVT pathway

```
Atg20_24*                          = Atg20 and Atg24
Lap4_PAS*                          = Lap4 and Atg19 and Atg20_24 and PAS
Ams1_PAS*                          = Ams1 and (Atg19 or Atg34) and PAS
Ape1*                              = preApe1

CVT_bodies*                        = (Lap4_PAS or Ams1_PAS) and Ape1 and Atg11
```

3.7.3 Assembly of autophagosome precursors

Induction of autophagy leads to assembly of several factors at a site, commonly refered to as the pre-autophagosomal site (PAS). Interestingly, Atg9 seems to shuttle between PASes and mitochondria, fetching membrane from the latter, to enable PAS growth. Listing 20 summarizes our model for PAS assembly, however, PASes are constitutively present in the cell, so their underlying mechanism is not interesting, with respect to possible targets of stress effectors or mitophagy.

Listing 20: Boolean model of the preparation of pre-autophagosomes

```
PAS*                       = Atg1_13_17 and Atg29 and Atg31 and
                             Atg8_PE and (Atg9 or Atg9_mito)
```

3.7.4 Vacuolar engulfment, breakdown and lysis

There exists differing interpretations of the term ”autophagosome”. We would like use it to refer to the ”mature” autophagosome, so a pre-autophagosome, that has engulfed a target. We speculate, that microtubule play a role in active transport of autophagosomes to the vacuole via Atg8. We can not confirm this hypothesis, however it seems likely to us, that either PASes or targets or both can actively move to each other together for efficient autophagy. We therefore introduced Atg8, which is bound to microtubule. Fusion also does not occur spontaneously, at leat not efficiently, several factors play a role. Interestingly, the content of autophagosomes is not immediately endocyted in the vacuole, but the autophagosome is completely taken up, including it’s intact membrane. Since no mechanism is known, that can excrete autophagosomes from the vacuole, the intravacuolar

degradation may be subject to undiscovered regulation. In listing 21, we present our model.

Listing 21: Boolean model of the vacuolar breakdown of autophagosomes

```
autophagosome*                  = bulk_autophagy or pexophagy or ribophagy or aggrephagy or mitophagy
transport_to_vacuole*           = autophagosome and Atg8_mtub

#
# autophagic vacuole fusion (GO:0000046)
#
fusion*                         = transport_to_vacuole and Ccz1 and Vam3 and Atg8_PE

#
# autophagic membrane disassembly (GO:0030399)
#
# MVB: multi-vesicular bodies
#
Atg15*                          = Atg15_ER and MVB

vacuolar_catabolism*            = fusion and CVT_bodies and Atg15
```

3.8 Mitophagy

We have provided a brief introduction to mitophagy in chapter 1.4. In the following chapter, we would like to examine more detailled, how mitophagy works, how it is induced and which proteins are involved. We will take literature knowledge, but also protein domain predictions and our interpretation of their respective function into account.

3.8.1 Mitophagy is a reproduceable behaviour

Nitrogen starvation as well as culturing cells to stationary phase on a non-fermentable carbon source are reliable methods to reliably induce mitophagy in wild-type yeast [24]. Besides this, mitophagy has been observed upon treatment with rapamycin, and has been linked to mitochondrial quality control [24].

As we discussed in chapter 3.4.1, rapamycin acts via simulation of nitrogen starvation. Additionally to the nitrogen starvation induced mitophagy pathway, we expect a mechanism promoting mitophagy for reasons of quality control. It is also not obvious, whether mitophagy in the stationary phase is to be accounted to mitochondrial quality control, to nitrogen starvation, to both, or to a third mechanism.

3.8.2 Why is mitophagy necessary upon nitrogen starvation?

While, to maintain their quality, degradation of dysfunctional mitochondria is important under any nutritional condition, the degradation of mitochondria as a reaction to the absence of nitrogen is not easy to explain. It seems, however, that yeast is eager to degrade it's mitochondria upon nitrogen starvation.

One possible explanation could be the breakdown of mitochondrial protein, to make the bound nitrogen available for the cellular metabolism. The favor for mitochondrial protein could e.g. be explained, by the cell, considerring mitochondria an especially lucrative source of nitrogen. If this idea is the case, we would expect also other mechanisms of protein degradation to be up-regulated. However, this hypothesis is in contradiction with the fact, that

protein catabolism requires the OAC, which takes place in the mitochondria and should therefore rather inhibit mitophagy, than induce it.

A different explanation could be an increased need for α-ketoglutarate: This compound is required for nitrogen assimilation from ammonia (see Fig. 13). α-ketoglutarate is a component of the OAC, therefore increased cellular levels would be degraded by the mitochondria in the OAC, shooting down cellular efforts to increase ammonia assimilation efficiency via α-ketoglutarate. If this idea is the case, the question would remain, why mitophagy is required to interrupt the OAC. Intuitively, inhibition or degradation of enzymes, that are involved in the OAC, would be sufficient to prevent it from stepping forward. Mitophagy, to increase cellular levels of α-ketoglutarate seems a rather drastic measure.

Another idea is based on the conclusion, that the specific degradation of mitochondria implies, that they become disadvantageous. From what we know about mitochondria, the only disadvantage they can represent, is the generation of ROS. Assuming, the ROS production increases upon nitrogen starvation, it would be reasonable to induce mitophagy [16].

Another finding, concerning the cellular pH, may support this idea: When cells grown in a rich medium were transferred to a synthetic nitrogen starvation media, secreted metabolites lowered the extracellular pH below 3 and autophagy-defective mutants mostly died [22]. So, the cell degrades it's mitochondria, to prevent a pH dropdown. The question remains, why pH is expected to drop when nitrogen becomes limited and why this is bad.

Glycolysis produces pyruvate, which is converted to ethanol in the absence of OAC, so glycolysis does not result in production of acids. OAC activity, on the other hand, does, since CO_2 is set free (we already addressed this in chapter 3.2.2). pH lowering thus is a hallmark of OAC. Under stress-free conditions, the pH lowering in OAC is compensated by an equal amount of counteracting redox reactions in the RC, which reduces oxygen, assimilating two protons, thereby raising the pH. The dropdown upon nitrogen starvation can thus *only* be explained by a deficiency in RC activity.

The question remains, why nitrogen is essential for the RC, but not for the OAC. There are two important differences between OAC and RC:

- RC genes are encoded in the mitochondrion, while the entire OAC is nuclearly encoded.
- RC proteins have a higher turnover rate, due to a higher frequency of ROS-damage.

These differences can explain mitophagy: When the nitrogen sources fade out, the cell, having access to a bigger nitrogen pool / reserve, continues OAC gene expression, which sustains longer, than the RC gene expression in the mitochondrion. Additionally, the higher RC protein turnover rate, demands higher concentrations of nitrogen in the mitochondrion, making it even more susceptible to a decrease in nitrogen levels. Moreover, when the RC efficiency decays, the pH drops, which could further reduce RC efficiency, since the pH may leave the range of optimal protein efficiency. ROS production increases, leading to a vicious circle of additional RC damage, quickly completely abolishing all respiratory processes, severly dropping the pH and causing damage to the cell.

If this idea is the case, it would actually be understandable, why cells consider mitochondria a hazard during nitrogen starvation, and are eager to precautionarily degrade them.

3.8.3 Whi2 is probably not a mitophagy-specific factor

As discussed in chapter 3.4, mechanisticly, the Tor network is responsible for nitrogen sensing.

The fact, that nitrogen starvation induced mitophagy (and also other stress responses) is diminished in Δwhi2 mutants [1], leads to the conclusion, that STREs are activated in response to nitrogen starvation in the wild-type.

Since STREs are also active, e.g under conditions of heat or osmotic stress, not promoting increased mitophagy, the induction of STRE is insufficient to explain mitophagy.

Since, up to our model, mitophagy upon nitrogen starvation requires a quick response, the indirect way via induction of gene transcription anyway seems unlikely to us. It seems more likely, that one the involved kinases or phosphatases reponds to nitrogen starvation and activates an already

present mitophagy-specific factor, which leads to mitophagosome formation and degradation, using an already present machinery.

We are aware, that this hypothesis is in contradiction with the findings, linking mitophagy to Whi2 [1]. Nevertheless, from the data we are aware of, we can find no other explanation than, that nitrogen starvation indeed, but not only, promotes STRE acitvation via Whi2, which indeed increases mitophagy, but via increased expression of general autophagy-related factors. The specific influence on mitophagy must be accounted to a parallel, Whi2-independent pathway, involving nitrogen-responsive (probably Tor-mediated) kinase or phosphatase activity on a mitophagy-specific factor.

We will now try to present candidates for this kinase or phosphatase, as well as for the mitophagy-specific factor.

3.8.4 Atg32 is the central mitophagy promoting factor

Intuitively, we would have expected, that quality control and nitrogen starvation induced mitophagy act via distinct mechanisms at the mitochondria, since they respond to different upstream signals and fulfill a different purpose in the cell. However, both mechanisms seem to be conferred via Atg32-Atg11-dependent selective autophagy:

In cells deleted for Atg32, mitophagy did not rise above 7% of wild-type mitophagy levels, even under stress [16]. The low level of basal mitophagy probably explains from their physical size: mitochondria seem to be too big for efficient autophagosome engulfment via bulk autophagy. Also the fact, that mitochondria are connected to a mitochondrial network, may make it difficult, to catch them via a non-directed mechanism, although fission was shown, not to be required for mitophagy [1]. We conclude, that all types of mitophagy depend on selective autophagy, conferred exclusively via Atg32.

Atg32 is a mitochondrially anchored transmembrane receptor, that recruits Atg8 and Atg11 to the mitochondrial surface [69].

The resulting initiator complex (${}^{8}Atg_{32}^{11}$) links mitophagy to the selective autophagy machinery. ${}^{8}Atg_{32}^{11}$ attracts pre-autophagosomes to the marked mitochondrion, which begin engulfing it to form a mitophagosome, that is

subsequently targeted to the vacuole for degradation.

3.8.5 Atg32 activity is controlled via phosphorylation

In chapter 3.8.3, we hypothesized, that the Tor network is responsible for induction of mitophagy. Very recent findings seem to support our hypothesis: When mitophagy is induced, Ser^{114} and Ser^{119} in the Atg32 amino acid sequence are phosphorylated [70].

3.8.6 Slt2 and Hog1 are the kinases controlling Atg32

Under nitrogen starvation, Atg32, Bck1, Mkk1/2, Slt2, Pbs2 and Hog1 are required for mitophagy [71]. Δbck1, Δmkk1 Δmkk2, Δslt2, Δpbs2 and Δhog1 mutants all exhibited half of the wild-type mitophagy.

Together with the upstream protein kinase C homolog Pkc1, Bck1, Mkk1/2 and Slt2 constitute a linear pathway, that was previously linked to cell wall remodelling. In listing 22 we present it's mechanistical logic.

Listing 22: Boolean model of the "PKC pathway"

```
Bck1*                   = Pkc1
Mkk1*                   = Bck1
Mkk2*                   = Bck1
Slt2*                   = Mkk1 or Mkk2
```

Similarly, Pbs2 and Hog1 also constitute a linear pathway, that was previously linked to osmostress response. In listing 23 we present it's mechanistical logic and how we derive regulation of mitophagy.

Listing 23: Boolean model of the "PKC pathway"

```
Hog1*                   = Pbs2

Atg32*                  = Slt2 or Hog1
Atg32_8_11*             = Atg32 and Atg8 and Atg11

#
# PAS = pre-autophagosomal sites/complexes
#
mitophagy*              = PAS and Atg32_8_11
```

3.8.7 Atg33 is also related to mitophagy

Additionally to Atg32, Atg33 has been found to be required for efficient mitophagy.

Atg33 is a comparably small protein (197 aa, 20,4 kDa). After peptide cleavage, only the tiny region between aa 102-171 (70 aa) remains, to potentially fulfill a function. Atg33 consists mainly of transmembrane domains:

- aa 13 - 35 = 23 aa
- aa 55 - 72 = 18 aa
- aa 79 - 101 = 23 aa
- aa 172 - 194 = 23 aa

Mgm1, for comparison, is five times heavier (881 aa, 99,2 kDa), not fulfilling any active function, we are aware of. Notably, Atg32 contains only one transmembrane domain (23 aa; aa 389-411), which renders Atg33, with four transmembrane domains, a rather strongly anchored protein. Atg32 contains one 388 aa cytosolic and one 119 aa intramitochondrial domain (amino-terminal, respectively carboxy-terminal), the first one acting as Atg11 adaptor region, the latter not having any apparent function. Therefore it would be cryptic, how the tiny Atg33 region could become active in induction of mitophagy. We rather believe, that there exists a currently unidentified Atg33-binding protein, which is conferring this activity.

3.8.8 Atg33 is regulated via phosphorylation

PhosphoGrid lists two CK-1 phosphorylation motifs, found by Li X in a large-scale assay. These sites lie inside the previously mentioned 70 aa region. We presume, that this region exhibits a regulatory function.

Overexpression of CK-1 isoforms has been shown to rescue starved, stationary-phase cells, deleted for Gcs1, which is required to re-enter mitotic cycle after stationary phase. This suggests, that there exists a CK-1, responding to nutrient availability, phosphorylating Atg33.

3.8.9 Lge1 was suggested as Atg33-binding factor

Lee et al. claim to have detected Atg33 in complex with Lge1 [72]. Lge1 is a protein of unknown function. Null mutants display delayed premeiotic DNA

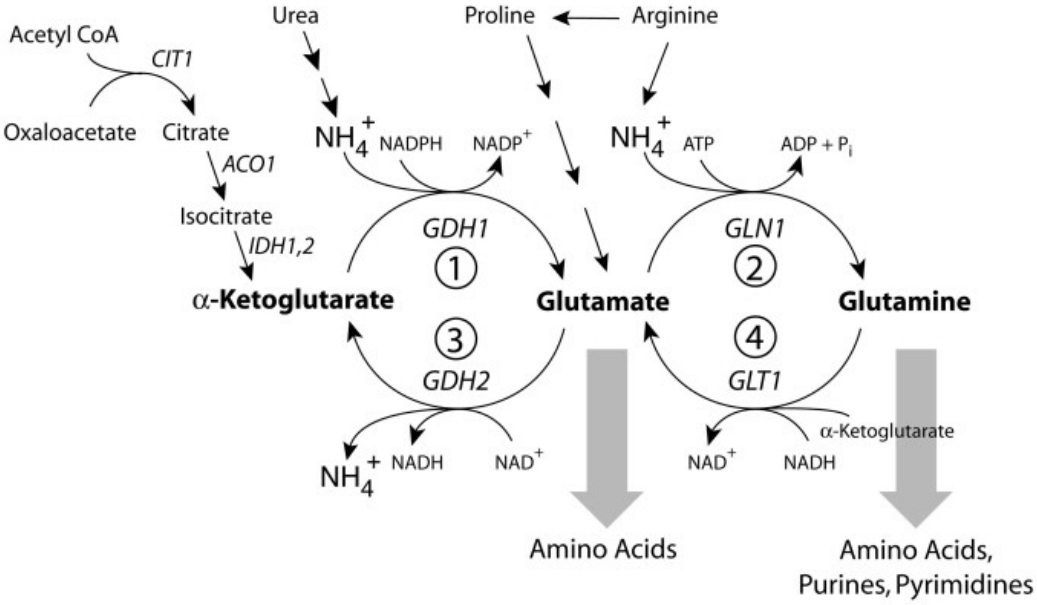


Figure 13: *Saccharomyces cerevisiae*'s nitrogen metabolism, as presented by Magasanik and Kaiser [58].

Protein Sequence

```
  1 MSVCLAITKG IAVSSIGLYS GLLASASLIT STTPLEVLTG SLTPTLTTLK NAATALGAFA
 61 STFFCVSFFG APPSLRHPYL LYGMLVAPLS SFVLGCASNY QSRKYSKVSK ESSLFPEDSK
121 LAASELSDSI IDLGEDNHAS ENTPRDGKPA ATTVSKPAEA LHTGPPIHTK NLIAATAIAI
181 VGFVQAVIGV YGEGQFI
```

Protein Kinase Motifs

124 - 127: Casein Kinase I (CKI, CK-1)	140 - 143: Casein Kinase I (CKI, CK-1)

Figure 14: Sites, that have been encountered phosphorylated in Atg33

synthesis and reduced efficiency of meiotic nuclear division [7]. If further experiments can confirm the binding of Lge1 to Atg33, this could render Lge1 an import factor in mitochondrial quality control. The binding itself can only occur on Atg33's regulatory region (see below), as we expect Lge1 to be a cytoplasmatic protein. Without experimental confirmation, of course, the role of Lge1 remains a weak hypothesis.

3.8.10 Atg33 contains a signal peptide sequence

According to protein sequence analysis (Fig. 15), Atg33 contains several transmembrane domains, suggesting localization to the mitochondrial outer membrane (23 aa mitochondrial membrane anchor).

Additionally, Atg33 contains a signal peptide subsequence, irreversible cleaved by a signal peptidase I.

Signal peptides have previously been reported to mediate allocation of proteins to their designated target organelles. It is thus possible, that Atg33's signal peptide is responsible for Atg33's integration into the mitochondrial membrane. Atg32, though, does not contain such a sequence, so we favor a different explanation, which we discuss in the following subchapter.

3.8.11 Atg33 links mitochondrial quality to mitophagy

Deletion of Atg33 blocks mitophagy to half the level of wild type yeast during nitrogen starvation, whereas it almost completely inhibits mitophagy in stationary phase [24]. Additionally, it was observed, that only a fraction of the total mitochondrial pool is degraded by mitophagy in stationary phase, wherefore it is reasonable to assume, that Atg33 confers the selective degradation of inefficient mitochondria.

Menon et al report, that the Δgcr1 Δatg33 double mutantion is lethal. This goes together with the assumption, that Atg33 is required for mitochondrial quality control (and thus for sustained mitochondrial energy efficiency), since Gcr1 is a transcriptional activator, required for efficient glycolysis. Thereby outfading mitochondrial efficiency, combined with diminished glycolysis, could well explain the double mutant's lethality.

In our model, Atg33 is constitutively expressed by the cell and integrates into the mitochondrial membrane. An unidentified mitochondrial membrane protein cuts the signal peptide in an energy-/ATP-dependent manner, promoting the removal of Atg33 from the mitochondria. Mitochondria with a diminished production of the ATP, fail to efficiently remove Atg33 from their membrane. Atg33 therefore accumulates on the mitochondrial surface in a quality dependent manner and presents inefficient mitochondria for mitophagy. The stringency with respect to the efficiency, the cell demands from it's mitochondria, could be regulated by the phosphorylation of Atg33's regulatory region. We consider it probable, that cells can inhibit (cloak) Atg33 in situations, that demand a larger pool of mitochondria. Such situations include high nutrient concentrations, that cause an increase in mitochondrial metabolism, as it is sensed by Ras. It would therefore not be surprising, if Atg33 would be encountered phosphorylated by the PKA. As we suggested in subchapter 3.3.7, it may also be dephosphorylated by Cyr1.

We conclude, that Atg33 is specifically involved in mitophagy of inefficient mitochondria. It remains the question, whether Atg32 and Atg33 function parallel, upstream or downstream of each other.

3.8.12 Atg33 acts via Atg32

We have concluded, that Atg32 is essenial for mitophagy, in chapter 3.8.4. Consequently, Atg33 must act upstream of Atg32. But, Atg33 is not essential for mitophagy [24]. With this in mind, we are able to present a mechanistical logic for Atg33's function (Lst. 24).

Listing 24: Boolean model of Atg33's function

```
Atg33*                          = not ATP

Atg32*                          = Atg33
Atg32_8_11*                     = Atg32 and Atg8 and Atg11
```

3.8.13 Conclusion

Control of mitophagy does not require STRE activation, but is regulated on the protein level, presumably via phosphorylation. Listing 25 summarizes,

how we derive induction and regulation of mitophagy on the Atg level.

Listing 25: Boolean model of mitophagy

```
Atg33*                          = not ATP

Atg32*                          = Atg33 or Hog1 or Slt2

Atg32_8_11*                     = Atg32 and Atg8 and Atg11

mitophagy*                      = PAS and Atg32_8_11
```

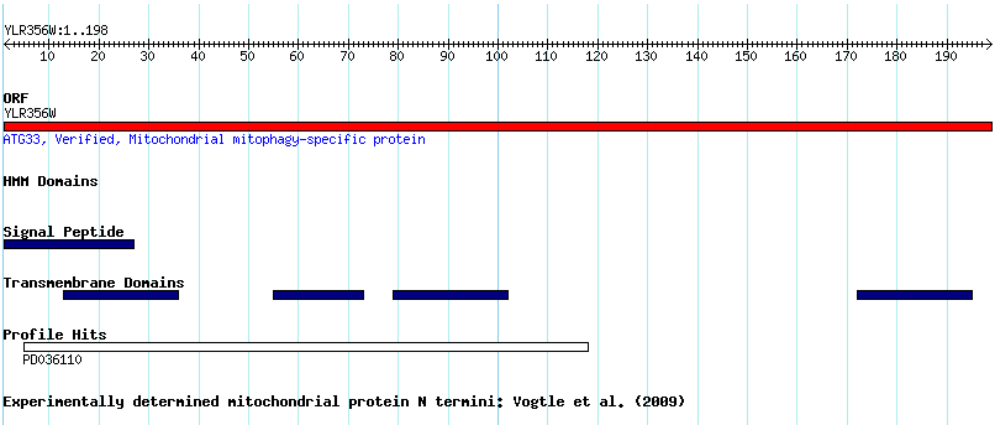


Figure 15: Atg33 protein domains, as predicted by SGD

3.9 Summary

In this work we have shed light on stress signalling, especially with respect to Whi2 and it's partners, Psr1/2, Msn2/4 and Rim15. We modelled the mechanisms underlying autophagy, suggested functional mechanisms for mitophagy. For the latter we have re-inspected the mitochondrial metabolism. We encountered several kinase pathways, of which we especially analyzed PKA and the Tor network. We have derived a Boolean model, in which, with respect to the involved players, we tried to incorporate all knowledge, that is available from literature. As a result, we have gained a model, by which we were able to reproduce previously established pathways. To some degree (the Ras chapter), we felt safe, to provide alternative explanations, contradicting established opinions. Also, we derive complete pathways for previously unexplainable phenotypes mainly from theoretical conclusions (Ras, Atg32, Atg33), which we present with the suggestion to review them, keeping in mind, that they are hypothesises:

Ras, is usually presented as upstream signaller of the "Ras-cAMP-PKA" pathway, sensing glucose presence. However, here we derive, that neither is Ras a glucose signaller, nor is the term "pathway", in our opinion, appropriate for the interaction of Ras and PKA. Instead we suggest, that the action of Ras on PKA is of secondary nature, compared to the importance of Ras as a detector for mitochondrial quality. We also "re-address" adenylate cyclase Cyr1, which we suggest, to be one of the missing links between mitochondrial energy efficiency and mitophagy, due to it's ATP concentration-dependent activity and it's localization to the mitochondrial surface. We presume, that the cellular ratio of cAMP to ATP reflects the activity of mitochondria.

With respect to mitophagy-specific factors, we have analyzed all 36 autophagy-related proteins (Atgs), and suggest, Atg32 and Atg33 to be responsible for all encountered phenotypes, related to mitophagy. Moreover, we present a possible mechanism, by which Atg33 mediates selection of mitochondria in dependence of their quality, while we assign the function of pre-autophagosome recruiting to Atg32, that, as it has already been suggested, via Atg11 links mitochondria to the autophagy machinery.

Inspecting the interaction partners of Whi2, we obtained a negative result with respect to the suggested specific link to mitophagy. Although we found phosphorylation sites, that could be targeted by mitophagy-specific kinases, we in general consider it unlikely, that mitophagy is induced via transcriptional activation, especially with respect to the alternative mechanism, we provide for the control over mitophagy. Still, however, we have derived a pathway, that is likely, to be responsible for induction of transcription of stress-responsive elements (STREs). It includes Psr1, Psr2, Whi2, Msn2, Msn4 and Rim15 and is controlled by at least three different kinases: protein kinase A, a glycogen type 3 kinase and a casein kinase 1. Induction of stress via this pathway can upregulate or even induce non-specific autophagy.

As we inspected interactions with Tor, we encountered a complex, fine-tuned network, that links phosphorylation of special amino acid motifs to sensory of nutrients and stress. The Tor network is very interesting, but also too complex, to be addressed in the context of this work. However, we are certain, that at least one Tor1 complex exists, that links glutamin/glutamate recognition to (de)phosphorylation of Atg32 or 33. With respect to the essential function, we expect the involved proteins to be highly conserved.

Also, we have encountered several molecules, that are related to aging, respectively lifespan extension. Among these are Sch9, Ras and rapamycin. Therefore we believe, that our model contains at least some of the mechanisms, that are capable of modulating lifespan in yeast. All important factors with respect to lifespan had one in common: They were related to mitochondrial metabolism, specifically the respiratory chain. Mutant longevity phenotypes could all be explained by either reduction of mitochondrial metabolism, an increase of mitochondrial quality control or increased resistance to oxidative stress. We discuss the importance of reactive oxygen species in detail.

As a more general conclusion, we would like to point to the complexity of the machinery, that is involved in the control of mitochondrial quality, that makes it evident to us, that reactive oxygen species, that leake from the mitochondrial respiratory chain complexes, must have a drastic impact on cells' viability. Nevertheless, as we presumed initially, the gain of huge amounts of energy from the mitochondria seems to justify the trade for lifespan.

4 Discussion

All of our modelling was carried out using a Boolean approach. While other approaches, including linear, exponential, Michaelis-Menten or Hill kinetics are the methods of choice to get quantitative, rather than qualitative results, Boolean networks are interesting, when few is known about a network, e.g. about efficiency of involved transmembrane transporter, enzyme expression pattern and rate constants, diffusion constants or active transport inside the cell, transcription rates and so on. Also, when inferred interactions are unsure and the modeller wants to test many different hypothesises. They are also interesting and reliable, when the actual kinetics can be expected to be of secondary importance for the functionality of the modelled system, e.g. when modelling signalling pathways, where it mainly interests, that signals are passed on, rather than the actual kinetics of protein interaction.

Still, as soon, as modelling a state requires more, than two possible states, Boolean networks are at their limits, thinking of a protein, that could e.g. exist in an inactive, uncomplexed state, lateron complexing a partner protein, then becoming phosphorylated and subsequently being imported to the nucleus, all of which are possible, realistic scenarios.

Boolean networks also do not include stochiometry: Boolean update rules can either include an input signal or not, quantification is not possible. Additionally, they can have only one output. Modelling (bio)chemical reactions therefore requires further efforts, namely introduction of abstract process nodes, to which several output nodes can be connected.

With respect to chemical reactions, although most compounds in a cell are subject to equillibrium reactions, there are, of course, also processes, where educts are almost entirely consumed. Since the states of nodes, included in an update rule are not modified, only the state of the output node, Boolean networks can't model consumption.

But, we believe, that the error, we consequently introduced, by choosing the Boolean approach, did not harm the confidency of the derived conclusions, since most of the modelled processes are either signalling pathways or metabolic reactions, we assumed to be either active or abolished.

Also, when it comes to bidirectional reactions, Boolean networks need two rules to discriminate foward and backward reaction.

Still, since little is known about the modelled protein interactions and the possibility to quickly test many different, possible scenarios, was of crucial importance, we believe, that the decision for the Boolean network approach was appropriate.

Concerning the data, we used for modelling, we clearly favored experimental data, rather than predictions, e.g. for protein domains, although bioinformatics are nowadays capable of producing confident results with respect to protein sequence alignment and proper conclusions about properties, like transmembrane region probability. When it comes to pure recognition of previously identified motifs of different proteins, as we used it, e.g. to predict the transmembrane regions of Atg33 to be mitochondrial outer membrane anchors, confidency is especially high.

Although, we mostly relied on information from literature and tried to favor reviews as well as more recent articles for our conclusions, we also took database knowledge into account, since we had to. The BioGRID lists many protein interactions, physical as well as genetical. One of the problems thereby is, that, of course, different methods are used to infer interactions for different proteins, which may only to some extend be comparable, in addition to systematic experimental weaknesses, like the false positives, frequently encountered in Two-Hybrid screens. Such screens, although mostly acquired via high-throughput assays, also include data from literature, which to some degree already implies the established or the authors' opinion about a certain field or protein, which is disadvantegous, especially when trying to elucidate pathways, that are not yet elucidated.

Data from PhosphoGrid is not predicted, but actually eperimentally encountered, so it is reliable.

With respect to literature, we have to mention, that inspection of data, rather than relying on respective authors' conclusions, is sometimes required. At least to confirm author conclucions, we have found, that it is always a good idea. More than once we encountered data, that either lead us to conclusions not at all addressed in the paper, or even lead us to completely

different explanations of data. We tried to avoid such papers, since we are not always confident enough to contradict the opinion of authors, which of course have more experience and usually know about their presented topic.

So, author conclusions, especially when relying on summaries/abstracts can obfuscate the "truth". However, whenever searching the "needle in the haystack", as in the present work, of course, a lot of data is required and so all these considerations can not always be applied, if one wants to produce results.

Unfortunately, it is not always possible to take all data into account, without introducing interpretation and thereby maybe unintentionally obfuscate the clear view to "the truth". To some degree an author has to include also his own ideas, in order to present something "new", which may after presentation be critically reviewed. Knowing, that all authors have to consider such thoughts, presented data must always be critically read and inspected for interpretations, that actually do not necessarily follow from the presented data:

In the present work we have hypothesized especially in the chapters regarding Ras function and Atgs, however, after all, we believe, that we have always made it clear, when we were presenting data and when we were hypothesizing.

5 Acknowledgements

I want to say Thank you! at first to Edda Klipp, for guiding and assisting me during the development of this work, to Matthias Müller and Andreas Reichert from Frankfurt, collaborators in GerontoMitoSys, for the meetings and fruitful discussions about Whi2 and mitophagy, to Till Scharp, for his assistance, especially with respect to Boolean networks, to Thomas Handorf, the initiator of the biographer project in Google's Summer of Code 2011, to Chaitanya Talnikar, for his advancements to the simulator in the context of Google's Summer of Code 2012, to all the members of TBp, for having a nice and relaxed atmosphere, to my parents, for giving birth to me, as well as all of my family, for supporting me during the last months, and finally to Ludwig van Beethoven, for his motivating, imposant symphonies.
I also greatly acknowledge the funding by the Bundesministerium für Bildung und Forschung BMBF (Projektnummer KFZ 0315584).

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Eigenständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Masterarbeit erstmalig einreiche, selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Berlin, den 1. Juli 2012