UNIVERSITY OF LATVIA

Faculty of Biology

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Doctoral Thesis

# Impact of purine starvation on physiology of budding yeast *Saccharomyces cerevisiae*

# Purīna badināšanas ietekme uz maizes rauga *Saccharomyces cerevisiae* fizioloģiju

Submitted for the degree of PhD of Natural sciences

Subfield of Microbiology

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Dr. Biol. Jānis Liepiņš

“Pētīt.”

Raiņa Kastaņolas perioda (1906.-1920.)
dienasgrāmatās bieži lietots mudinājums

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## ABSTRACT

The budding yeast *Saccharomyces cerevisiae* is a commonly used eukaryotic model organism. It has evolved to live in environments with rapid influx of nutrients, which can be followed by nutrient scarcity. These conditions have created cellular mechanisms for nutrient uptake and sensing and allows metabolic adjustment for nutrient availability. In conditions where nitrogen, carbon or other macro nutrients are lacking, bakers yeast stops the metabolism and cell cycle, leaving the cells in a "fasting phenotype".

Because budding yeast is often used in research due to its relatively simple maintenance, mutations in a number of amino acid or nucleotide biosynthetic pathways are commonly found in laboratory strains - auxotrophs, which serve as metabolic markers to facilitate genetic engineering in these strains. Several authors have shown that yeast cells are unable to "perceive" the lack of auxotrophic nutrients, so the lack of auxotrophic factors does not result in a fasting phenotype.

This thesis deals with the physiology of budding yeast if it has a mutation in the pathway of adenine biosynthesis (*ade8 or ade2*). Adenine auxotrophy is a relatively common metabolic marker because mutants of the *ade1* and *ade2* genes accumulate red pigment in cells that can be used in cell selection. Our results show that adenine mutants in the absence of adenine are phenotypically more similar to cells with a "fasting phenotype." The cells successfully stop the cell cycle, their stress resistance increases, and a transcriptomic response is observed where adenine-starved cells are quite similar to cells in the stationary phase. The shift of cellular carbon metabolism from fermentation to the production of other metabolites - glycerol, acetate - is also observed.

The thesis shows how several frequently used strains with different mutations in the adenine synthesis pathway respond to the inactivation of the adenine synthesis pathway and the consequent adenine deficiency. The results show a similar metabolic response, indicating that this is a general phenomenon. This is also indicated by the fact that several intracellular parasites have evolutionarily lost genes in the adenine biosynthetic pathway. One of these parasites (*Leishmania*) has been shown to have increased stress resistance in an adenine-free environment. This suggests that adenine deficiency in the cells are perceived in a wide range of living organisms and probably the mechanism behind this “sensing” is alike across many evolutionary distinct organisms.

## KOPSAVILKUMS

Maizes raugs *Saccharomyces cerevisiae* ir zinātnē bieži lietots eikariotu modeļorganisms. Tas evolucionāri ir attīstījies dzīvot vidēs ar fluktuējošu barības vielu pieejamību - straujam barības vielu pieaugumam var sekot barības vielu trūkums. Šie apstākļi radījuši raugā jutīgus mehānismus barības vielu daudzuma uztveršanai un vielmaiņas pielāgošanai barības vielu pieejamībai. Apstākļos, kuros trūkst slāpeklis, ogleklis vai citi makroelementi, maizes raugs aptur vielmaiņu un šūnas ciklu, šūnas nonāk "badošanās fenotipā".

Tā kā maizes raugu tā samērā vienkāršās uzturēšanas dēļ bieži izmanto arī zinātniskos pētījumos, laboratorijās izmantotajiem celmiem nereti ir sastopamas mutācijas vairākos aminoskābju vai nukleotīdu biosintēzes ceļos - auksotrofijas, kas kalpo kā metaboliskie marķieri, lai atvieglotu gēnu inženieriju šajos celmos. Vairāki autori ir pierādījuši, ka rauga šūnas nespēj "uztvert" auksotrofo barības vielu trūkumu, tādēļ pie auksotrofo faktoru trūkuma šūnas parasti nenonāk badošanās fenotipā.

Šajā darbā aplūkota maizes rauga fizioloģija, ja tam ir mutācija adenīna biosintēzes ceļā (*ade8* vai *ade2*). Adenīna auksotrofija ir samērā bieži izmantots metaboliskais marķieris, jo *ade1* un *ade2* gēnu mutanti uzkrāj šūnās sarkano pigmentu. Mūsu rezultāti norāda, ka adenīna mutanti adenīna trūkuma gadījumā fenotipiski drīzāk atbilst šūnām ar "badošanās fenotipu". Šūnas sekmīgi aptur šūnas ciklu, tām pieaug stresa izturība, novērojama transkriptomiskā atbilde, kas tuvina adenīna badinātas šūnas šūnām stacionārajā fāzē. Novērojama arī šūnas oglekļa metabolisma pārvirzīšana no spirta uz citu metabolītu ražošanu – glicerīnu, acetātu.

Darbā parādīts, kā uz adenīna sintēzes ceļa inaktivēšanu un tai sekojošu adenīna trūkumu reaģē vairāki pētniecībā bieži izmantoti celmi ar atšķirīgām adenīna sintēzes ceļa mutācijām. Rezultāti parāda līdzīgu metabolisko atbildi, norādot uz to, ka šis ir vispārīgs fenomens. Uz to norāda arī fakts, ka vairāki iekšsūnu parazīti ir evolucionāri zaudējuši adenīna biosintēzes ceļa gēnus. Vienam no šādiem parazītiem (*Leishmania*) novērota paaugstināta stresa izturība vidē bez adenīna. Tas ļauj spekulēt, ka adenīna trūkums šūnās tiek uztverts ar līdzīgiem mehānismiem plašā dzīvo organismu lokā.

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# Abbreviations

**(p)ppGpp** - Guanosine pentaphosphate and tetraphosphate, signalling molecules involved in stringent control in bacteria, leading to inhibition of RNA synthesis in the absence of amino acids

**ADE** - ADEnine requiring, genes whose recessive alleles determine the requirement for adenine in the culture medium

***ADH2*** **-** Alcohol DeHydrogenase - Glucose-repressible alcohol dehydrogenase II; catalyzes the conversion of ethanol to acetaldehyde

**ADP** - adenosine diphosphate

**AICAR** - 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranosyl 5'-monophosphate, a product of the Ade13p of the purine biosynthesis pathway

**AMP** - adenosine monophosphate

**AMP** - adenosine monophosphate, nucleotide

**ATP** - adenosine triphosphate - the cell's main energy cofactor

**Bas1p** - BASal transcription factor involved in regulation of basal and induced expression of genes of purine and histidine biosynthetic pathways; controls cellular ATP levels

**cAMP** - cyclic adenosine monophosphate - secondary messenger in eukaryotic cells

**CFU** - Colony Forming Unit - a cell of a microorganism that is capable of multiplying - forming a colony - on a solid medium

**CLS** - Chronological Life-Span - measures the length of time nondividing cells survive.

**ESR-** Environmental Stress Response - includes ~900 genes whose expression is stereotypically altered when yeast cells are shifted to stressful environments. The coordinated expression changes of these genes is a common feature of the responses to many different environments, however the regulation of these expression changes is gene-specific and condition-specific.

***FCY2*** **-** Purine-cytosine permease gene; mediates purine (adenine, guanine, and hypoxanthine) and cytosine accumulation

**FTIR** - Fourier Transformation Infrared Spectroscopy is used to obtain infrared absorption or emission spectra of a solid, liquid or gas.

**G0** - $G_0$ phase describes the state of the cell outside the replicating cell cycle.

**G1** - Gap1 phase or growth phase 1 is the first of the four phases of the cell cycle that occur during eukaryotic cell division.

**GAAC** - General Amino Acid Control - Gcn4p coordinated transcriptional response to amino acid depletion in the environment

**Gcn4p** - General Control Nonderepressible, a transcriptional activator of amino acid biosynthetic genes; the activator responds to amino acid starvation.

**Gis1p** - Histone demethylase and transcription factor; regulates genes during nutrient limitation.

***GLN3*** **-** GLutamiNe metabolism**-** Transcriptional activator in nitrogen catabolite repression system; localization and activity regulated by quality of nitrogen source

**GMP** - Guanosine monophosphate, nucleotide

**IMP** - inosine monophosphate, nucleotide

**Msn2p** - Stress-responsive transcriptional activator; when activated, migrates to the nucleus in response to different stress conditions; binds to DNA to stress response elements of genes.

**Msn4p** - Stress-responsive transcriptional activator; when activated migrates to the nucleus in response to various stress conditions; binds to the DNA to stress response elements of genes.

**mtDNA** - mitochondrial DNA is the DNA located in mitochondria

**NGS** - Next Generation Sequencing - a massively parallel sequencing technology that provides extremely high throughput, scalability and speed.

**NMR** - Nuclear Magnetic Resonance - Spectroscopy is a technique that exploits the magnetic properties of certain atomic nuclei and can be used to determine the physical and chemical properties of the atoms or molecules in which they are located.

**OD** - Optical Density, the absorption of light by a sample proportional to the density of cells in the sample

**Pho2p** - PHOsphate metabolism transcription factor; regulatory targets include genes involved in phosphate metabolism.

**Pho85p -** Cyclin-dependent kinase involved in regulating the cellular response to nutrient levels and environmental conditions and progression through the cell cycle

**PKA** - Protein Kinase A, also known as cAMP-dependent protein kinase. PKA has several functions in the cell, including regulation of glycogen, sugar and lipid metabolism.

**Ras** - GTPase encoded by the *RAS1* gene, involved in G-protein signalling in adenylate cyclase activation; plays a role in the regulation of cell proliferation; localised to the plasma membrane.

**Rim15p -** protein kinase encoded by the *RIM15* gene, regulates cell proliferation by changing the location between the nucleus and cytoplasm

**rRNA-** Ribosomal ribonucleic acid (rRNA) is a type of non-coding RNA which is the primary component of ribosomes, essential to all cells

**SAICAR** - 1-(phosphoribosyl)imidazole carboxamide, a product of the Ade1p of the purine biosynthesis pathway

**SAM** - S-adenosylmethionine is a common cosubstrate involved in methyl group transfer, transsulfuration and aminopropylation.

**SD** - Synthetic complete Dextrose, a type of synthetic medium with a known mineral content, glucose as a carbon source

**Snf1p -** AMP-activated protein kinase; required for glucose-repressed gene transcription, heat shock, sporulation, and peroxisome biogenesis

**TOR** - Target Of Rapamycin - a protein kinase involved in nutrient sensing and link to cell growth.

**tRNA** - transport RNA - ensures amino acid delivery to ribosomes.

**YPD** - Yeast Peptone Dextrose, a type of complete medium containing yeast extract, proteins hydrolysed by pepsin and glucose

# Introduction

Cells perceive available nutrients and tailor their metabolism accordingly. Starvation for basic nutrients elicits stress resistant phenotype. Purines are the basis of structure and functionality of every living cell, yet the response of the cells for lack of purines is scarcely described. This work **aim**s to describe how *Saccharomyces cerevisiae* cells react to the depletion of purines and place purine starvation with the respect of other starvations.

To achieve this aim following **tasks** were formulated:

- to characterise ade auxotrophic strain growth in rich (YPD) media;
- to describe metabolism, transcriptome and stress resistance of ade auxotrophic strains when starved for purines in defined media;
- to describe phenotype of purine starved auxotrophs with truncated transcription factors for elucidation of purine starvation signalling in the cells;
- to review the overall structure of the purine synthesis metabolic pathway and purine auxotrophy across all domains of life.

# 1. Literature review

## 1.1. Basic biology of *S. cerevisiae*

Yeast *Saccharomyces cerevisiae* is a species of unicellular *Ascomycota* fungi that replicates by budding. The life cycle of this yeast consists of haploid and diploid phases. In the case if conditions are optimal - it rapidly proliferates mitotically (either as haploid or diploid), while in the case of poor nutrient supply it sporulates. Two haploid cells mate and form asci with 4 spores. Bakers yeast – *S. cerevisiae* as the name suggests is widely used in the food industry where its ability to quickly ferment simple sugars is employed in bread and alcohol production. The genome studies of *S. cerevisiae* point to the emergence of it as a species in the end of the Cretaceous age when sugar rich fruits appeared (Friis et al., 1996). Whole genome duplication event allowed S. cerevisiae to develop a "make-accumulate-consume" lifestyle, where available sugar is quickly converted into alcohol, prohibiting growth of other microorganisms. Alcohol is later consumed (Piškur et al, 2006). While *S. cerevisiae* employed by humans is selected for its quick fermentation capabilities and genetic analysis shows interspecific genome regions from other yeasts, wild yeasts isolated on various trees and primaeval forests still retain "make-accumulate-consume" lifestyle (Liti, 2015). To achieve this lifestyle *S. cerevisiae* exhibits Crabtree effect – if sugar concentration is high, most of energy is produced with fermentation even if oxygen is present (Verdyun et al., 1984), that is possible due to the glucose repressing mitochondrial enzyme transcription. Genome duplication gave rise to several enzyme isoforms, f. ex., *ADH2* allowing the consumption of ethanol (Piškur et al, 2006; Thomson et al., 2005). It is worth noting that in both human generated or natural environments *S. cerevisiae* will have times of plenty when high quantities of nutrients are present and times of scarcity when nutrients are exhausted and cells must persist until the next nutrient influx (Lahue et al., 2020; Smets et al. 2010). In figure 1 is the scheme of central carbon metabolism adapted from Rintala 2010.

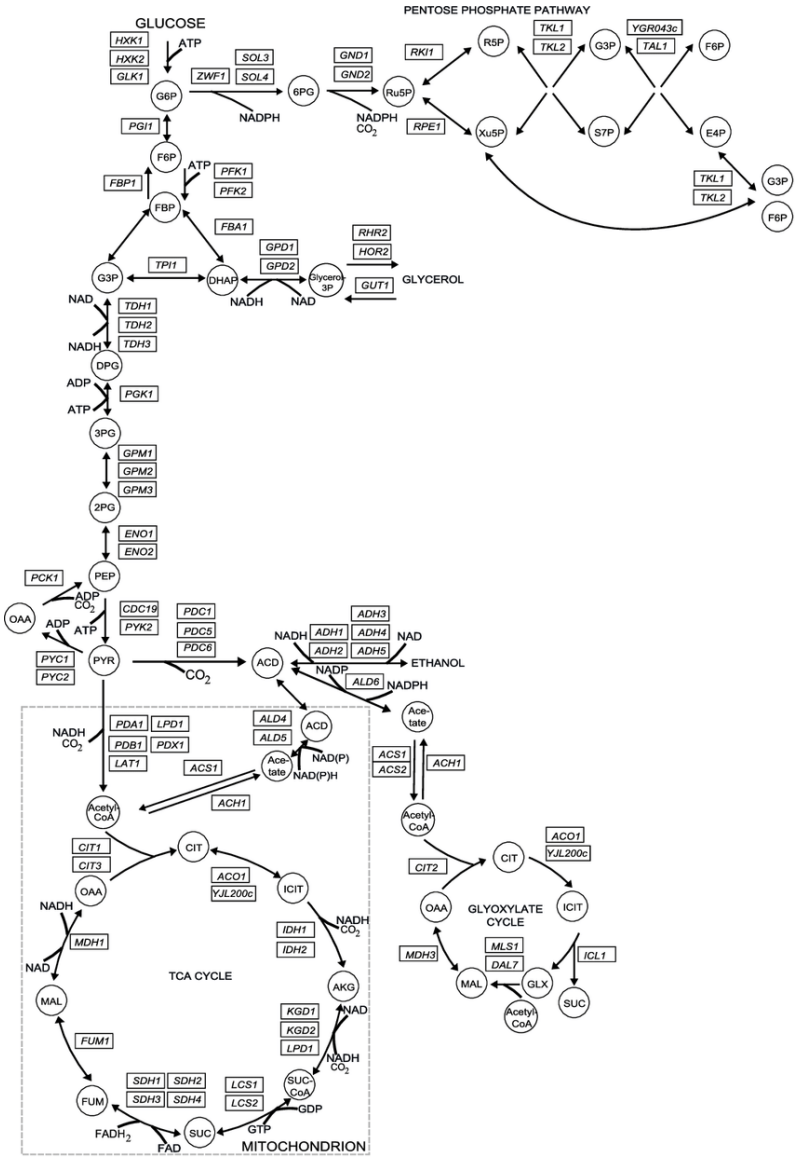


Figure 1. Carbon flow in the *S. cerevisiae* in presence of oxygen, in circles metabolites, in rectangles genes coding for respective enzymes (from Rintala, 2010). Note the amount of isoenzymes for many reactions.

*S. cerevisiae* is a heterotroph organism, meaning cells produce energy and biomass from organic molecules that are acquired from the environment**.** The empirical estimated biomass equation for *S. cerevisiae* is C:H(1.613):O(0.557):N(0.158). The varying amounts and quality of nutrients present in the environment requires yeast cells to be able to sense and tailor metabolism according to it, sustaining cell composition and viability. Sugars are the main energy supply and also carbon source for yeast. Yeast cells prefer glucose or fructose to other mono-, di- or trisaccharides. Fermentable carbon sources are consumed prior to substrates that would yield energy with oxidative phosphorylation (Broach, 2012).

Glucose repression of mitochondrial function is the basis of the Crabtree effect. When cells are in a glucose rich environment various signalling systems activate genes required for fast growth such as ribosome biogenesis genes, at the same time stress response genes and alternate carbon source utilisation genes are repressed. Same as for the carbon sources also nitrogen sources are prioritised - ammonia ions that can be easily converted into central intermediates of nitrogen metabolism in cell - glutamate or glutamine, but *S. cerevisiae* can also use less preferred nitrogen sources such as proline. Use of nitrogen sources is also regulated by nitrogen source repression mechanism prioritising easily metabolised ones (Broach, 2012). When cells are using less preferable sources of C and N growth is slower and stress resistance genes are activated. Eventually with exhaustion of available nutrients the cell ceases to divide and becomes dormant (Smets et al., 2010).

In full media *S. cerevisiae* has a relatively short doubling time – 1-2 h and is easily cultivated in laboratory conditions, but in difference to bacteria, yeast is a eukaryotic organism. This has led to usage of yeast as a "workhorse" of molecular biology. Several strains of yeast are developed that are widely used in research. W303 strain was constructed by Rodney Rothstein and is quite often seen in physiology related research such as ageing (Ralser et al., 2012). Another widely used strain is S288C or its derivatives. S288C was developed by Robert Mortimer for biochemical studies and was used to develop gene knock out collection and also serves as a reference genome (Mortimer, Jonston, 1986). W303 and S288C share more than 85% of their genome information (Ralser et al., 2012). System biology on other hand mostly uses strains derived from CEN.PK strain - yeast strain series developed by Michael Ciriacy and K.D. Entian (Entian, Kötter, 2007). Irrespective of field of studies quite often genetic manipulations are performed in these strains. To help in these manipulations strains harbour several mutations in biosynthesis genes of amino acids and nucleotides - W303 *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*, S288C derived BY4741 *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* (Branchman et al., 1998), and CEN.PK family strains usually carry *ura3-52 his3-Δ1 leu2-3,112 trp1-289*. By inserting a working copy of defective biosynthesis gene along with intended genetic modification it is easy to select modified strains.

In laboratory conditions yeast cells are usually grown in a synthetic medium that is rich in carbon source (glucose) and good nitrogen source (ammonia) and all other nutrients in excess to support rapid growth/ proliferation of the yeast.

## 1.2. Starvation response

When all nutrients are plentiful, yeast cells grow and proliferate until a certain nutrient is exhausted, afterwards growth halts until metabolism is reoriented to use any available alternative or stops completely if no substitute is available. If a microbial cell number in a fresh media would be recorded several population growth phases can be distinguished. In the beginning the population experiences a so-called lag phase where no or minimal growth is observed. After the lag phase and adapting to the environment cells proliferate exponentially giving the name of this phase - log phase. After a while nutrients are exhausted and a stationary phase where no population growth is observed will be reached. Due to the Crabtree effect *S. cerevisiae* populations grown aerobically on glucose exhibit so called diauxic growth, where the second log phase can be observed as cells switch from fermentation to the consumption of fermentation products (Figure 2).

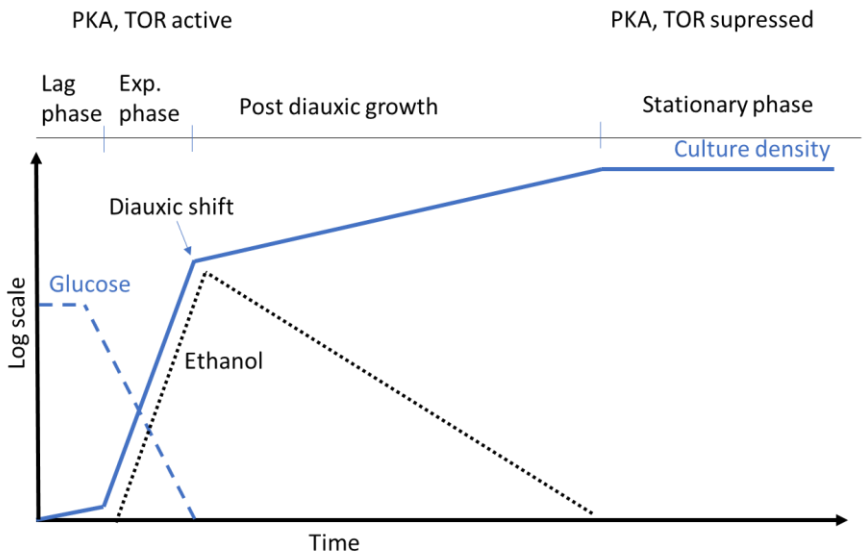


Figure 2. Typical growth curve of *S. cerevisiae* in aerated glucose rich environment. First growth phase is observed when glucose is used as the main carbon source, second growth observed on fermentation products.

Lack of nutrients is common in nature. Cells in later population growth phases coincident with slow or no growth show increased stress resistance and longer lifespans if compared to log phase cells. This helps cells to survive until the next supply of fresh nutrients is available. Growth is regulated by cellular signalling pathways that perceive the state of nutrients in the environment. After receiving signal from the receptor usually with the help of a secondary messenger or G protein signal is passed via kaskades of signal

transduction pathway most commonly ending with activation of protein kinase that in turn phosphorylates enzymes or transcription factors causing changes in cellular metabolism and gene expression. Glucose being the preferred carbon source will activate several cell signalling systems. Most of the glucose effects on biosynthetic capacity and stress responses are mediated by the protein kinase A pathway, while repression of genes involved in use of alternative carbon sources are mediated predominantly by Snf1p. Protein kinase A (PKA) pathway is activated by G protein Ras that causes synthesis of secondary messenger cAMP, that in turn will activate PKA that directly and indirectly will affect enzyme activity and variety of transcription factors. TOR (target of rapamycin) is the second main cell growth speed regulating signalling system. TORp is a phosphatidylinositol kinase-related protein kinase that controls cell growth in response to nutrients. Rapamycin is an immunosuppressive and anticancer drug that acts by inhibiting TORp. The modes of action of TORp and rapamycin are remarkably conserved from *S. cerevisiae* to humans (Crespo, Hall, 2002). While the Ras/PKA pathway mainly relates to the carbon availability and TOR is described in connection with nitrogen sensing, both pathways are interconnected and share many downstream targets - transcription factors (Plank, 2022). Both pathways regulate ribosome production and repress autophagy and stress response, thus a suppression of either of the pathways results in slow down of the growth (Figure 3).

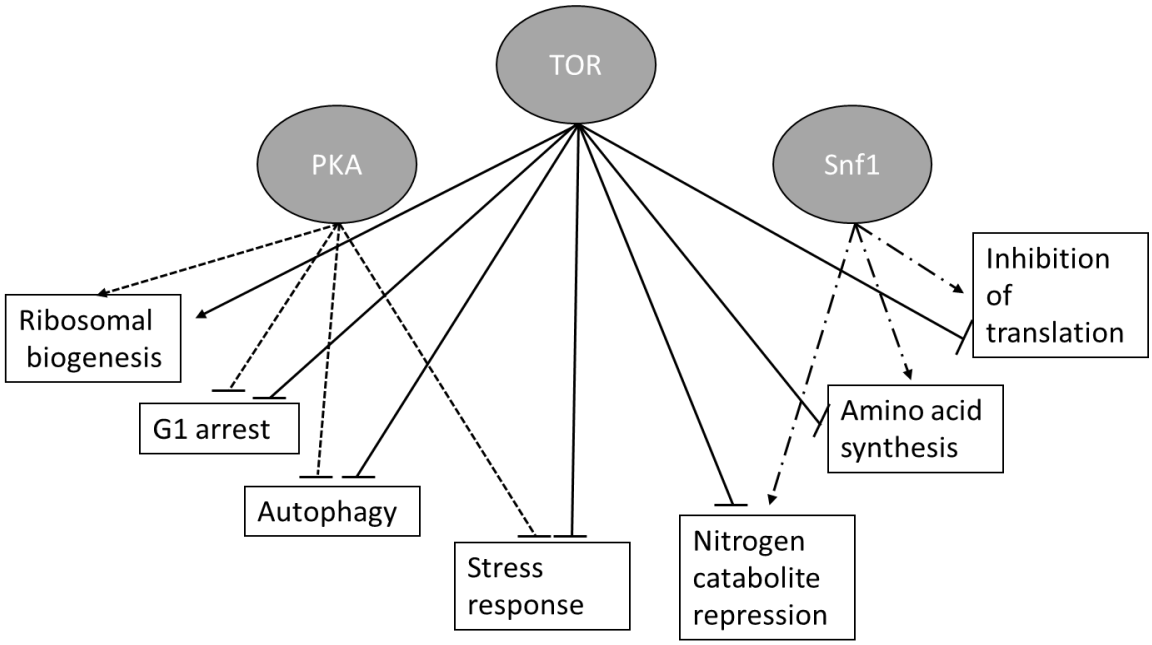


Figure 3. General signalling pathways in yeast cells influencing cell growth/resistance phenotype. Snf1 is a protein kinase required for glucose-repressed gene transcription. Figure adapted from Busti et al., 2010.

Starvation for the nutrients is known to induce quiescence in the budding yeast. Quiescence is a state of the cell where no proliferation happens yet after returning in a nutrient rich environment proliferation resumes. As of yet no definite single marker for achieving quiescent state has been defined, but there is a cluster of characteristics that are typical for quiescent cells - smaller, denser cells with thicker cell wall, these cells show reduced metabolic activity with smaller amounts of RNA and ribosomes. Quiescent cells arrest cell cycle at G0 stage (Sun, Gresham, 2021)

Yeast cells subjected to starvation for any nutrient exhibit a stereotypic pattern of gene expression changes, referred to as the environmental stress response (ESR). ESR is initiated also by a large number of environmental stressors, such as heat, oxidative stress, or high osmolarity (Gasch et al., 2000). The predominant components of the set of genes that are repressed in the ESR include those required for mass accumulation, primarily ribosome biogenesis genes. Stress resistance genes - such as catalase or heat shock protein expression is activated. The fact that other stresses also elicit a similar repression suggests that the individual stressors either engage nutrient signalling pathways, such as PKA and TOR, or interact with the same transcriptional regulatory apparatus that responds to nutrients (Broach, 2012).

It has been observed that in some cases if cells are starved for some auxotrophic agents - uracil, leucine - they do not enter quiescent state and lose viability rapidly. This had led to distinguishing two types of starvations, dependent on the missing nutrient: ‘natural limitations’, which sets in when basic nutrients (carbon, phosphorous, sulphur and nitrogen) are scarce, and ‘artificial limitations’, which sets in when particular metabolites or metabolic intermediates are insufficient (Saldanha et al., 2004).

## 1.3. Natural and artificial starvations

Cells starved for main nutrients carbon, nitrogen, phosphorus and sulphur share same characteristics - cell cycle arrest as unbudded cells, thickened cell walls, increased stress resistance and an accumulation of storage carbohydrates (Klosinska et al., 2011; Lillie & Pringle, 1980; Schulze et al., 1996). At the same time intracellular nutrients do change depending on the factor causing starvation and are not uniform across starvations. If cells experience a lack of nitrogen in the presence of carbon source, protein and RNA amount in cells drops, with almost no free amino acids in cytoplasm, but storage carbohydrates and

fats increase. Cells that are starved just for carbon, but have nitrogen present would have almost twice as much proteins and RNA and less storage molecules - carbohydrates (especially glycogen) and fats (Albers et al., 2007).

Boer and colleagues published results of amounts on intracellular metabolites during various starvations. Cells starved for carbon were mainly limited in metabolites of main energy generating pathways - glycolysis and Krebs cycle, nitrogen starved cells experienced lack of amino acids, whereas in phosphate starved cells phosphorylated intermediates of pentose phosphate pathway and triphosphates were found to be main metabolites concentrations of which were significantly reduced (Boer et al., 2010). Interestingly, similar reduced amounts of metabolites were observed also in *E. coli* starved for carbon and nitrogen (Brauer et al., 2006). Also recent research on involvement of transcription factors during starvation shows various pathways how starvation is sensed and communicated via Rim15p in carbon or nitrogen starvation in budding yeast (Sun et at., 2020).

Auxotrophy is a typical example of an artificial starvation that would not be experienced by prototrophic cells. Many common laboratory yeast strains (W303, S288C, CEN.PK and FY series) contain one or several auxotrophic markers. Histidine, leucine, uracil, adenine and tryptophan (*his, leu, ura, ade* and *trp*) are the most common auxotrophic markers of *Saccharomyces cerevisiae* strains used in physiology studies (Pronk, 2002; Da Silva, Srikrishnan, 2012). Insufficient concentration of an auxotrophic agent leads to artificial limitation that converts to starvation.

When cells were starved for leucine and uracil it was observed that their viability rapidly decreased, which led to the concept of artificial starvations (Saldanha et al., 2004; Gresham et al., 2011). Before coinage of this concept it was known that *fas1* cells starved for fatty acids die rapidly - cells lose viability by several folds of magnitude within 24h (Henry, 1973). Henry also observed survival of cells starved for lysine, tryptophan and adenine, where after 24h around 30% -50% were alive after starvation, that is considerably less as with fatty acids. Research with wine yeast shows that lack of vitamins in media also leads to the rapid loss of viability (Duc et al., 2017).

Methionine has been shown to be an exception of the rule where methionine starved cultures resemble more of the natural starvations. It is argued that lack of methionine is perceived as sulphur starvation (Unger, Hartwell 1976; Petti et al., 2011)

It seems that in most of these “artificial” starvations the cell signalling system is not “aware” of intermediates lacking and does not arrest cell cycle, but tries to proceed with fast growth eventually “running out” of building blocks. If for some other reason growth speed affecting signalling systems are affected, leucine starved cells do not die as rapidly, that can be seen that if cells are starved for leucine in non fermentable carbon sources cell life span increases (Boer et al., 2008). It is further shown with quiescence and chronological life span screens where low survivability in artificial starvations is suppressed by mutations in TOR pathway or its targets (Boer, 2008; Gresham et al., 2011). Cell cycle arrest and quiescence do not occur for an artificial starvation with either leucine or uracil. Inability to complete cell division and to halt subsequent cell cycle leads to a decrease in viability in addition to an observable “glucose wasting” phenomenon, where auxotrophic starved cells converted higher quantities of glucose to ethanol compared to the phosphate starved ones (Boer et al., 2008). Both cell cycle arrest and mitochondrial respiration is governed by cell signalling systems further pointing to involvement of those in “sensing” nutrient scarcity.

## 1.4. Purine biosynthesis in yeast

New purine bases in eukaryotic cells are obtained in two ways - salvage and *de novo* synthesis. In the salvage pathway, purine bases are taken up from the environment or recycled within the cell and attached back to the ribose 5-phosphate, thus restoring nucleotides. Most eukaryotes have two purine salvage enzymes - one that can produce AMP (adenine phosphoribosyltransferase, EC 2.4.2.7) and hypoxanthine phosphoribosyl-transferase (EC 2.4.2.8) that can produce GMP or IMP, and sometimes xanthosine monophosphate. Purines are actively transported across cell membrane with the help of purine-cytosine permease Fcy2p (Kurtz et al., 1999) and stored in the vacuole (Nagy, 1979) presumably with the help of a Fun26p transporter that is a passive transporter (Boswell-Casteel, 2014).

The purine synthesis pathway and its regulation are highly conserved in all eukaryotes from fungi to mammals (Agmon et al., 2020). Most probably, the last common ancestor had a pathway with the same structure that diversified into the now known three domains of life - *Bacteria*, *Archaea* and *Eukarya* (Armenta-Medina et al., 2014; Vázquez-Salazar et al., 2018).

In *S. cerevisiae*, the purine de novo synthesis pathway comprises sequential chain of reactions adding C and N atoms to ribose phosphate until inosine monophosphate (IMP) is formed, which is the common substrate for GMP and AMP synthesis. To synthesise IMP, the glycine backbone is fused with nitrogen provided by the amide groups of two glutamine molecules and one aspartate. Additional carbon atoms are provided by two formate and one $CO_2$ molecule (Pedley, Benkovic, 2017). All *de novo* pathway gene expression is regulated by Bas1/2p transcription factors that respond to the concentrations of pathway intermediates phosphoribosylaminoimidazolecarboxamide (AICAR) and phosphoribosylaminoimidazole-succinocarboxamide (SAICAR), metabolites that are products of reactions catalysed by Ade13p and Ade1p, respectively (Denis et al., 1998). The total flux of metabolites through the purine de novo synthesis pathway is regulated by the first enzyme Ade4p that is sensitive to ATP and ADP concentrations in the cell (Pinson et al., 2009; Rébora, Desmoucelles, Borne, Pinson, & Daignan-Fornier, 2001; Rébora, Laloo, & Daignan-Fornier, 2005). See simplified purine salvage and *de novo* synthesis scheme in figure 4. Note that IMP provides both AMP and GMP, thus a mutation in IMP synthesis pathway in a media without nucleotides would result not only in adenine, but also guanine auxotrophy. Thus further on we will refer to this starvation as purine starvation.

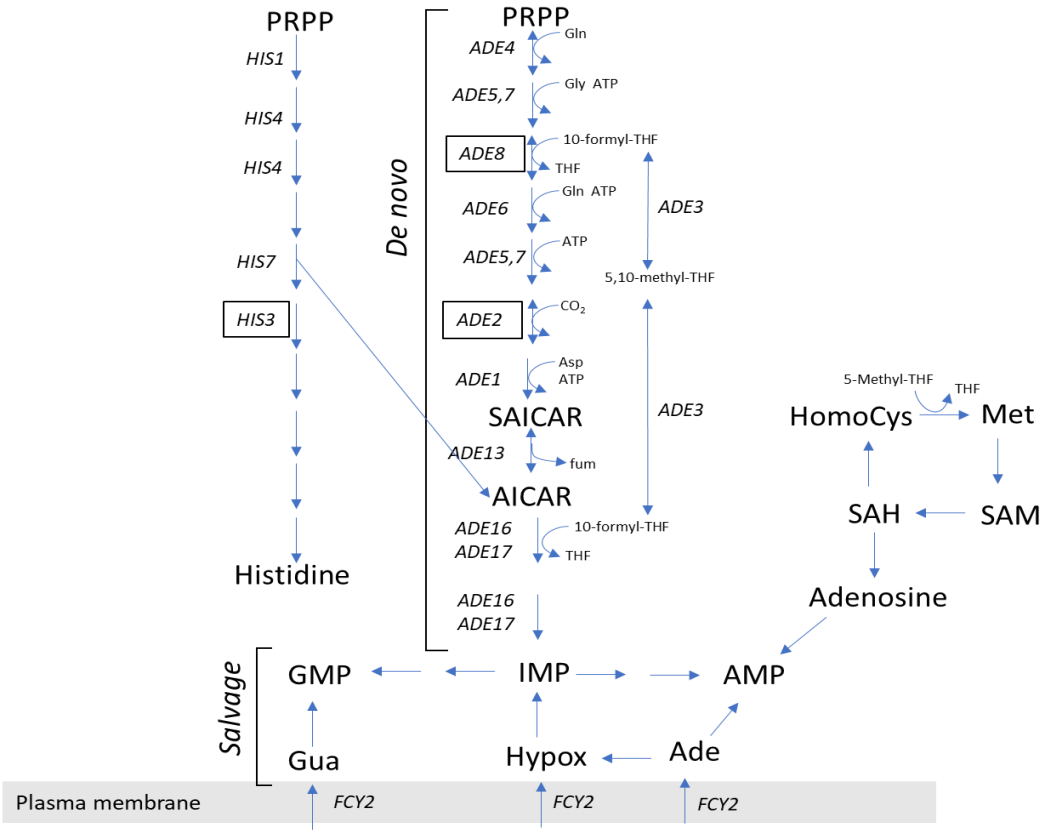


Figure 4. Simplified purine salvage and *de novo* synthesis scheme, all genes of *de novo* pathway and some intermediates shown. Genes shown in italics, genes in boxes - genes used in this thesis research. Adapted from Kowalski et al, 2008. Abbreviations: PRPP - 5-phospho-α-D-ribose 1-diphosphate, Gln - glutamine, Gly - glycine, Asp - aspartate, fum - fumarate, THF - tetrahydrofolate, Gua - guanine, Hypox - hypoxanthine, Ade- adenine, SAH - S-adenosylhomocysteine, SAM - S-adenosylmethionine, Met - methionine, HomoCys - homocysteine.

As purine synthesis is connected to histidine synthesis via AICAR some biosynthesis mutants are not only adenine auxotroph but also require histidine if the mutation is below the *ADE13* gene. AICAR and SAICAR both have regulatory roles. *ADE13* mutant is not viable due to SAICAR accumulation and *ADE16 ADE17* double mutant accumulates AICAR and shows slower growth (Tibetts, Appling, 2000). Histidine synthesis pathway reactions catalysed by *HIS1*, *HIS4* and *HIS7* are also affected by Bas1/2 transcription factors and adenine depletion in the cell (Denis et al., 1998).

*ADE3* gene is not directly involved in generation of purine rings but catalyses sequential reactions 10-formyl-THF synthetase (EC 6.3.4.3), 5,10-methenyl-THF cyclohydrolase (EC 3.5.4.9), and 5,10-methylene-THF dehydrogenase (EC 1.5.1.5), to supply forms of activated one-carbon units required for biosynthesis of purines, histidine,

methionine and pantothenic acid. Cells defective in the *ADE3* gene are adenine and histidine auxotrophs.

## 1.5. Purine auxotrophy in nature

Purine auxotrophy is a common phenomenon among monera, protozoans, and metazoans. For example, all parasitic protozoans and many intracellular bacterial pathogens are purine auxotrophs. Two of the well-known examples are *Toxoplasma gondii* (causes toxoplasmosis) and *Plasmodium falciparum* (causes malaria) (Downie et al., 2008; Weiss, Kim, 2011). Parasitic worm genome analyses revealed that purine auxotrophy is common among parasitic platyhelminths and roundworms (nematodes). Parasitic worms lack some or all de novo purine pathway enzymes (International Helminth Consortium, 2019). These auxotrophic organisms save resources for expensive purine *de novo* synthesis as one nucleotide "costs" ~50 ATP molecules (cost calculated including energy spent for synthesis of all intermediate metabolites) (Lynch, Marinov, 2015).

Many apicomplexan parasites lack all the genes encoding enzymes of *de novo* purine synthesis and rely only on purines harvested from their host. Using salvage pathways, parasites collect different species of purines - adenine, xanthine, hypoxanthine, adenosine, and inosine. Parasitic organisms absorb purine sources through specific nucleotide transporters (Chaudhary et al., 2004; Major et al., 2017). However, the inability to synthesise purine is not specific to parasites. For example, *Tetrahymena* is a genus of free-living protozoans that requires exogenous purine and pyrimidine supply to sustain growth. It has not lost its entire purine synthesis pathway, but several steps are missing (Hill, 1972).

As purine supply of parasitic organisms is dependent on host cells, purine starved cells will show specific phenotype. *Leishmania* cells will arrest cell cycle, increase stress resistance and reorient cell metabolism to deal with both purine deprivation and general stress. (Carter et al., 2010; Martin et al., 2014).

Purine auxotrophy in yeast is not naturally occurring and is caused by genetic manipulations. Literature on effects of purine auxotrophy is scarce. It is known that purine depletion will stimulate Gcn4p - transcription regulator protein, responsible for increased transcription of more than 30 different amino acid biosynthetic genes in response to starvation for a single amino acid. (Rolfes, Hinnebusch, 1993). There are reports on Gcn4p

dependent purine biosynthesis gene activation (Mösh et al., 1991), as the *ADE4* gene promoter contains three sequences ATGA (C/G)TCAT that bind Gcn4. This motive is shared with Bas1p binding site GAGTCA, which is proven to be involved in *ADE4* transcription initiation (Som et al., 2005), which causes competition between these two transcription factors. Purine synthesis genes are also mentioned to be involved in determination of chronological life span (CLS), but reports are contradictory. Matecic describes the effect of purine *de novo* mutations on CLS that is comparable to glucose restriction. Mutations in *GLN3*, *TOR1* and *FCY2* also extend CLS but to a smaller extent than mutations in *de novo* synthesis pathway genes. CLS extension in ade *de novo* synthesis mutants is suppressed by adding extra adenine in media, but not in *gln3*, *tor1* or *fcy2* mutants (Matecic et al., 2010). Garay and colleagues on the other hand identify adenine *de novo* synthesis pathway gene mutations as CLS shortening ones. It is worth noting that in both cases strains harbouring several auxotrophies and synthetic media were used (Garay et al., 2014). Analysis of several quiescence studies stresses the fact that differences in strains used and media employed may explain vast discrepancies in genes affecting CLS (Smith et al., 2016).

There are also reports on influence of adenine starvation cells on retrotransposon activity, where starvation induces transcription of retrotransposons (Todeschini et al., 2005; Servant et al., 2008). Stress induced transposon activity has been connected to the possibility of genome evolution (Fedoroff, 2012).

# 2. Materials and methods

## 2.1. Strains and Cultivation Conditions

Two genetic backgrounds were used: W303 and CEN.PK. Strains used in research are summarised in table 1. All cultures were maintained on YPD agar and kept at 4° C. Fresh YPD agar plates were regularly reinoculated from stock cultures kept at − 80° C.

Table 1. Strains used in research used for theses. 1 - Kokina et al., 2014, 2 - Kokina et al., 2022, 3 - Ozoliņa et al., 2017

| Strain name used in text | Genotype | Source | Used in research in |
|---|---|---|---|
| W303 ade2 | W303-1A *MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15* | Dr. Peter Richard | 1 |
| W303 ADE2 | W303-1A *ADE2* | Dr. Arnold Kristjuhan | 1 |
| W303 prototroph | 2832 – 1B *MATa can1* | Dr. Frederick R. Cross | 1 |
| CEN.PK prototroph | CEN.PK 113-1A | Dr. Peter Richard | 1,2 |
| CEN.PK ADE8 | CEN.PK2 *MATa leu2-3/112 ura3-52 trp1-289 his3-1, MAL2-8c SUC2* | Dr. Peter Richard | 1,2 |
| CEN.PK ade8 | CEN.PK2 *MATa leu2-3/112 ura3-52 trp1-289 his3-1, ade8Δ0, MAL2-8c SUC2* | Our research | 1,2,3 |
| msn2 | CEN.PK ade8 *msn2::KanMX* | Our research | 3 |
| msn4 | CEN.PK ade8 *msn4::KanMX* | Our research | 3 |
| rim15 | CEN.PK ade8 rim15::KanMX | Our research | 3 |

The *ade8* knockout was induced by the *ura3-URA3* 5-FOA toxicity knockout technique, using *ade8* knockout construct plasmid (Sadowski et al., 2008).

Transcription factors were truncated by transforming yeast with linear PCR fragment containing flanking homologous sequences of the respective transcription factor and 6xHis

tag and G418 marker in between (Janke et al., 2004). After transformation strain identity was confirmed by colony PCR using gene specific test primers and internal primer (Nat_ctrl_Hb) from the insert (Lõoke et al., 2011). All the details regarding plasmids and primers are given in Table 2. All primers were synthesised by Sigma Aldrich.

Table 2. Plasmid and primers used for generation of strains with truncated transcription factors used in Ozoliņa et al, 2017

| Name | Description or sequence | Source |
|---|---|---|
| pYM46 | PCR template for C-terminal myc tag plus 7 His residues: marker pAgTEF-kanMX-tAgTEF, selectable phenotype: G418 resistance | Janke et al. 2004, EUROSCARF |
| MSN4_S2 | CTTGTCTTGCTTTTATTTGCTTTTGACCTTATTTTTTTCAATCGATGAATTCGAGCTCG | Our research |
| MSN4_S3 | GCATTCAGACGCAGTGAGCACTTGAAAAGGCATATAAGATCGTACGCTGCAGGTCGA | Our research |
| MSN2_S3 | GAAATTTAGTAGAAGCGATAATTTGTCGCAACACATCAAGCGTACGCTGCAGGTCGA | Our research |
| MSN2_S2 | TGAAGAAAGATCTATCGAATTAAAAAAATGGGGTCTATTAATCGATGAATTCGAGCT | Our research |
| RIM15_S2 | CAGTTATTTTTTTTAATTATCTTTATCTTAAAATTTATCAATCGATGAATTCGAGTCCG | Our research |
| RIM15_S3 | CAGGAGGCGGCAACCAGTAGAGTCTTTGACGATGTTTTAGCGTACGTCGCAGGTCGA | Our research |
| MSN4_test_F01 | AGAAGGCATTCAGACGCAGT | Our research |
| RIM15_test_F01 | CCAATTGTGGCCATAACAAA | Our research |
| MSN2_test_F01 | CCATTATCGCCTGCATCATCAT | Our research |
| NAT-HgB_ctrl | ACGAGGCAAGCTAAACAGATCT | Our research |

In Kokina et al., 2014 strains were cultivated in YPD - 10 g $L^{-1}$ of yeast extract (Biolife), 20 g $L^{-1}$ of peptone (Biolife), 20 g $L^{-1}$ of dextrose (Sigma) or SD media. In Kokina et al. (2022) and Ozoliņa et al. (2017) cells were cultivated exclusively in Synthetic Defined (SD) media (Saldanha et al., 2004) with 80 mg tryptophan, 100 mg uracil, 480 mg leucine, 100 mg histidine, and 100 mg adenine added per litre, as suggested in (Pronk, 2002). For purine starvation exponentially grown cells were transferred to fresh SD media with adenine omitted but other additives same as previous.

To ensure that yeast cultures were in the exponential growth phase, we reinoculated overnight cultures (grown from a single colony) into fresh media, where at least 6 doublings occurred and $OD_{600}$ 0.5–1, corresponding to $1–2 \cdot 10^7$ cells $mL^{-1}$, was reached. Cultures in the exponential growth phase ($OD_{600}$ 0.5–1) were washed with distilled water twice and resuspended at $OD_{600}$ 0.5 in full SD media (SD) or SD media with adenine omitted (SD ade−).

All cultures used for further measurements were incubated on a rotary shaker at 30 ˚C and 180 rpm in flasks where broth volume does not exceed 20% of total volume. To demonstrate changes in optical density during starvation, 96-well Tecan Infinite M200 multimode reader was used with the following cultivation cycle: orbital (3.5 mm) shaking for 490 s, waiting for 60 s, optical density measurement at 600 nm. Alternatively, culture growth dynamics was measured with a Z2 Cell and Particle Counter (Beckman Coulter, Brea, CA, USA).

## 2.2. Analysis of Extracellular Amino Acids and Purines

In Kokina et al. (2022) NMR spectroscopy was used. To obtain samples cell-free culture media was mixed with DSS (sodium 4,4-dimethyl-4-silapentane sulfonate) in $D_2O$ to obtain a final DSS internal standard concentration of 1.1 mM and transferred to a 5 mm NMR sample tube. NMR analysis was performed at 25 ˚C on a 600 MHz Bruker Avance Neo spectrometer equipped with a QCI quadruple resonance cryoprobe. The noesypr1d pulse sequence was used with water suppression during a recycle delay of 10 s. The spectral width was 11.9 ppm, and 128 scans were collected into 32K data points using an acquisition time of 2.3 s. The acquired 1H NMR spectra were zero-filled once, and no apodization functions were applied prior to Fourier transformation.

Phase and baseline corrections were applied manually. Spectra were referenced to DSS (at 0.00 ppm). The identification and quantification of sample components were performed using Chenomx NMR Suite professional software (version 5.11; Chenomx Inc., Edmonton, AB, Canada).

In Kokina et al., 2017 the concentration of adenine in media was determined enzymatically following a modified protocol from Zhang et al. (2003). More specifically, the concentration of adenine in media was quantified fluorometrically by hypoxanthine

oxidase (Sigma X4500)-coupled assay using horseraddish peroxidase (HRP; Sigma) and Amplex UltraRed dye (Molecular Probes, ex/em 530/590 nm). The reaction mix contained 20 μL of sample, 2 mL 0.1 M, pH 7.5 sodium phosphate buffer, 0.02 U xanthine oxidase, and 2 U HRP. Reaction mixtures were incubated at 30 °C for 30 min at which point the emission at 590 nm (ex 530) was measured with a FluoroMax-3 (Yvon Horiba) spectrofluorometer.

## 2.3. Cell Morphology Measurements

Cell samples were fixed in formaldehyde 0.5% and examined with an optical microscope (Olympus BX51, Tokyo, Japan). Microphotographs (1360 × 1024 pixels) were obtained with a digital camera (Olympus DP71, Tokyo, Japan). Cell size and budding index were determined by microphotography analysis in the ImageJ program. Budding index was defined as the proportion between the number of cells with buds and the total cell number. Bud was defined as a cell with a cross-section area less than half the mother cell size. Cell size was determined as the cell cross-section area measured from the microphotographs using ImageJ. Cells were defined as ellipses, with area measured in pixels and recalculated to square micrometers (1 μm = 5.7 pixels). For each sample, at least 500 cells were measured.

## 2.4. Flow Cytometry

Cell DNA content was determined by flow cytometry as described in (Sein et al., 2018). Briefly, 0.5 mL of yeast culture was fixed in 10 mL of ice-cold 70% ethanol for at least 15 min and washed once with 50 mM citric acid. RNA was degraded using RNase A (10 μg $mL^{-1}$) in 50 mM citric acid overnight at 37 °C. DNA was stained with 10 × SYBR Green (Invitrogen, Waltham, MA, USA) in 50 mM citric acid for 30 min. Cells were analysed with a FACSAria device (Becton Dickinson, Franklin Lakes, NJ, USA). Cell cycle distribution was analysed with Cyflogic software.

## 2.5. Fermentation and Metabolite Flux Measurements

Fermentation was done in a Sartorius Q-plus fermentation system with working volume of 0.3 L, gas flow 0.25 L · $min^{-1}$, mixing rate 400 rpm, media pH set to pH 5.5. Biomass concentration was determined as absorbance in 590 nm (WPA Colorimeter Colourwave CO7500, Biochrom, Cambridge, UK). The following coefficient to convert

absorbance units to dry weight was used: 1 $OD_{590}$ = 0.278 g · $L^{-1}$. Carbon dioxide evolution was recorded by an exhaust gas analyzer (Infors Gas Analyser, InforsHT, Basel, Switzerland) in parallel with harvesting metabolite samples.

The contents of extracellular glucose, ethanol, acetate, and glycerol were measured simultaneously by an Agilent 1100 HPLC system with a Shodex Asahipak SH1011 column, and they were quantified with a refractive index detector (RI detector RID G1362A). The flow rate of the mobile phase (0.01 N $H_2SO_4$) was 0.6 mL $min^{-1}$ and the sample injection volume was 5 µL. Biomass from fermentations was centrifuged and intracellular nucleotide pools were extracted via cold methanol extraction. ATP, ADP, and AMP were quantified by HPLC-MS-TOF analysis, as described in (Valgepea et al., 2010).

## 2.6. FTIR Analysis

For cell macromolecular content analysis, Fourier-transform infrared (FTIR) spectroscopy was used as described in (Grube et al., 2002). For this analysis, 2 mL of cells ($OD_{600}$ 1–4) was harvested by centrifugation and washed 3 times with distilled water. Cell pellets were diluted with 50 µL of distilled water, and samples were spotted on 96-well spot-plates. Absorbance data were recorded by a Vertex 70 device with HTS-XT microplate extender, interval 4000–600 $cm^{-1}$, resolution 4 $cm^{-1}$. For data collection and control, OPUS/LAB 6.5 software was used.

## 2.7. Cell Carbohydrate Extraction and Quantification

Fractional cell polysaccharide purification for quantitative assays was done as described in (Stewart, 1975). Total carbohydrate content of each fraction was determined by anthrone assay, and results were expressed as glucose equivalent mg · $gDW^{-1}$ biomass (Dubois et al., 1956).

## 2.8. Transcriptomics

Total yeast RNA after 4-h cultivation in synthetic dextrose (SD) or SD media with adenine omitted (SD ade−) was isolated with a RiboPureTM RNA Purification Kit for yeast (Thermo Scientific, Waltham, MA, USA). RNA samples for each condition were harvested in triplicate. Cell pellets from 50 mL suspensions were frozen in liquid $N_2$ and stored at −80 °C. RNA samples were prepared using 3.0 mRNA-Seq Library Prep Kit (Lexogen,

Vienna, Austria) according to the manufacturer's protocol. Yeast transcriptome was analysed using MiSeq (Illumina, San Diego, CA, USA) NGS data analysis. Sequencing reads were quality filtered (Q = 30), Illumina adapters and poly-A tails were removed,and reads at least 100 nt in length were selected for further processing using cutadapt. S288C reference genome from yeastgenome.org was used to identify gene transcripts.

Genes with lower than 1 count per million (CPM) in fewer than 2 samples were filtered out. The Benjamini and Hochberg method was used to calculate multiple comparison adjusted p-value as false discovery rate (FDR). FDR < 0.001 with logFC > 2 was set as a threshold for significance. Expression data set were submitted to the European Nucleotide Archive (ENA) database, under accession no. PRJEB40525.

## 2.9. Sublethal Stresses

Cells were grown in SD media until the exponential phase, washed with distilled water twice, and inoculated in SD or SD ade − with cell density of $1 \cdot 10^7$ cells $\cdot$ mL$^{-1}$. After 4 h incubation, cells were harvested by centrifugation, washed with distilled water once, and aliquoted in 1 mL, with $OD_{600} = 1$ (corresponding to $2 \cdot 10^7$ cells $\cdot$ mL$^{-1}$). Three aliquots were exposed to each stress.

For thermal stress, cells were kept at 53 °C for 10 min.

For oxidative stress, cells were incubated in 10 mM $H_2O_2$ for 50 min, then washed with distilled water.

For desiccation, cells were sedimented by centrifugation, the supernatant was removed, and the pellet was air-dried in the desiccator at 30 °C for 6 h. After drying, distilled water was added to resuspend cells.

After all stress treatments, treated cells were serially diluted, and dilutions were spotted on YPD plates to assess CFU $\cdot$ mL$^{-1}$. To check for cell loss during washing steps, the OD of the suspension was measured and CFU $\cdot$ mL$^{-1}$ corrected for OD value. Survival is expressed as % assuming that $OD_{600} = 1$ corresponds to $2 \cdot 10^7$ cells $\cdot$ mL$^{-1}$.

To test weak acid stress resistance, cells were spotted on YPD plates supplemented with 0.1 M acetic acid, with pH of agar media set to 4.5 (Martynova et al., 2016).

# 3. Results

## 3.1. Adenine auxotrophy – be aware: some effects of adenine auxotrophy in Saccharomyces cerevisiae strain W303-1A

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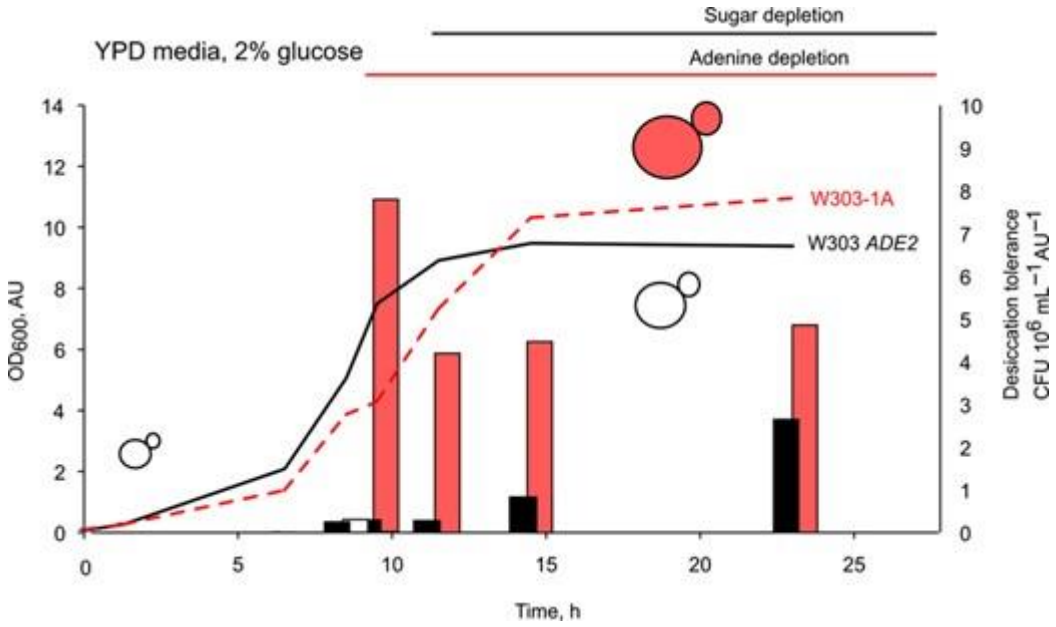
Key points:

- When cultivated in YPD media strains with ade auxotrophy exhaust adenine before glucose.
- After adenine exhaustion apparent increase in optical density of the culture is due to the cell swelling.
- Long term survival places adenine starvation between carbon and leucine starvations.
- Purine starved cells gain desiccation tolerance

Graphical abstract



FEMS YEAST RESEARCH

RESEARCH ARTICLE

# Adenine auxotrophy – be aware: some effects of adenine auxotrophy in *Saccharomyces cerevisiae* strain W303-1A

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**Keywords**
auxotrophy; starvation; adenine; W303; desiccation tolerance; trehalose.

## Abstract

Adenine auxotrophy is a commonly used genetic marker in haploid yeast strains. Strain W303-1A, which carries the *ade2-1* mutation, is widely used in physiological and genetic research. Yeast extract-based rich medium contains a low level of adenine, so that adenine is often depleted before glucose. This could affect the cell physiology of adenine auxotrophs grown in rich medium. The aim of our study was to assess the effects of adenine auxotrophy on cell morphology and stress physiology. Our results show that adenine depletion halts cell division, but that culture optical density continues to increase due to cell swelling. Accumulation of trehalose and a coincident 10-fold increase in desiccation stress tolerance is observed in adenine auxotrophs after adenine depletion, when compared to prototrophs. Under adenine starvation, long-term survival of W303-1A is lower than during carbon starvation, but higher than during leucine starvation. We observed drastic adenine-dependent changes in cell stress physiology, suggesting that results may be biased when adenine auxotrophs are grown in rich media without adenine supplementation.

## Introduction

Like all micro-organisms, baker's yeast cells respond to environmental changes and adapt their growth and proliferation accordingly. A drop in nutrient availability is a signal for the onset of nutrient limitation and impeding starvation. Even a slight drop in the concentration of a critical nutrient is sufficient to induce alterations in cell physiology such as the initiation of a 'preconditioning programme', which prepares the cell for harsh conditions (Smets *et al.*, 2010).

Two types of limitations or starvations, dependent on the nutrient, can occur: 'natural limitations', which sets in when basic nutrients (carbon, phosphorous, sulphur and nitrogen) are scarce, and 'artificial limitations', which sets in when particular metabolites or metabolic intermediates are insufficient (Saldanha *et al.*, 2004). Additionally, depending on nutrient supply mode, distinction is made between starvation and limitation. Starvation for certain nutrient is defined if it is absent, whereas limitation occurs when certain nutrient is added in scarce amounts and thus limits the growth. Starvation is a typical phenomenon of batch cultivations, and limitation is usually attributed to chemostat cultivations. Auxotrophy is a typical example of an artificial limitation. Many common laboratory yeast strains (W303, S288C, CEN.PK and FY series) contain one or several auxotrophic markers. Histidine, leucine, uracil, adenine and tryptophan (his, leu, ura, ade and trp) are the most common auxotrophic markers of *Saccharomyces cerevisiae* strains used in physiology studies (Pronk, 2002; Da Silva & Srikrishnan, 2011). Insufficient concentration of an auxotrophic agent leads to artificial limitation. Depending on the type of limitation, the yeast cells exhibit different responses. Cell cycle arrest and subsequent quiescent state constitute a typical response to natural limitations (Boer *et al.*, 2008). However, cell cycle arrest and quiescence do not occur for an artificial limitation with either leucine or uracil. On the other hand, inability to complete cell division and to halt subsequent cell cycle leads to a decrease in viability in addition to an observable 'glucose wasting' phenomenon (Boer *et al.*, 2008).

The adenine auxotrophic marker, *ade2-1*, is common to the *S. cerevisiae* strain W303-1A and its derivatives. These strains are well known for the fact that they acquire red colouration during culture growth. This red pigment is the oxidised form of ribosylaminoimidazole,

an intermediate of the adenine *de novo* synthesis pathway. The adenine auxotrophy-dependent red colouration is used in white red mutant screens (Weng & Nickoloff, 1997) and synthetic lethality assays (Barbour & Xiao, 2006). In addition to *ade2*, *ade1* is also used in white red mutant screens, and *ade8*, another adenine *de novo* synthesis pathway gene, has been used as an integration site yielding moderate expression levels of heterologous genes (Sadowski *et al.*, 2007).

External adenine supplement in synthetic media is needed to promote proliferation of adenine auxotrophs. However, availability of adenine is sometimes ignored in rich media (e.g. yeast extract-based media) because it is assumed that all nutrients are present in sufficient levels. Several researchers point out that in rich media, adenine levels vary from batch to batch and the adenine is often depleted before exhaustion of the carbon source (VanDusen *et al.*, 1997; Zhang *et al.*, 2003).

Besides hampering proliferation, adenine auxotrophy might have other adverse effects on yeast physiology. Thus far, adenine auxotrophy has been associated with a decrease in heterologous protein expression. Interestingly, this has been observed for both low and high external adenine levels (VanDusen *et al.*, 1997; Zhang *et al.*, 2003).

W303-1A and its derivatives have been exploited in basic physiology research for 30 years (Carlson & Botstein, 1982; Ralser *et al.*, 2012). However, to our knowledge, no research on the effects of adenine auxotrophy on physiology of this particular strain has been performed. Many physiological studies are performed in batch mode using rich media where the effects of adenine limitation can become pronounced, obfuscating the physiological phenomenon of interest. The findings from the studies herein can help to minimize these undesired effects. In the present work, we report some basic culture physiology and cell morphology studies using W303-1A batch cultivation. We find that adenine depletion has a direct impact on the cell size, trehalose content and subsequent desiccation stress tolerance. Taken together, our results serve as a basis for new interpretations of some previous results regarding yeast stress physiology as well as a warning against assuming that adenine auxotrophy provides a neutral background for physiology studies.

## Materials and methods

### Strains

Laboratory strains used in this study are shown in Table 1. CEN.PK *ade8* disruption was created by homologous recombination using a *URA* cassette and screening for *ura*$^-$ mutants on 5-FOA as described in Sadowski *et al.* (2007).

### Growth media

YPD [10 g $L^{-1}$ of yeast extract (Biolife), 20 g $L^{-1}$ of peptone (Biolife), 20 g $L^{-1}$ of dextrose (Sigma)] was used for yeast cell physiology studies: growth dynamics, cell morphology, trehalose content and desiccation stress tolerance. Synthetic dextrose (SD) media [1.7 g $L^{-1}$ of yeast nitrogen base w/o amino acids and ammonium sulphate (Difco), 5 g $L^{-1}$ of $(NH_4)_2SO_4$, 20 g $L^{-1}$ of dextrose] supplemented with leucine (260 mg $L^{-1}$), of tryptophan (80 mg $L^{-1}$), of uracil (100 mg $L^{-1}$), histidine (100 mg $L^{-1}$) and adenine (100 mg $L^{-1}$) was used for adenine titration experiments. For starvation experiments, SD media with either adenine, leucine or glucose omitted depending on starvation type investigated were used.

### Cultivation

Yeasts were cultivated in shake flasks at 180 r.p.m. in 30 °C with broth volume not exceeding 20% of the flask volume.

### Morphological measurements

Optical density (OD) was measured at 600 nm with Ultrospec 2100 pro (Amersham Biosciences), diluting cultures below 0.3 absorbance units.

The cells dry weight was determined by harvesting biomass from 20 to 40 mL of cultivation broth by centrifugation, washing twice with distilled water, and drying at

**Table 1.** Yeast strains used in this study

| Strain name in text | Strain genotype | Source |
|---|---|---|
| W303 *ade2* | W303-1A *MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15* | Dr. Peter Richard |
| W303 *ADE2* | W303-1A *ADE2* | Dr. Arnold Kristjuhan |
| W303 prototroph | 2832 – 1B *MATa can1* | Dr. Frederick R. Cross |
| CEN.PK prototroph | CEN.PK 113-1A | Dr. Peter Richard |
| CEN.PK *ADE8* | CEN.PK2 *MATa leu2-3/112 ura3-52 trp1-289 his3-1, MAL2-8c SUC2* | Dr. Peter Richard |
| CEN.PK *ade8* | CEN.PK2 *MATa leu2-3/112 ura3-52 trp1-289 his3-1, ade8, MAL2-8c SUC2* | This study |

105 °C until reaching constant weight. For cell counts and morphology assessment, cells were fixed in 1% glutaraldehyde and then sonicated briefly. Cell number was counted by hemocytometer.

Cell size (for a sample size of $n > 300$) was determined by analysing micrographs with ImageJ software, approximating cell shape as an ellipse and calculating the area of the acquired ellipses (Jorgensen *et al.*, 2007). Buds, if they exceeded half the size of the mother cell, were defined as separate cells. Budding index, defined as the proportion of cells with buds, was determined for the population from a sample size of $n > 500$.

### Metabolite measurements

Anthrone assay was used to determine trehalose and media glucose concentrations (Terevelyan & Harrison, 1956). For trehalose measurements, cells were washed with distilled water twice, disintegrated in 5% TCA with glass beads, and then the supernatant (diluted with water when necessary) mixed with anthrone (2 g $L^{-1}$ in 75% $H_2SO_4$) in a 1 : 6 ratio. The mixture was heated at 100 °C for 10 min, and absorbance at 626 nm was measured. The same procedure was used for media glucose quantification.

The concentration of adenine in media was determined enzymatically following a modified protocol from Zhang *et al.* (2003). More specifically, the concentration of adenine in media was quantified fluorometrically by hypoxanthine oxidase (Sigma X4500)-coupled assay using horseradish peroxidase (HRP; Sigma) and Amplex UltraRed dye (Molecular Probes®, ex/em 530/590 nm). The reaction mix contained 20 μL of sample, 2 mL 0.1 M, pH 7.5 sodium phosphate buffer, 0.02 U xanthine oxidase, and 2 U HRP. Reaction mixtures were incubated at 30 °C for 30 min at which point the emission at 590 nm (ex 530) was measured with a FluoroMax-3 (Yvon Horiba) spectrofluorometer.

### Stress tolerance assessment

Desiccation tolerance was assayed by estimating CFU $mL^{-1}$, before and after dehydration. One millilitre of culture at $OD_{600} = 1$ was washed with distilled water twice, diluted serially and spotted on YPD plates. The remaining cell suspension was centrifugated, and the pellet was left to desiccate for 10 h at 30 °C in a desiccator and then rehydrated for 10 min in room temperature in distilled water. The suspension of rehydrated cells was serially diluted and spotted on YPD plates. The viability was calculated by dividing the number of CFU $mL^{-1}$ before and after desiccation, as performed in Calahan *et al.* (2011).

To assess starvation stress tolerance, cells were grown in full SD media up to exponential phase, washed with distilled water, and re-suspended to $OD_{600} = 1$ in SD media lacking either sugar, leucine or adenine with all other broth components added in surplus. The yeasts were incubated for 10 days in a rotary shaker, and samples were taken upon inoculation (day 0) and on the 1st, 2nd, 4th, 7th and 10th day. Samples were diluted serially and plated on YPD plates to assess viability. Undiluted sample was fixed with glutaraldehyde and later used for budding index, cell size and count $mL^{-1}$ measurements.

### Statistical treatment of data

All the represented values are means from biological triplicates. Error bars and variation depict standard errors. Two-tailed, two-sample unequal variance Student's *t*-test or Wilcoxon rank-sum test (for cell size comparison) were used to compare means of physiological parameters. *P*-values $< 0.05$ were considered statistically significant.

## Results

### Growth characteristics and cell morphology

The effects of adenine auxotrophy on the *S. cerevisiae* strain W303-1A (from here on called W303 *ade2*) were explored during cell growth in YPD (glucose content 2%) media. A W303-1A-derived adenine prototroph (W303 *ADE2*) strain was used as a control. External adenine depletion was monitored by xanthine oxidase coupled to horseradish peroxidase assay. Also, red colouration of W303 *ade2* cells served as a signal for adenine depletion. The red pigment in yeast cells was observed after adenine became depleted in the media, as detected by xanthine oxidase assay. However, adenine auxotrophs tend to accumulate a vast amount of adenine and then use it upon depletion of the external adenine pool (VanDusen *et al.*, 1997). The adenine synthesis pathway and subsequent red pigment accumulation in adenine auxotrophs are induced after adenine *de novo* synthesis is started, which occurs when no free adenine remains inside the cell (Rebora *et al.*, 2001). Therefore, we assumed that pigment development is a physiologically more reliable signal for adenine starvation in the cell than the concentration of adenine in the media.

We assessed growth of W303 *ade2* and its corresponding adenine prototroph W303 *ADE2* using yeast extract from only one producer due to known variance in adenine content across different commercial suppliers (VanDusen *et al.*, 1997; Zhang *et al.*, 2003). For all experiments, yeast extract from the same producer and single batch was used.

First, we compared growth dynamics of W303 adenine auxotroph and prototroph in YPD media to determine

whether adenine content in media influences general cell physiology.

OD measurements at 600 nm were used to monitor growth (Fig. 1a). Throughout the exponential growth phase (from 0 to 10 h), both strains, W303 adenine auxotroph and prototroph, grew similarly. The adenine content in the media, measured by the xanthine oxidase assay, was depleted at the same time for both strains (see Fig. 1). Moreover, specific glucose consumption rates and biomass yields were similar for both strains during the exponential growth phase, before exhaustion of external adenine.

Biomass yields per substrate consumed ($Y$, $x/s$, g $g^{-1}$) for W303 *ade2* and W303 *ADE2* were 0.097 and 0.099, respectively. Specific substrate uptake rates ($q$) were 0.98 and 0.95 g $g^{-1}$ $h^{-1}$ for W303 *ade2* and W303 *ADE2*, respectively.

There was still some sugar left in the growth medium after external adenine was exhausted (Fig. 1b). Surprisingly, adenine depletion did not interrupt an increase in OD for the W303 *ade2* strain, a metric that suggests uninterrupted growth. This observation conflicted with the prediction that adenine exhaustion would halt proliferation and an $OD_{600}$ increase (Fig. 1a). When the ratio of dry weight to OD of the culture was analysed (Supporting Information, Fig. S1), both W303 strains, *ade2* and *ADE2*, maintained linear OD/dry weight ratios both before and after external adenine was depleted. As expected, W303 *ade2* formed red pigment as a response to external adenine depletion.

To resolve the discrepancy between the expected proliferation cessation and the clear increase in OD for W303 *ade2* in culture after adenine depletion, we compared optical densities with the corresponding cell counts $mL^{-1}$ of both cultures (Fig. 2).

There was a notable difference in cell number $mL^{-1}$ when comparing the adenine auxotroph and prototroph. While the cell number $mL^{-1}$ grew steadily in adenine prototroph, its increase ceased for the auxotroph after adenine depletion. To show that this effect was not strain specific, but purely adenine dependent, we added extra adenine (100 mg $L^{-1}$) to W303 *ade2* strain in YPD media. As a result of not reaching adenine starvation through supplementation, the behaviour of W303 *ade2* cells followed the pattern of the W303 *ADE2* strain. Therefore, we concluded that an increase in $OD_{600}$ after adenine depletion in adenine auxotrophic cultures is not caused by cell multiplication but most likely due to changes in the cells' optical properties.

Two hypotheses were proposed to explain the $OD_{600}$ increase after adenine depletion: either pigment induced by adenine auxotrophy has absorption that overlaps 600 nm or increased light scattering occurs due to increase in cell size.

The red pigment does not have specific absorption around 600 nm (Smirnov *et al.*, 1967). Therefore, we concluded that red pigment had negligible, if any, impact on W303 *ade2* OD measurements at 600 nm. Beauvoit *et al.* (1993) have reported that yeast cell size affects culture suspension light scattering and attributed it to a special case of general light scattering Mie theory.

To determine whether cell size was contributing to the increase in $OD_{600}$, mean cell size in W303 *ade2* and W303 *ADE2* cultures was measured after 27.5 h of growth in YPD medium. Size was measured as an elliptical approximation of the cell's cross-section in microphotographs. The adenine auxotrophic cells were significantly ($P < 0.0001$) bigger than those of the prototrophs: 22 ± 14 vs. 16 ± 5 μm$^2$, respectively (Fig. 3).

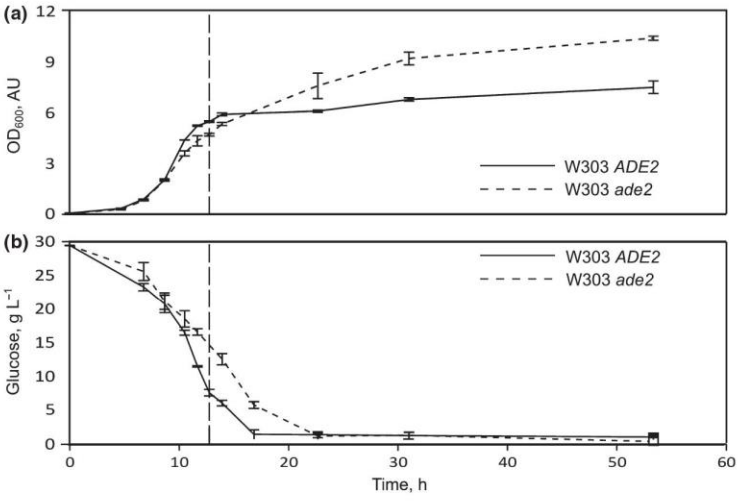


**Fig. 1.** W303 *ADE2* and W303 *ade2* culture growth (a) and glucose consumption (b) when cultivated in YPD media. Error bars represent standard deviation from three independent cultivations. Dashed vertical line indicates adenine depletion in the media.

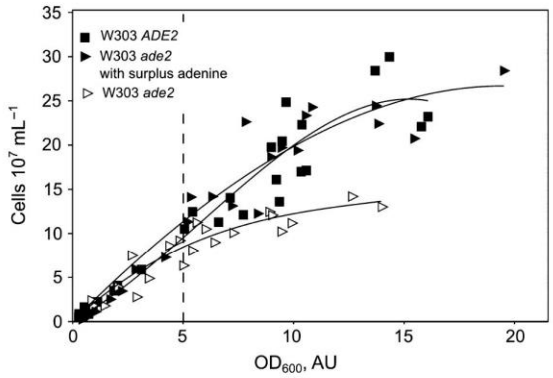


**Fig. 2.** W303 strain cell count $mL^{-1}$ depending on $OD_{600}$. Yeast strains were grown in YPD media with (100 mg $L^{-1}$) and without extra adenine supplement. Cell number $mL^{-1}$ was determined by hemocytometer. Vertical dashed line indicates adenine depletion during W303 *ade2* cultivation in YPD media without extra adenine supplement. Approximations of third-order polynomial functions were used for visualization of cell number $mL^{-1}$ data. However, it does not fully account for relationship between the variables.

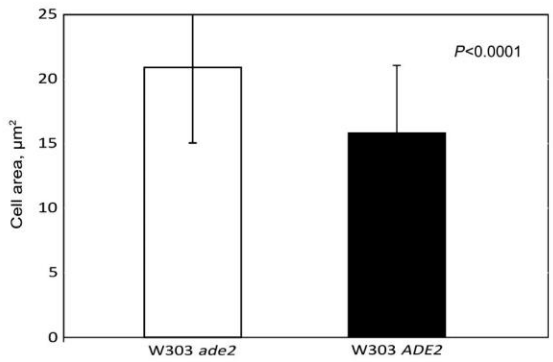


**Fig. 3.** W303 strain mean cell size. Cells were harvested after 27.5 h of cultivation, and their cross-section areas were measured from micrographs as described by Jorgensen *et al.* (2007). Cell number exceeded 300 for each strain. Means of both distributions were compared by Wilcoxon signed rank-sum test. Mean cell area of W303 *ade2* and W303 *ADE2* revealed to be significantly different ($P < 0.0001$).

Based on these data, we concluded that the increase in cell size has led to elevated turbidity of the cell suspension and, therefore, to the misleading impression of proliferation of adenine auxotroph cells after adenine depletion.

Our results indicate that growth of the W303 *ade2* strain in rich medium differs significantly from that of the adenine prototroph when adenine is depleted. VanDusen *et al.* (1997) reported on high variability of adenine content among yeast extracts of different vendors and different batches (ranging from 0.34 up to 1.91 mg $g^{-1}$ yeast extract). The YPD media used in experiments described here contained 13 mg $L^{-1}$ of adenine.

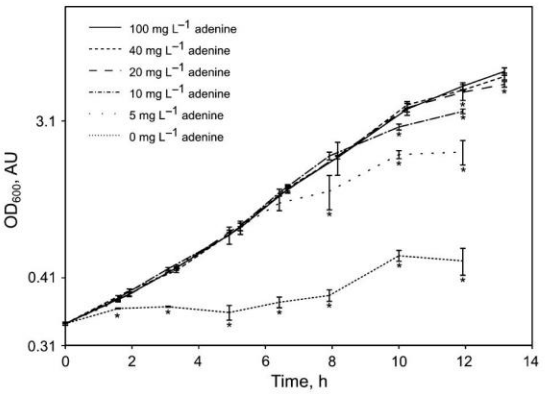


**Fig. 4.** Growth curves of W303 *ade2* strain in SD media with different adenine content (0, 5, 10, 20, 40 and 100 mg $L^{-1}$); all other nutrients were added in surplus. Each growth curve is mean of biological triplicates; error bars represent standard deviations. Statistical comparison between OD measurements during cultivation in medias of different adenine concentrations and 100 mg $L^{-1}$ (positive control) was made. Asterisks below data point denote significant difference from positive control ($P < 0.05$).

Due to vast variability of adenine content among different manufacturers' yeast extracts, and obvious adenine insufficiency observed in our rich media, we decided to determine optimal adenine concentration for the W303 *ade2* strain cultivation. To ensure exact adenine concentrations, SD media were used. We were interested in changes in growth rate, which, besides red pigmentation, would indicate adenine exhaustion. We inoculated exponentially growing, freshly washed W303 *ade2* cells to flasks with media of different adenine content. We used an adenine concentration of 100 mg $L^{-1}$ as a positive control (physiologically 'safe' after Pronk, 2002) against which growth curves of all other adenine concentrations (0, 5, 10, 20 and 40 mg $L^{-1}$) were compared (Fig. 4). Results are plotted on log axis to demonstrate growth rate changes. W303 *ade2* culture growth profiles when cultivated in media with 0, 5, 10 and 20 mg $L^{-1}$ adenine diverged from 100 mg $L^{-1}$ curve at different time points, and cells started to accumulate red pigment, thus indicating depletion of external adenine. Student *t*-test revealed significant differences for $OD_{600}$ between 100 mg $L^{-1}$ and each of these cultivations. At the time points when growth rate changed, glucose persisted in ample amounts (20, 15.4, 14.2, 8.68 g $L^{-1}$ for flasks with 0, 5, 10, and 20 mg $L^{-1}$ adenine supplement, respectively) further indicating adenine depletion. No statistically significant

($P > 0.05$) differences between culture $OD_{600}$ were observed when comparing cultures containing 40 mg $L^{-1}$ with cultures of 100 mg $L^{-1}$ adenine supplement.

Based on our results, we concluded that at least 40 mg $L^{-1}$ supplement should satisfy uninterrupted W303 *ade2* mutant growth.

## Desiccation and starvation responses

Reserve carbohydrate accumulation (glycogen and trehalose) is reported to occur in yeast under certain limitations (Lillie & Pringle, 1980; Klosinska *et al.*, 2011). Studies of different limitations in chemostats have revealed that trehalose accumulation is inversely related to the culture's growth rate, but independent of the nature of limitation, whether it be natural or artificial (Boer *et al.*, 2010). Due to the observed halt of proliferation after the onset of adenine depletion (Fig. 2), we decided to measure trehalose accumulation in a series of W303 strains (W303 *ade2*, W303 *ADE2*, and W303 prototroph) during cultivation in a YPD medium. A fully prototrophic strain was added to the analysis to assess possible pleoitropic effects from other auxotrophies present in W303 *ade2* and W303 *ADE2* cells. Cells were sampled at exponential growth phase, shortly before and after exhaustion of adenine, after exhaustion of glucose, and during stationary phase. Both (W303 *ADE2* and W303 prototroph) strains had consumed all the glucose after 13 h of growth. At the same time, we observed accumulation of red pigment and cessation of W303 *ade2* growth. Glucose measurements revealed that there was still 8 g $L^{-1}$ glucose left in the media. Trehalose content in exponential phase cells, of all three strains, was close to zero. After adenine exhaustion, W303 *ade2* started to accumulate trehalose and its content increased with time. After glucose exhaustion, prototrophic strains began to accumulate trehalose as well, but at a slower rate. Interestingly, trehalose content continues to increase with time in a fully prototrophic strain but not in W303 *ADE2* (Fig. 5a). Trehalose content of adenine-starved W303 *ade2* cells differed significantly ($P < 0.05$) from W303 prototroph and W303 *ADE2*.

Traditionally, the increase in trehalose content has been linked to elevated tolerance to stress (e.g. desiccation). We assessed desiccation stress tolerance in cells sampled during various stages of culture growth, both adenine sufficient and adenine starved (Fig. 5b). All cell cultures sampled during exponential phase showed low desiccation stress tolerance, but desiccation tolerance of W303 *ade2* cells increased sharply after adenine depletion. Desiccation tolerance over time roughly corresponded to the pattern of trehalose accumulation, and for adenine-starved W303 *ade2* cells, desiccation tolerance differed significantly ($P < 0.05$) from W303 prototroph and W303 *ADE2*.

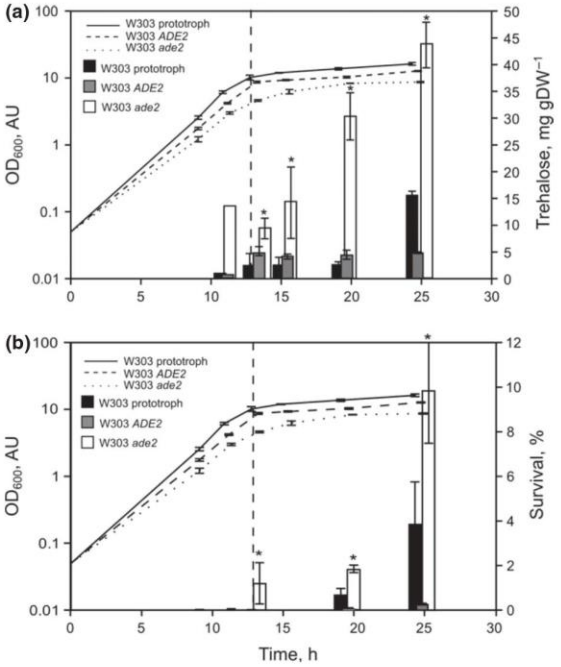


**Fig. 5.** Changes in accumulated trehalose (a) and desiccation tolerance (b) during W303 prototroph, W303 *ADE2* and W303 *ade2* cultivation in YPD media. Cells were desiccated in +30 °C for 10 h. Desiccation tolerance was quantified as in Calahan *et al.* (2011). In both (a) and (b), lines denote strain growth curves in logarithmic scale and bars trehalose (a) or survival (b). Error bars depict standard deviation from biological triplicates. Viability in first two time points (8 and 12 h) is close to zero (see b). Asterisks depict statistically significant difference ($P < 0.05$) between trehalose content of W303 *ade2* and both W303 prototroph and W303 *ADE2* strains (a). Also it depicts difference between W303 *ade2* and both W303 prototroph and W303 *ADE2* strains desiccation tolerance as statistically significant ($P < 0.05$) (b).

A distinction has previously been made between starvation for natural and artificial nutrients (Saldanha *et al.*, 2004). Cells starved for natural (C, P, N) nutrients survive for longer periods than cells under artificial starvations and do so by arresting the cell cycle. In contrast, auxotrophically starved cells have elevated glucose consumption rate and reduced survival and do not arrest their cell cycle (Brauer *et al.*, 2008). To place adenine starvation in the landscape of natural and artificial starvations, W303 *ade2* cells were incubated in synthetic media lacking either leucine, adenine or carbon source. While carbon-starved cells clearly showed high and stable survival rates when compared to both auxotrophies, survival of leucine and adenine differed significantly ($P < 0.05$) when measured at day 4, 7 and 10. Adenine starvation

led to higher culture viability than leucine starvation except for first 2 days where survival did not differ significantly between leucine and adenine starvation (Fig. 6a).

To find out whether survival of adenine- and leucine-starved cultures can be explained by differences in the cells' ability to arrest their cell cycle, we estimated the budding index of the starved cultures. The number of cells with small buds was counted, and the relative fraction for all cells calculated. Presence of a small bud indicates that the given cell is in the beginning of S phase and is not arrested (Smets *et al.*, 2010). Typically, a large percentage of cells are in a budded state while starving for leucine or uracil (Brauer *et al.*, 2008). Adenine-starved W303 *ade2* cells showed a smaller percentage of budded cells. Differences between leucine and adenine starvations were established in the first 2 days of starvation and remained relatively unchanged through the remainder of the experiment (Fig. 6b). When comparing the viability and budding index results, we noticed that higher mortality during starvation corresponds to a higher percentage of budded cells. That confirmed the possible role of cell cycle arrest in viability during auxotrophic starvation.

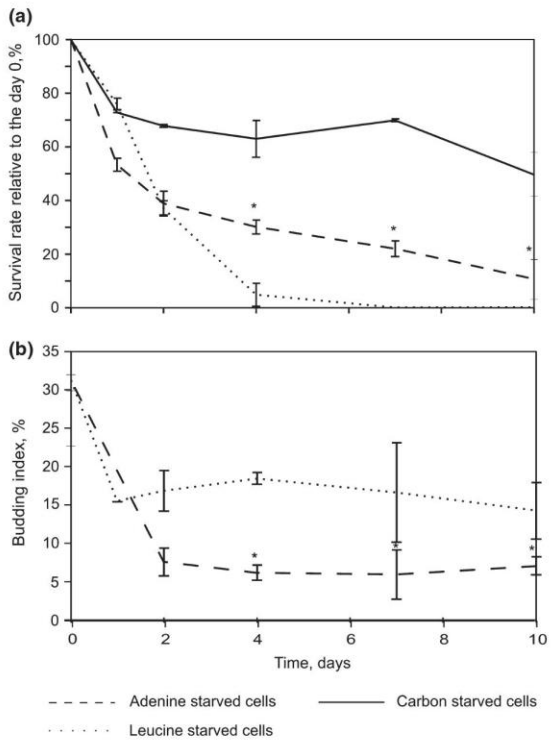


**Fig. 6.** Survival rate (a) and budding index (b) during prolonged W303 *ade2* auxotrophic (leucine or adenine) or carbon source starvation. Cells were grown in full SD media up to exponential phase, washed with distilled water and re-suspended to OD600 = 1 in SD media lacking sugar, leucine or adenine; all other broth components were added in surplus. Error bars represent standard deviation from three independent cultivations. Viability in (a) depicted as percentage of CFU from OD600 = 1 in the beginning of experiment. Budding index is calculated as ratio of cell number with small bud against total cell number. Asterisks depict statistically significant difference ($P < 0.05$) between adenine and leucine and carbon starvations.

Although exponentially growing, double-washed cells were used for SD adenine- and leucine-deficient media inocula, a two- to fourfold increase in OD of the culture was observed during the first days of cultivation (Fig. S2a). This might be explained either by cell multiplication due to accumulated resources within the cells or by cell size increase. We estimated cell number $mL^{-1}$ during cultivation (Fig. S2b). Almost no increase in cell numbers was seen. When using the real cell density (cell number $mL^{-1}$) instead of $OD_{600}$ measurements, viability curves showed no statistically significant ($P < 0.05$) difference in cell survival between adenine- and leucine-starved cultures, during the first 2 days of starvation (Fig. S2c).

Because differences in cell size and cell number per optical unit were noted during W303 *ade2* and W303 *ADE2* cultivation in YPD medium, we expected a similar effect during long-term adenine or leucine starvation. Mean cell size of the culture increased for both cultures, albeit to a different degree (Fig. 7). The increase in the mean cell size for leucine-starved cultures stopped after 2 days, whereas it continued to increase in adenine-starved culture. These differences are statistically significant for each day of cultivation ($P < 0.001$). When the distribution of the frequencies of cell size was plotted (Fig. S3), the distributions were shown to widen with each starvation day. While some of the starved cells slowly lost viability and retained their size, living cells became increasingly larger. It seems plausible that the increase in cell size is caused by metabolite (e.g. trehalose) accumulation.

On the basis of our results, we conclude that adenine auxotrophy is distinctively different from leucine auxotrophy. Although cell viability drops over time relative to carbon starvation, adenine starvation exhibits characteristics similar to those of other natural starvations: higher survival rates and ability to arrest cell cycle more efficiently when starved in comparison with artificial starvations such as leucine.

## Discussion

Auxotrophy is a common property of haploid laboratory strains. Traditionally, amino acid and purine/pyrimidine auxotrophic markers are used in yeast strain genetic

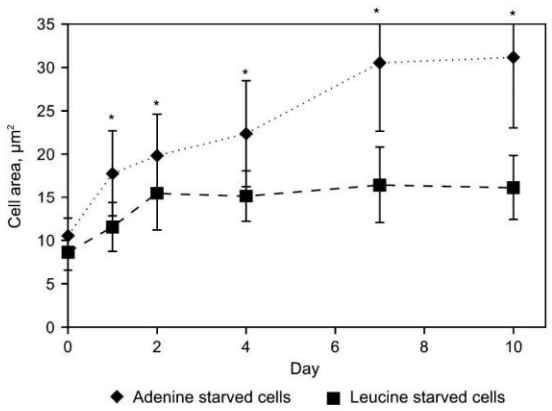


**Fig. 7.** W303 *ade2* culture mean cell size dynamics during prolonged adenine or leucine starvation. Cell cross-section areas were measured from micrographs as described by Jorgensen *et al.* (2007). Cell number exceeded 300 for each data point. Asterisks depict statistically significant difference ($P < 0.0001$) between cell sizes of adenine and leucine starvations, as determined by Wilcoxon rank-sum test.

engineering. Leucine, tryptophan, adenine, uracil, methionine, and histidine auxotrophies are typical markers and targets for complementation with plasmids or integration constructs (Pronk, 2002; Da Silva & Srikrishnan, 2011). However, questions have been raised regarding how this common strain property affects the general physiology of cells (Mülleder *et al.*, 2012; Liu *et al.*, 2013). Problems with auxotrophic strains might arise for two reasons: depletion of the auxotrophic agent in the growth medium may occur with great regularity if the concentration of the agent is insufficient and there may exist pleiotropic interactions between multiple auxotrophies in the same strain. Several protocols define different amounts of auxotrophic agents needed in synthetic media, and yeast cell physiologists have raised concerns that the amounts usually used might not be sufficient for uninterrupted cell growth (Pronk, 2002). However, rich media have been considered safe in respect to auxotrophies, and additional amino acids or nucleotides are seldom added.

Our results show that additional adenine (at least 40 mg $L^{-1}$ or more), even to the rich media, should be added to avoid adenine starvation. Concerns are raised regarding growth attenuation effects of auxotrophic supplements, if added in excess (Mülleder *et al.*, 2012). Our results on synthetic media (Fig. 4) show no statistically significant differences in growth if 40 or 100 mg $L^{-1}$ adenine is added. Furthermore, no difference between growth parameters of W303 prototroph and W303 *ade2* on YPD with extra 100 mg $L^{-1}$ adenine supplement is seen ($\mu = 0.45\ h^{-1}$ for both strains). Similarly, VanDusen *et al.* (1997) and Zhang *et al.* (2003) reported on sufficient adenine concentration to be 50–80 mg $L^{-1}$.

Auxotrophic markers can have pleiotropic effects on *S. cerevisiae* physiology. There are many examples of the effects of tryptophan, methionine and histidine autotrophy on yeast physiology. For example, any gene deletion in the tryptophan biosynthesis pathway (*trp1-5* genes) leads to decreased growth in the presence of rapamycin, caffeine and SDS. Notably, wild-type characteristics are not regained after the respective gene complementation (González *et al.*, 2008). Also, depending on the length of the *HIS3* gene deletion (200 bp or 1 kbp) used to generate the histidine auxotrophy, various levels of respiration deficiency at 37 °C can be observed (Young & Court, 2008). Criticism has also been raised regarding the use of methionine auxotrophs because the need for methionine supplement masks the effects of other gene deletions as seen in *zwf1* strains (Thomas *et al.*, 1991; Pronk, 2002).

In contrast to the above auxotrophies exhibiting pleiotropic impact on physiology, our results on adenine auxotrophy show purely adenine-dependent effects. When ample amount of adenine is available in the media, adenine auxotrophs and prototrophs are physiologically indistinguishable: autotroph and prototroph cell size, OD and dry weight ratio, biomass yield, glucose consumption, and desiccation tolerance are similar. On the other hand, when adenine concentration in medium is limiting, cell morphology and physiology change significantly. Additionally, adenine-dependent effect is not strain or specifically *ADE2* gene dependent. We repeated desiccation tolerance experiments with strains of CEN.PK series: full CEN.PK prototroph, CEN.PK *ADE8* (CEN.PK2 *MATa leu2-3/112 ura3-52 trp1-289 his3-1, MAL2-8c SUC2*) and CEN.PK *ade8* deletion (made on CEN.PK *ADE8* background). Growth rate, trehalose accumulation and desiccation tolerance were measured. Results follow the same pattern as in W303 – the adenine auxotroph accumulates trehalose and shows significantly ($P < 0.05$) elevated desiccation tolerance when adenine becomes depleted (results are shown in Fig. S4). From this, we conclude that the observed phenomenon is not strain or *ade2* gene specific; instead, it relates to adenine auxotrophy generally.

When comparing fully prototrophic strains with their respective *his*, *leu*, *trp*, *ura* auxotrophs, a decrease in trehalose accumulation and desiccation tolerance can be seen; however, differences are not statistically significant ($P > 0.05$). Some or even all of these common auxotrophies could decrease desiccation resistance in prototrophs. To fully understand the phenotypical interplay between all auxotrophic markers, each auxotrophy alone and its combination with the others should be tested in otherwise prototrophic strains. Some interplay between adenine, histidine and tryptophan auxotrophies could take

place as they share some common elements in their biosynthesis pathway. Moreover, multiple stress-related phenotypic traits of tryptophan auxotrophs have been described before (González *et al.*, 2008). We did segregant analyses of crosses between $ade^{+}trp^{-}$ and $ade^{-}trp^{+}$ strains and found that increased desiccation stress tolerance and trehalose accumulation after adenine depletion occurs in $ade^{-}$ segregants independently of *trp* gene functionality (data not shown). However, adenine auxotrophy is usually accompanied by other four auxotrophic markers in W303 series strains or through *ade8* disruption by an integration vector. Statistically significant differences among adenine auxotrophs and prototrophs that we observed in two strain backgrounds, W303 and CEN.PK, indicate that the observed effects are adenine auxotrophy specific and thus should be carefully considered when doing physiological studies with those strains.

We explored this phenomenon further to determine the extent of biases in yeast physiology that can be produced due to insufficient adenine in the medium. Measurements of OD, a common indicator of cell growth used in microbiology (Madrid & Felice, 2005), can be misleading in adenine auxotrophs due to the swelling of cells – $OD_{600}$ increases even after cell proliferation has stopped. One of the reasons why *ade* markers are widely used in genetic research is because the accumulation of red pigment is a convenient visual marker used when distinguishing segregants. The same pigment is autofluorescent and hinders cell visualization (Weisman *et al.*, 1987), so usually care is taken to use adenine-enriched media when growing cells for visualization studies. On the other hand, stress physiology research is quite often performed on adenine auxotrophs after exponential growth phase in rich media, without any additional supplementation (Carrasco *et al.*, 2001; Petrezselyova *et al.*, 2010). In our opinion, increased stress tolerance of stationary-phase adenine auxotrophs in rich media is due to adenine depletion and not because of other strain characteristics.

Desiccation is a multifactorial stress that challenges cells with hyperosmolarity, hyperoxidation, hyperionicity and protein misfolding/aggregation during dehydration and rehydration (Chakrabortee *et al.*, 2007; França *et al.*, 2007). Trehalose is a widely discussed storage carbohydrate, which accompanies various stress conditions (Crowe *et al.*, 1998). Still, whether trehalose is an important desiccation stress protector or just a metabolite that accumulates during slow growth remains unclear (Paalman *et al.*, 2003; Ratnakumar & Tunnacliffe, 2006). Although trehalose accumulation in W303 *ade2* coincides with the increase in desiccation tolerance, adenine prototroph strain does not show the same relationship between trehalose accumulation and desiccation tolerance. This indicates that trehalose accumulation could serve as a signal for elevated desiccation tolerance, but it might not be a prerequisite for it. Previously, rate of trehalose accumulation has been attributed to the growth rate (Paalman *et al.*, 2003). Adenine auxotroph cells indeed accumulate far greater amounts of trehalose after they cease to proliferate, and dynamic trehalose levels that change over time in arrested cells indicate that there may be additional regulatory mechanisms.

Trehalose levels in cells are determined as an outcome of dynamic equilibrium of trehalose synthesis and hydrolysis (Hohman & Mager, 2003). The activity of trehalase and trehalose synthase is regulated by the cAMP-PKA pathway, which is upregulated in the presence of glucose (Winderickx *et al.*, 1996). A great increase in trehalose levels in adenine-starved cells could indicate downregulation of cAMP-PKA pathway and consistent downregulation of trehalase activity and upregulation of trehalose synthase complex activity. Research shows that yeast cells, when starved for carbon, nitrogen or phosphorous, upregulate both sides of the trehalose metabolism – synthesis and hydrolysis. However, trehalose accumulates only in nitrogen-starved cells (Klosinska *et al.*, 2011). We have measured trehalase activity in *ade8* deletion strain in CEN.PK background, and it shows the same tendency – trehalose accumulation is accompanied by elevated trehalase activity during adenine starvation (data not shown). Additional research is needed to clarify dynamics of trehalose accumulation during adenine starvation to determine whether they are similar to those observed in nitrogen starvation.

The recent work of Welch *et al.* (2013) elucidates the role of TOR and RAS pathway in acquiring desiccation stress resistance. These two pathways regulate cell growth rate by monitoring external carbon and nitrogen supplies (Smets *et al.*, 2010). Here, we show that adenine depletion does stop cell division and increases cell viability after desiccation, indicating a possible role for these two signalling pathways in physiological changes observed during adenine starvation. The increase in desiccation tolerance in response to carbon, nitrogen and phosphorus starvation has already been reported (Welch *et al.*, 2013).

Although adenine and uracil are nucleotides, different physiological responses to starvation can be observed for them. It is possible that either yeast cells sense external adenine levels or, as adenine becomes limited, the cells sense limited or imbalanced adenylate levels intracellularly. Yet there are no reported transcriptional response mechanisms to disturbed nucleotide balance (Ljungdahl & Daignan-Fornier, 2012). Little is known about adenylate levels in cells undergoing adenine starvation, so further studies are required to elucidate possible cell response mechanisms. Existence of specific transcriptional response leading to a quiescent state for yeast is under debate (Klosinska *et al.*, 2011).

To summarize our findings, the physiological state of W303-1A changes dramatically after adenine is exhausted in YPD media. Cell proliferation ceases while cells increase in size and accumulate trehalose. Prominent increase in desiccation stress tolerance follows. Trehalose accumulation and elevated desiccation tolerance imply that adenine auxotrophs change their carbon flow and internal signalling after adenine depletion. In prolonged starvation experiments, adenine-deficient cells show increased ability to arrest cell cycle and are viable for longer period of time compared with cells starved for leucine. These facts distinguish adenine starvation from other auxotrophies described thus far. To avoid unwanted phenotypic changes due to adenine depletion in rich media, we suggest adding extra adenine for *ade*$^{-}$ if cell physiology will be studied after exponential growth phase, to avoid adenine exhaustion before glucose depletion.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** W303 adenine auxotroph and prototroph dry weight dependence on optical density measurements when cultivated in YPD medium.
**Fig. S2.** Optical density (a), cell count $mL^{-1}$ (b) and relative survival rate (c) during W303 *ade2* adenine (diamonds) and leucine (squares) starvation.
**Fig. S3.** Cell size distribution during adenine (upper panel) and leucine starvation (lower panel).
**Fig. S4.** Changes in accumulated trehalose (a) and desiccation tolerance (b) during CEN.PK prototroph, CEN.PK *ADE2* and CEN.PK *ade2* cultivation in YPD media.

**Supplementary figures from article**

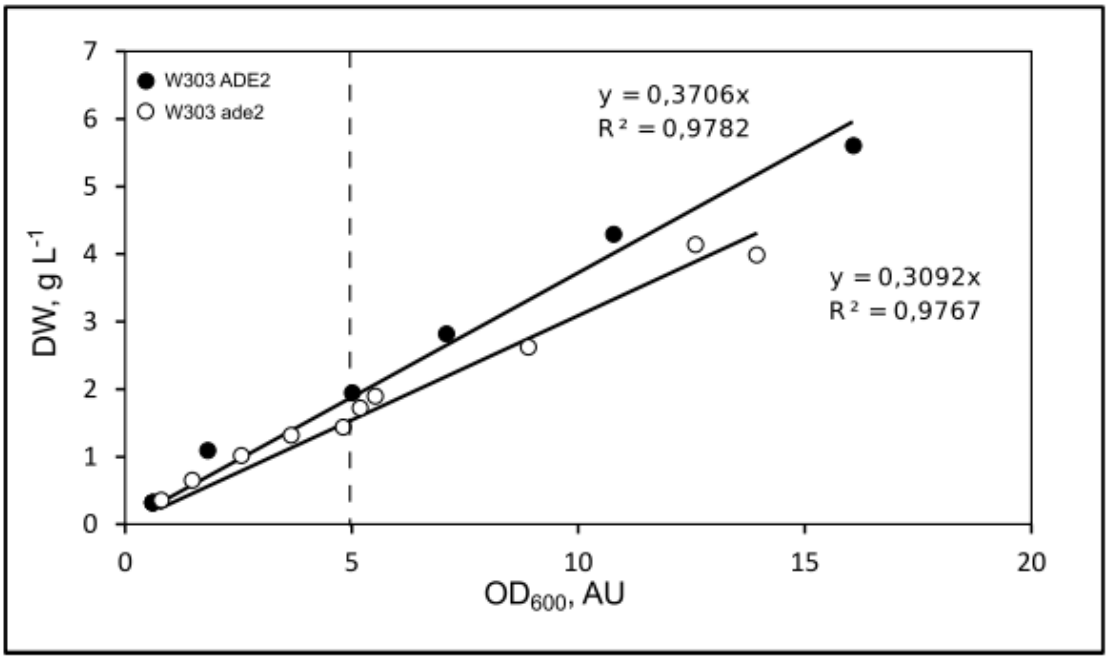


Fig. S1. W303 adenine auxotroph and prototroph dry weight dependence on optical density measurements when cultivated in YPD medium. Cells were harvested by centrifugation, washed and left to dry at 105°C. Vertical dashed line indicate adenine depletion during cultivation.

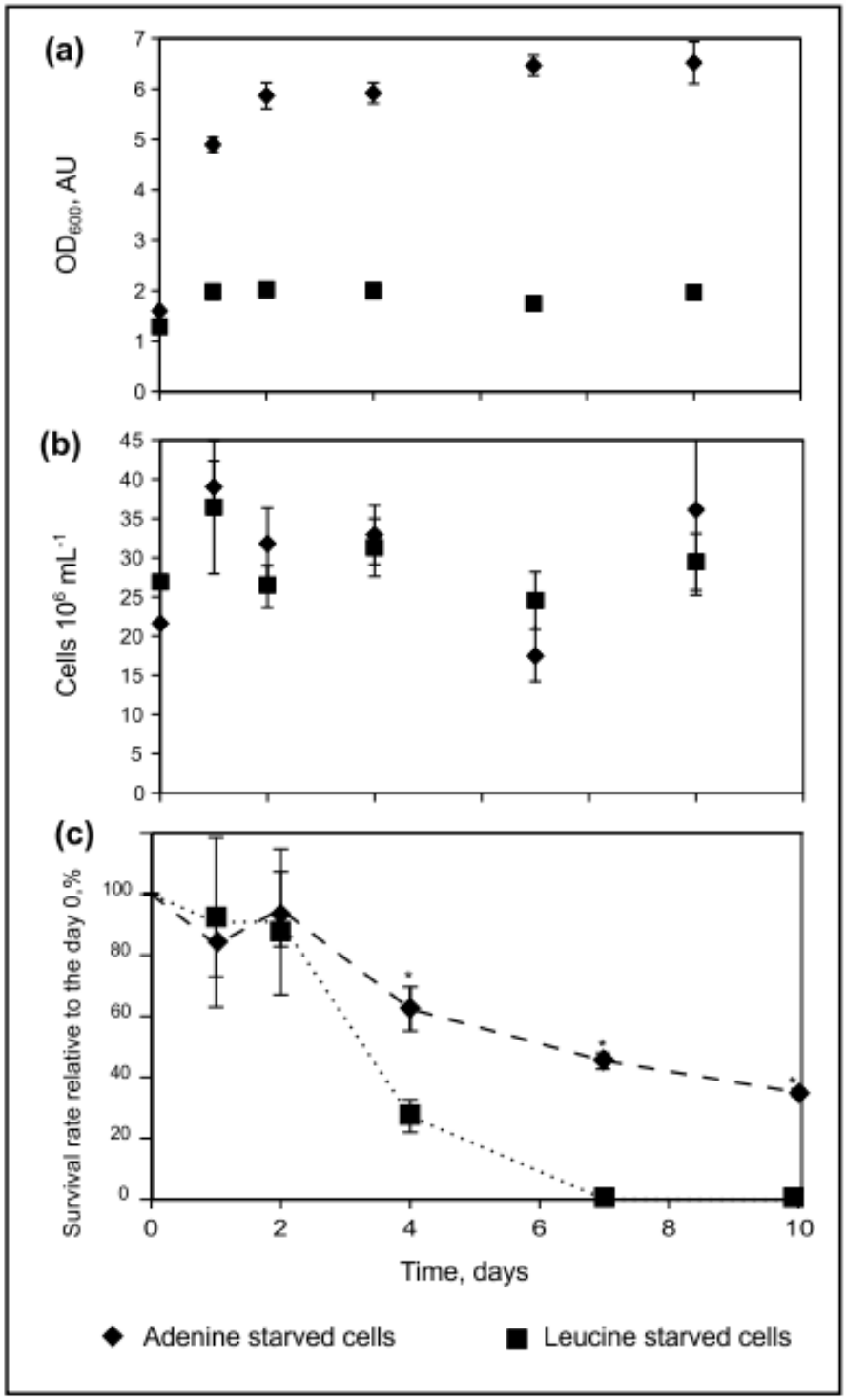


Fig. S2 Optical density (a). Cell count mL-1 (b) and relative survival rate (c) during W303 *ade2* adenine (diamonds) and leucine (squares) starvation. Cells were grown in full SD media up to exponential phase, washed with distilled water and re-suspended to OD600 = 1 in SD media lacking leucine or adenine; all other broth components were added in surplus.
Viability here is shown relative to cell number per millilitre at the beginning of experiment (day 0).

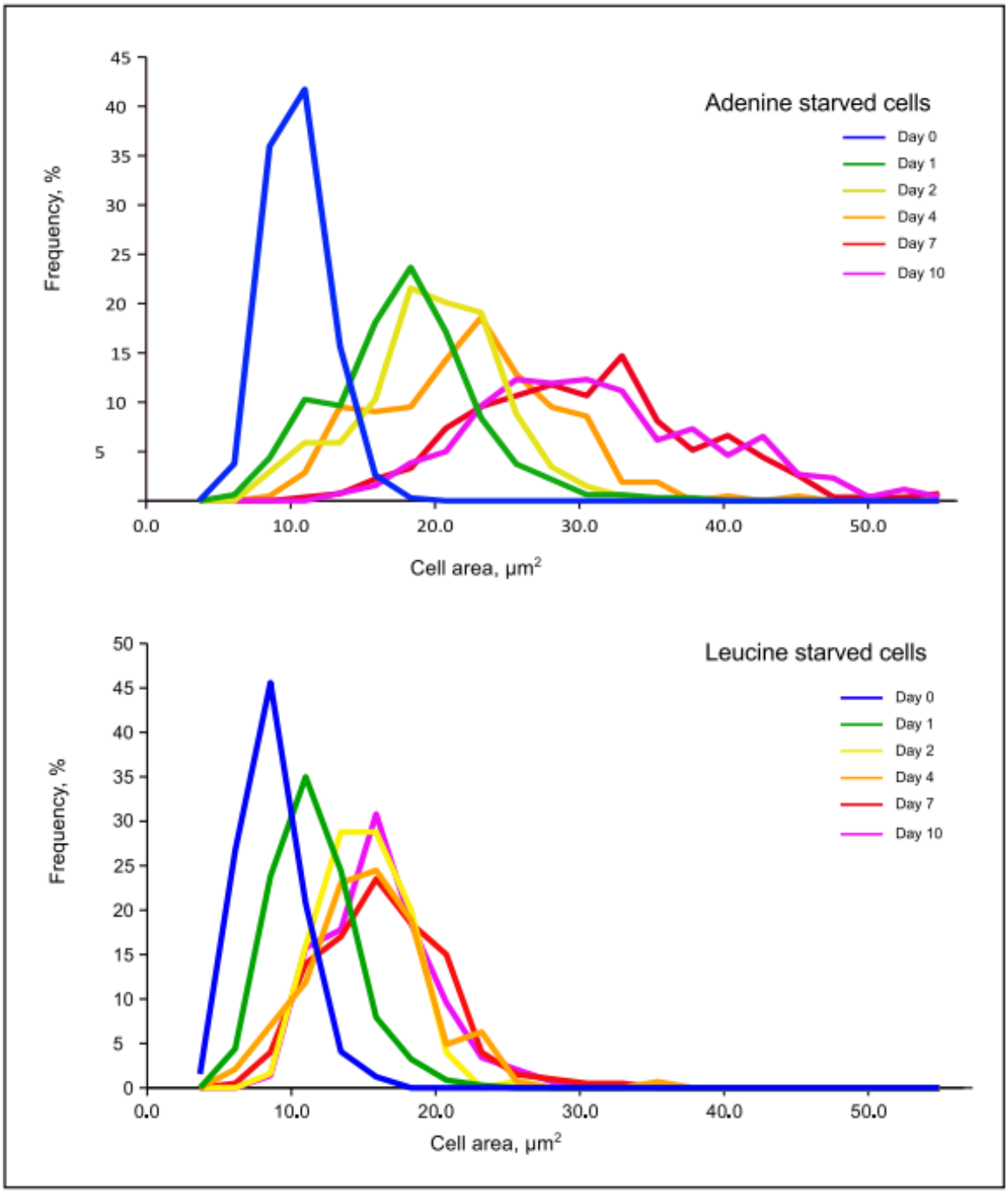


Fig. S3. Cell size distribution during adenine (upper panel) and leucine starvation (lower panel).

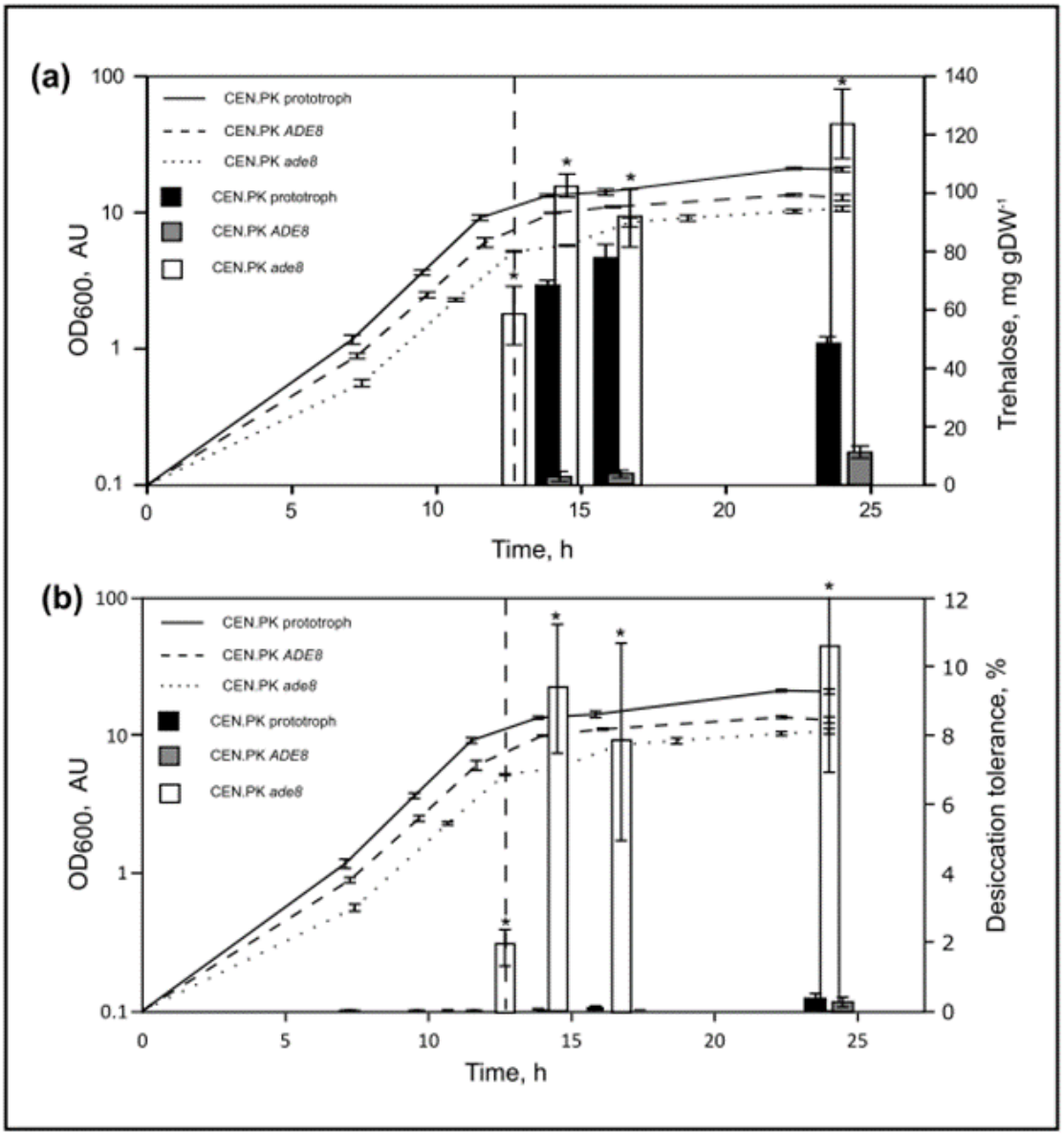


Figure S3 Changes of accumulated trehalose (a) and desiccation tolerance (b) during CEN.PK prototroph, CEN.PK ADE8 and CEN.PK ade8 cultivation in YPD media
Cells were desiccated in +30°C for 10 hours. Desiccation tolerance was quantified as in Calahan et al. (2011).
Error bars depict standard deviation from 3 independent cultivations.
In both panels lines denotes strain growth curves in logarithmic scale and bars trehalose (a) or survival (b). Error bars depict standart deviation from biological triplicates
Viability in first two time points (8 and 12 h) is close to zero (see panel b).

## 3.2. Purine Auxotrophic Starvation Evokes Phenotype Similar to Stationary Phase Cells in Budding Yeast

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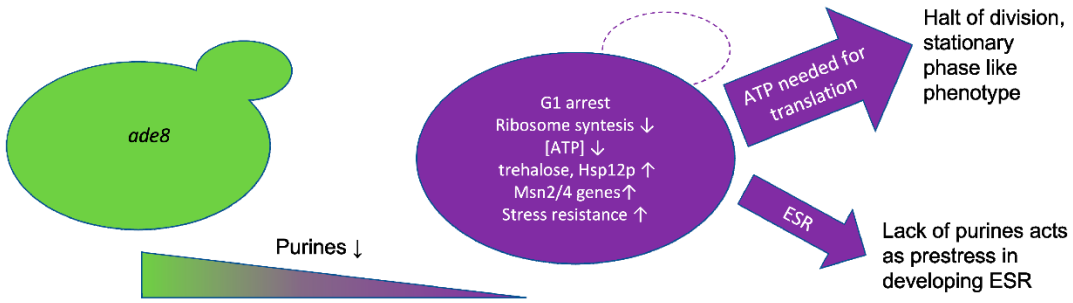
Key points:

- Purine starved yeast cells arrest their cell cycle at G1/G0 phase in the first two hours of purine starvation.
- During the first four hours of purine starvation cells accumulate reserve carbohydrates and reduce glucose flow, reorienting more towards production of glycerol and acetate.
- Transcription analysis shows that 4h purine starved cells downregulate transcription and translation processes.
- Purine starved cells acquire stress resistance that depends on translation and is higher than rapamycin elicited resistance.
- Pattern of gene regulation of purine starved cells highly overlaps with cells entering stationary phase.
- Transcription analysis indicates involvement of environmental stress response programm, that is signalled through Msn2p and Msn4p.

Graphical abstract



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# Purine Auxotrophic Starvation Evokes Phenotype Similar to Stationary Phase Cells in Budding Yeast

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**Abstract:** Purine auxotrophy is an abundant trait among eukaryotic parasites and a typical marker for many budding yeast strains. Supplementation with an additional purine source (such as adenine) is necessary to cultivate these strains. If not supplied in adequate amounts, purine starvation sets in. We explored purine starvation effects in a model organism, a budding yeast *Saccharomyces cerevisiae ade8* knockout, at the level of cellular morphology, central carbon metabolism, and global transcriptome. We observed that purine-starved cells stopped their cycle in G1/G0 state and accumulated trehalose, and the intracellular concentration of AXP decreased, but adenylate charge remained stable. Cells became tolerant to severe environmental stresses. Intracellular RNA concentration decreased, and massive downregulation of ribosomal biosynthesis genes occurred. We proved that the expression of new proteins during purine starvation is critical for cells to attain stress tolerance phenotype Msn2/4p targets are upregulated in purine-starved cells when compared to cells cultivated in purine-rich media. The overall transcriptomic response to purine starvation resembles that of stationary phase cells. Our results demonstrate that the induction of a strong stress resistance phenotype in budding yeast can be caused not only by natural starvation, but also starvation for metabolic intermediates, such as purines.

**Keywords:** *Saccharomyces cerevisiae*; starvation; purines; stress resistance



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## 1. Introduction

Budding yeast *Saccharomyces cerevisiae* is a unicellular fungus that has evolved for fast growth in the presence of abundant nutrients [1]. Deprivation of certain nutrients could be interpreted as a prelude to potentially more serious stressors to come. A lack of nutrients drives cells to enter a stationary phase, in which they do not proliferate, but become more stress resistant. In the case of carbon, nitrogen, or phosphorus depletion (so-called natural starvation, as yeast cells can experience these in the wild), yeast cells stop the cell cycle and activate specific gene expression patterns, thus forming a general stress-resistance phenotype [2–4]. In large genome-scale phenotyping screens, many mutations have been identified that increase budding yeast fitness when they are starved of carbon or nitrogen [5].

When setting up cultivation experiments, it is often important to reach an appropriate growth rate and biomass yield and avoid unexpected phenotypic side effects. Therefore, proper concentrations of all nutrients in the media should be ensured [6]. Alternatively, the sudden depletion of nutrients could initiate nutrient-specific starvation and thus induce a number of phenotypic effects in yeast cells [7–10]. Due to introduced auxotrophic markers in laboratory strains, specific "synthetic starvation" could set in when a specific auxotrophic

nutrient is not supplied or is exhausted. Gomes and colleagues show that limiting essential auxotrophic amino acids decreases the final biomass yield and stress resistance of yeast [11]. In the case of uracil or leucine starvation, cells fail to enter the stationary phase and mostly die in the exponential growth phase [3,9]. On the other hand, methionine starvation has shown signs similar to the natural starvation response [8,12], which is explained by methionine being a source of sulfur. This shows that not all auxotrophies are the same, and starvation for these nutrients can have dramatically different effects on yeast metabolism. In addition, the genetic background of the strain can affect the phenotypic response to environmental changes [5,11,13–15]. Therefore, to continue exploiting model yeast strains in fundamental or applied research, detailed knowledge on their physiology in every possible environmental or laboratory setting is invaluable.

Although adenine auxotrophic strains are widely used, their phenotypic response to purine starvation has not been studied in detail. Purine is a ubiquitous molecule in the cell and forms DNA, RNA (adenine and guanine nucleotides), and cofactors (NAD, FAD). Purine synthesis is highly conserved among eukaryotes. In budding yeast, it consists of a linear chain of 10 sequential reactions coded by *ADE1/2/4/5,7/6/8/12/16,17* genes. This pathway produces inosine monophosphate (IMP), which is a branching point to adenine and guanine. *ADE8* codes for phosphoribosylformylglycinamidine synthase, which is the third enzyme in the chain. Currently, no specific regulation activity of *ADE8* on IMP production is known [16]. If *ade8* mutant is placed in adenine-deficient media, it cannot produce IMP, thus neither adenine nor guanine is produced, and purine auxotrophic starvation sets in.

Until now, we have demonstrated some purine starvation effects on *ade2* strain in the W303 strain background. In the case of purine starvation, W303 *ade2* became desiccation tolerant, the budding index decreased, and trehalose content increased [17].

In this study we examined the global effects caused by purine starvation in *ade8* knockout in CEN.PK2-1D strain. We analyzed various aspects of the cell phenotype: cell growth, cell cycle state, changes in central carbon metabolism, cell ATP content, sublethal stress resistance, and genome-wide transcriptomic response. Our results imply that purine auxotrophic starvation initiates the formation of a stress-resistance phenotype and reroutes the energetic metabolism toward fermentative growth. The transcription pattern of purine-starved cells resembles that of stationary phase cells starving for carbon.

## 2. Materials and Methods

### *2.1. Strains and Cultivation Conditions*

Wild-type strain CEN.PK2-1D *MATalpha his3Δ1*; *leu2-3_112*; *ura3-52*; *trp1-289*; *MAL2-8c*; *SUC2* was a gift from Peter Richard, VTT Biotechnology, Finland. The *ade8* CEN.PK2-1D *ade8Δ0*. *ade8* knockout was induced by the *ura3-URA3* 5-FOA toxicity knockout technique, using *ade8* knockout construct plasmid from [18]. Cultures were maintained on YPD agar and kept at 4 °C. Fresh YPD agar plates were regularly reinoculated from stock cultures kept at −80 °C.

Strains were cultivated in Synthetic Defined (SD) media [19] with 80 mg tryptophan, 100 mg uracil, 480 mg leucine, 100 mg histidine, and 100 mg adenine added per liter, as suggested in [6]. To ensure that yeast cultures were in the exponential growth phase, we reinoculated overnight cultures (grown from a single colony) into fresh media, where at least 6 doublings occurred and $OD_{600}$ 0.5–1, corresponding to 1–2·$10^7$ cells $mL^{-1}$, was reached. Cultures in the exponential growth phase (OD 0.5–1) were washed with distilled water twice and resuspended at OD 0.5 in full SD media (SD) or SD media with adenine omitted (SD ade−).

All cultures were incubated on a rotary shaker at 30 °C and 180 rpm. To demonstrate changes in optical density during starvation, 96-well Tecan Infinite M200 multimode reader was used with the following cultivation cycle: orbital (3.5 mm) shaking for 490 s, waiting for 60 s, optical density measurement at 600 nm. Alternatively, culture growth dynamics was measured with a Z2 Cell and Particle Counter (Beckman Coulter, Brea, CA, USA).

### 2.2. NMR Analysis of Extracellular Amino Acids and Purines

Cell-free culture media was mixed with DSS (sodium 4,4-dimethyl-4-silapentane sulfonate) in $D_2O$ to obtain a final DSS internal standard concentration of 1.1 mM and transferred to a 5 mm NMR sample tube. NMR analysis was performed at 25 °C on a 600 MHz Bruker Avance Neo spectrometer equipped with a QCI quadruple resonance cryoprobe. The noesypr1d pulse sequence was used with water suppression during a recycle delay of 10 s. The spectral width was 11.9 ppm, and 128 scans were collected into 32K data points using an acquisition time of 2.3 s. The acquired 1H NMR spectra were zero-filled once, and no apodization functions were applied prior to Fourier transformation. Phase and baseline corrections were applied manually. Spectra were referenced to DSS (at 0.00 ppm). The identification and quantification of sample components were performed using Chenomx NMR Suite professional software (version 5.11; Chenomx Inc., Edmonton, AB, Canada).

### 2.3. Cell Morphology Measurements

Cell samples before and after 4-h cultivation in media with (SD) or without adenine (SD ade−) were fixed in formaldehyde 0.5% and examined with an optical microscope (Olympus BX51, Tokyo, Japan). Microphotographs (1360 × 1024 pixels) were obtained with a digital camera (Olympus DP71, Tokyo, Japan). Cell size and budding index were determined by microphotography analysis in the ImageJ program. Budding index was defined as the proportion between the number of cells with buds and the total cell number. Bud was defined as a cell with a cross-section area less than half the mother cell size. Cell size was determined as the cell cross-section area measured from the microphotographs using ImageJ. Cells were defined as ellipses, with area measured in pixels and recalculated to square micrometers (1 μm = 5.7 pixels). For each sample, at least 500 cells were measured.

### 2.4. Flow Cytometry

Cell DNA content was determined by flow cytometry as described in [20]. Briefly, 0.5 mL of yeast culture was fixed in 10 mL of ice-cold 70% ethanol for at least 15 min and washed once with 50 mM citric acid. RNA was degraded using RNase A (10 μg $mL^{-1}$) in 50 mM citric acid overnight at 37 °C. DNA was stained with 10× SYBR Green (Invitrogen, Waltham, MA, USA) in 50 mM citric acid for 30 min. Cells were analyzed with a FACSAria device (Becton Dickinson, Franklin Lakes, NJ, USA). Cell cycle distribution was analyzed with Cyflogic software.

### 2.5. Fermentation and Metabolite Flux Measurements

Fermentation was done in a Sartorius Q-plus fermentation system with working volume of 0.3 L, gas flow 0.25 L·$min^{-1}$, mixing rate 400 rpm, media pH set to pH 5.5. Biomass concentration was determined as absorbance in 590 nm (WPA Colorimeter Colourwave CO7500, Biochrom, Cambridge, UK). The following coefficient to convert absorbance units to dry weight was used: 1 $OD_{590}$ = 0.278 g·$L^{-1}$. Carbon dioxide evolution was recorded by an exhaust gas analyzer (Infors Gas Analyser, InforsHT, Basel, Switzerland) in parallel with harvesting metabolite samples.

The contents of extracellular glucose, ethanol, acetate, and glycerol were measured simultaneously by an Agilent 1100 HPLC system with a Shodex Asahipak SH1011 column, and they were quantified with a refractive index detector (RI detector RID G1362A). The flow rate of the mobile phase (0.01 N $H_2SO_4$) was 0.6 mL $min^{-1}$ and the sample injection volume was 5 μL. Biomass from fermentations was centrifuged and intracellular nucleotide pools were extracted via cold methanol extraction. ATP, ADP, and AMP were quantified by HPLC-MS-TOF analysis, as described in [21].

### 2.6. FTIR Analysis

For cell macromolecular content analysis, Fourier-transform infrared (FTIR) spectroscopy was used as described in [22]. For this analysis, 2 mL of cells ($OD_{600}$1–4) was

harvested by centrifugation and washed 3 times with distilled water. Cell pellets were diluted with 50 µL of distilled water, and samples were spotted on 96-well spot-plates. Absorbance data were recorded by a Vertex 70 device with HTS-XT microplate extender, interval 4000–600 $cm^{-1}$, resolution 4 $cm^{-1}$. For data collection and control, OPUS/LAB 6.5 software was used.

### 2.7. Cell Carbohydrate Extraction and Quantification

Fractional cell polysaccharide purification for quantitative assays was done as described in [23]. Total carbohydrate content of each fraction was determined by anthrone assay, and results were expressed as glucose equivalent mg·$gDW^{-1}$ biomass [24].

### 2.8. Transcriptomics

Total yeast RNA after 4-h cultivation in synthetic dextrose (SD) or SD media with adenine omitted (SD ade−) was isolated with a RiboPure™ RNA Purification Kit for yeast (Thermo Scientific, Waltham, MA, USA). RNA samples for each condition were harvested in triplicate. Cell pellets from 50 mL suspensions were frozen in liquid $N_2$ and stored at −80 °C. RNA samples were prepared using 3′ mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria) according to the manufacturer's protocol. Yeast transcriptome was analyzed using MiSeq (Illumina, San Diego, CA, USA) NGS data analysis. Sequencing reads were quality filtered (Q = 30), Illumina adapters and poly-A tails were removed, and reads at least 100 nt in length were selected for further processing using cutadapt (see File S4 for details). S288c reference genome from yeastgenome.org was used to identify gene transcripts.

Genes with lower than 1 count per million (CPM) in fewer than 2 samples were filtered out. The Benjamini and Hochberg method was used to calculate multiple comparison adjusted *p*-value as false discovery rate (FDR). FDR < 0.001 with logFC > 2 was set as a threshold for significance. Expression data set were submitted to the European Nucleotide Archive (ENA) database, under accession no. PRJEB40525.

### 2.9. Sublethal Stresses

Cells were grown in SD media until the exponential phase, washed with distilled water twice, and inoculated in SD or SD ade− with cell density of $1 \times 10^7$ cells·$mL^{-1}$. After 4-h incubation, cells were harvested by centrifugation, washed with distilled water once, and aliquoted in 1 mL, with $OD_{600} = 1$ (corresponding to $2 \times 10^7$ cells·$mL^{-1}$). Three aliquots were exposed to each stress. For thermal stress, cells were kept at 53 °C for 10 min. For oxidative stress, cells were incubated in 10 mM $H_2O_2$ for 50 min, then washed with distilled water. For desiccation, cells were sedimented by centrifugation, the supernatant was removed, and the pellet was air-dried in the desiccator at 30 °C for 6 h. After drying, distilled water was added to resuspend cells. After all stress treatments, cells were serially diluted, and dilutions were spotted on YPD plates to assess CFU·$mL^{-1}$. To check for cell loss during washing steps, the OD of the suspension was measured and CFU·$mL^{-1}$ corrected for OD value. Survival is expressed as % assuming that $OD_{600} = 1$ corresponds to $2{\cdot}10^7$ cells·$mL^{-1}$. To test weak acid stress resistance, cells were spotted on YPD plates supplemented with 0.1 M acetic acid, with pH of agar media set to 4.5 [25].

## 3. Results

To characterize global changes initiated by purine starvation, we constructed *ade8* knockout in laboratory yeast strain CEN.PK2-1D background (*ade8* strain). To investigate the physiological effects imposed by purine starvation in this background, we cultivated *ade8* strain in SD media with all necessary auxotrophic supplements present in surplus media (SD) and media with adenine omitted (SD ade−).

### 3.1. Growth of CEN.PK2-1D ade8 Ceases in the Absence of Purine

CEN.PK2-1D strain, which is often used in laboratory experiments, contains several auxotrophic markers: histidine (*his3*), leucine (*leu2*), tryptophan (*trp1*), and uracil (*ura3-52*). We introduced additional purine auxotrophy by "clean" (antibiotic marker-free) *ade8* knock-out, as suggested by [18] The lack of any single supplement necessary to complement the metabolic needs of this strain leads to growth cessation (Figure 1a). When exponentially growing CEN.PK2-1D *ade8* cells were washed with distilled water and inoculated in SD ade− media, increased cell numbers were observed in the first two hours, but then cell numbers ($mL^{-1}$) remained stable (Figure 1b). For most of our experiments, we chose cells that had been starved in SD ade− for 4 h, as that is the time when ade− specific phenotype appears. To ensure that missing purine is the only factor influencing cell phenotype, we made sure that all other auxotrophic agents were still in media after 4 h of cultivation in SD or purine starvation media (Figure 1c).

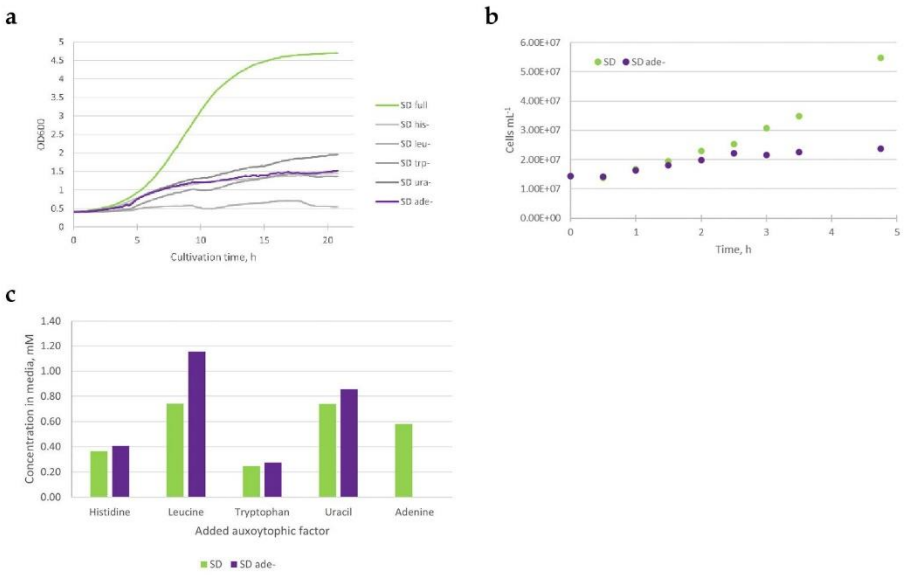


**Figure 1.** (**a**) Growth of *ade8* (CEN.PK2-1D *MAT alpha his3Δ1; leu2-3_112; ura3-52; trp1-289; MAL2-8c; SUC2 ade8Δ0*) strain in SD media with all auxotrophic factors added in surplus or one auxotrophic factor omitted to starve cells for that particular nutrient. Slight increase in optical density can be observed in most starvation conditions. (**b**) Cell number per mL during first 4 h of growth in SD media or adenine starvation. It can be seen that cells stop increasing in number after 2 h of purine starvation. (**c**) Concentration of auxotrophic factors in growth media after 4 h of *ade8* cultivation. In purine starvation media, all other auxotrophic factors except adenine are still in surplus.

Therefore, we conclude that our media composition can induce starvation specifically for adenine (purine), and the subsequent physiological effects observed are solely due to the lack of an external purine supply.

### 3.2. Cell Cycle Arrests in G1/0 during Purine Starvation

Although cell number·$mL^{-1}$ did not increase after the second hour of *ade8* cultivation in purine starvation media, elevated optical density over time was observed (Figure 1a,b). This led us to hypothesize that specific changes in cell morphology occur, increasing light dissipation and accounting for increase OD during *ade8* purine starvation. We quantified the budding index, analyzed the DNA content of the cell using FACS, and measured cross-sections of cells of the *ade8* grown in SD and SD ade− media.

The budding index is an indicator of culture progression through the cell cycle. We defined the budding index as the ratio (%) of the number of cells with buds and the total number of cells (see Figure 2a).

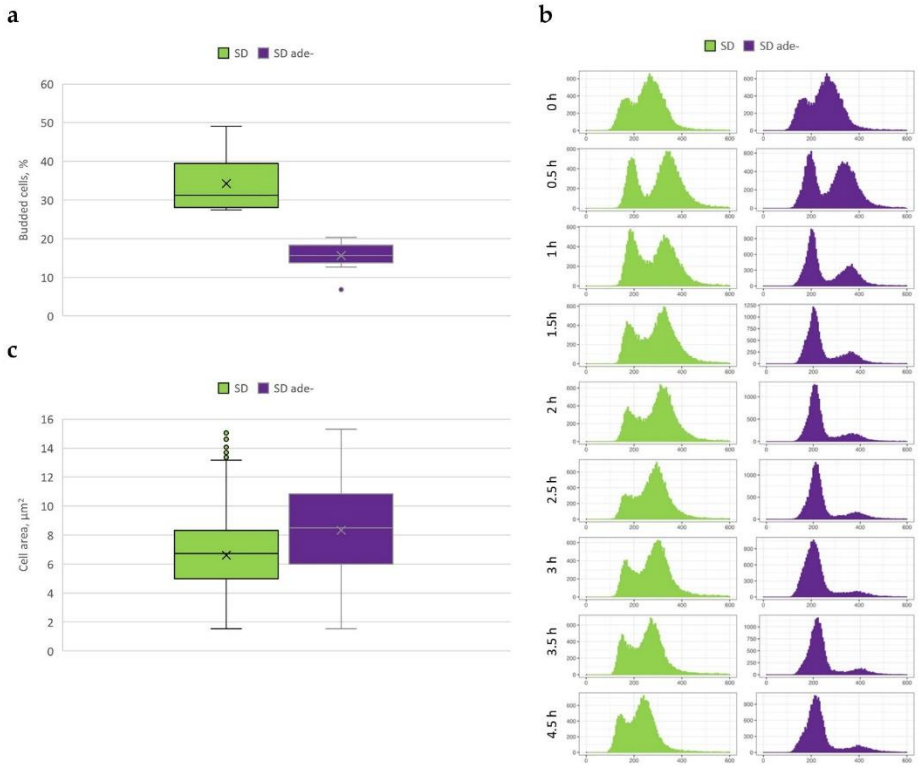


**Figure 2.** *ade8* cell morphology changes when grown in full or adenine-deficient media. (**a**) Cell budding index. Data from 200 cells analyzed from microscopic images. (**b**) *ade8* strain cell DNA copy dynamics over time in SD and SD ade− media. (**c**) Cell size analysis as determined by area of cross-section in microscopic images. Data from at least 500 cells from each cultivation.

We observed that the budding index was approximately 30% when cells were cultivated in SD media, while in purine starvation media it significantly decreased (down to 15%). To complement budding index data and test DNA content in the cells of a population grown in SD or SD ade− media, we performed FACS analysis. We found that the purine starvation culture became enriched with cells harboring N copies of DNA per cell, which occurred within the first 2 h of cultivation. After 4 h, the *ade8* cell population, when cultivated in SD media, contained both N and 2N DNA copies per cell, while cells cultivated in SD ade− contained mostly N copies of DNA (comparison shown in Figure 2b).

We tested whether cell size changes could contribute to OD increase during purine starvation. We measured the average cell cross-section after 4 h of cultivation in SD and SD ade− media. Indeed, purine-starved *ade8* cells were larger than cells growing in SD media, as shown in Figure 2c. Therefore, we conclude that purine starvation leads to a drop in the budding index accompanied by increased cell population of cells with N copies of DNA and significantly increased size (as demonstrated by increased cross-section). These morphological markers demonstrate that purine-starved cells are morphologically different from cells growing in SD media.

### 3.3. Purine Starvation Slows Glycolysis

Changes in culture growth parameters (optical density or cell concentration) are probably the most obvious phenotypic markers of auxotrophic starvation. To further investigate purine starvation effects, we measured several metabolic markers when *ade8* was cultivated in SD or SD ade− media. We measured glucose consumption and production of major carbon metabolites (ethanol, $CO_2$, glycerol, acetate, and biomass) and determined intracellular ATP, ADP, and AMP concentrations.

The specific growth rate of *ade8* in SD media was 0.4 $h^{-1}$ and in SD ade− media was 0.15 $h^{-1}$. The glucose-specific uptake rate q during purine starvation was two times smaller than in SD media: 43 +/−3.6 and 81+/−5.4 mCMol $\cdot gDW^{-1} \cdot h^{-1}$, respectively. Meanwhile, the specific $CO_2$ production rate was five to six times higher in SD media than in SD ade− media (see Figure 3b). Since glucose was the sole carbon source and we could account for more than 90% carbon in total, we calculated flux distribution as carbon % of glucose consumed for *ade8* cultivated in SD or SD ade− (see Figure 3a and raw flux data in File S1). Part of the carbon was rerouted away from biomass growth, and glycerol and acetate accumulated instead. Additionally, amount of $CO_2$ released from the purine-starved cells was equimolar to the ethanol produced. This, in turn, means that other pathways where $CO_2$ is produced (pentose phosphate pathway, mitochondria TCA) might be suppressed in purine-starved cells.

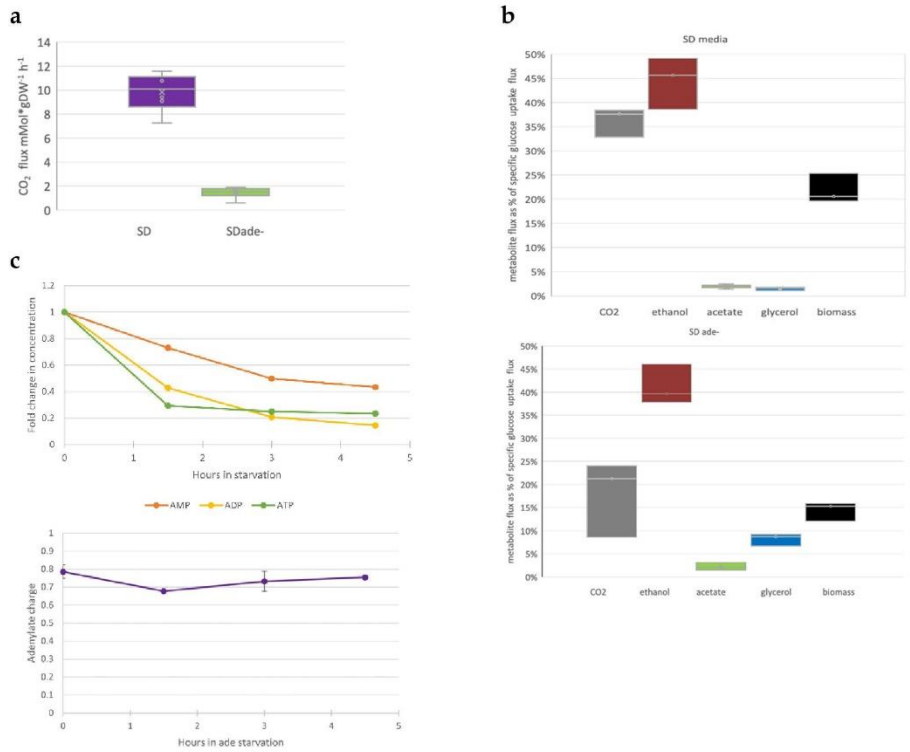


**Figure 3.** (**a**) Specific $CO_2$ production in *ade8* strain cultivation in SD or SD ade− media. (**b**) Carbon flux distribution in *ade8* strain cultivation in full SD media or with adenine omitted. Average $CO_2$ flux was measured as produced (mM $\cdot gDW^{-1} \cdot h^{-1}$) from SD and SD ade− media. All cultivations were performed in batch mode, in 400 mL Sartorius Qplus fermentation system. Starting volume was 300 mL. $CO_2$ was measured by infrared sensor (GasAnalyser, InforsHT). Box plot depicts standard deviations, error bars indicate min and max values from three independent bioreactors. (**c**) Top panel, changes of AXP amount in ade− starved *ade8* cells. Bottom panel, adenylate charge during purine starvation.

When cell growth is suspended due to the lack of an essential metabolite (purine), not only are main carbon fluxes affected (Figure 3a,b), but so are concentrations of intracellular purine nucleotides. We measured the concentrations of purine-containing moieties (ATP, ADP, and AMP) to find out if the intracellular concentration of these molecules changed if the external supply of precursor adenine was diminished (see Figure 3c).

Indeed, already 1.5 h after shifting the SD media to SD ade−, the intracellular concentrations of ADP and ATP dropped more than half of the initial values (Figure 3c). Interestingly, while intracellular concentrations of ATP, ADP, and AMP dropped significantly during purine starvation, energy charge throughout purine starvation remained almost constant. There was a slight drop in the beginning of starvation, but adenylate charge reached pre-starvation levels in the cells after that. It should be kept in mind that in the first two hours of purine starvation, cells were still proliferating (see Figure 1b).

Although *ade8* growth in SD ade− media stopped, cells continued to metabolize glucose. However, specific glucose uptake dropped significantly, from 81+/−5 to 43 +/−3 mCmol ·g DW·$h^{-1}$. We think this is related to the decreased intracellular adenine nucleotide concentration (Figure 3c), which does not allow rapid glucose metabolism [26]. The distribution of other carbon fluxes was also altered. In SD media, most of the energetic needs seem to be fulfilled with the help of fermentation; still, there is also $CO_2$ production that does not come from ethanol production, which points to the involvement of mitochondrial activity and respiro-fermentative growth. In SD ade− conditions, all $CO_2$ can be attributed to ethanol production. Interestingly, glycerol production significantly increased.

It seems that instead of biomass, a significant amount of carbon is redirected to glycerol synthesis, which points to an increase of cell glycerol and/or lipid content, as glycerol is the backbone of triacylglycerols (TAGs).

We checked whether the macromolecule content of the biomass was affected when cells were cultivated in SD media or purine starved. Relative amounts of proteins and nucleic acids decreased, while carbohydrates and lipids increased (see Figure 4a).

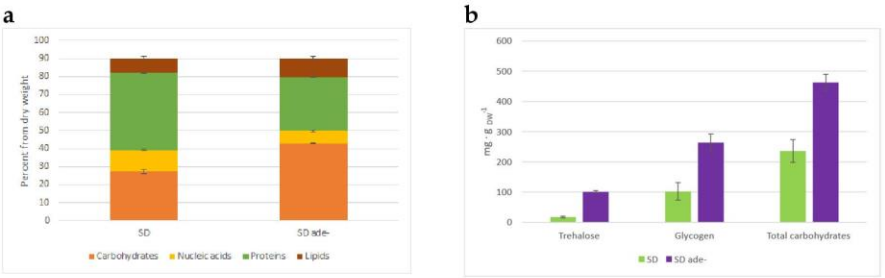


**Figure 4.** (**a**) Distribution of macromolecules in cell biomass as assessed by FTIR. Data are averages of two biological replicates; error bars show standard deviation among technical replicates. (**b**) Amount of carbohydrates in biomass assessed by anthrone method. Data shown are averages from three biological replicates. Error bars indicate standard deviation of biological replicates.

We extracted fractions of the main budding yeast reserve carbohydrates, trehalose, and glycogen, and quantified the carbohydrate content of each fraction by the anthrone method. The results show that during 4 h of purine starvation, *ade8* cells accumulated 100 mg trehalose and 263 mg glycogen per g DW, while cells growing in SD media had 17 mg and 102 mg, respectively. We also measured the concentrations of other cellular carbohydrates that make up most of the yeast biomass, mannans, and beta-glucans. Increased carbohydrate fraction during purine starvation is due to the accumulation of reserve carbohydrates, while the amount of structural carbohydrates does not change (Figure 4b). Purine-starved yeast biomass accumulated more reserve carbohydrates by 244 mg·$gDW^{-1}$, and total carbohydrate content increased by 227 mg·$gDW^{-1}$, which correlates to an increased carbohydrate fraction in the biomass macromolecular composition.

The carbohydrate fraction in purine-starved cell biomass almost doubled, as revealed by FTIR analysis (see Figure 4a).

To verify the biomass macromolecular composition data (Figure 4a), we estimated the RNA content of the cells. We assessed RNA quality (Qiaxcell capillary electrophoresis) before expression analysis by comparing the 18S and 28S rRNA ratio. The ratio between 18S and 28S did not change (whether it was an SD or ade− sample), therefore RNA quality was good, and no degradation was observed. At the same time, the amount of extracted RNA per unit of biomass was three times smaller in adenine-starved cells, thus reinforcing the biomass macromolecular content obtained by FTIR: decreased nucleic acid amount during purine starvation.

### 3.4. Purine Starvation Elicits Strong Stress Resilience

Carbon flux distribution away from energy production toward storage metabolites increased glycerol production, decreased glucose uptake, and decreased intracellular adenine nucleotide content. This indicates that energy and carbon in purine starvation might be redirected to other functions, away from biomass synthesis and growth.

Stress resistance is an important phenotypic feature of microbial cells. It is known that the general environmental stress resistance (ESR) phenotype is induced in slowly growing, stationary, or quiescent cells (reviewed in [27]). Previously, it was shown that methionine auxotrophic starvation can lead to elevated stress resistance [8]. To test if cultivation in purine starvation media would affect culture stress resistance, we tested cell viability after exposure to harsh environmental stresses (sublethal stress resistance). We tested whether purine-starved cells would have higher resistance to short thermic or oxidative stress and weak acid stress (growth on plates containing acetic acid at pH 4.5, to see long-term stress resistance). Additionally, we tested desiccation tolerance as an example of a multicomponent stressor. The time of exposure of each stress was adjusted so that the survival of exponentially growing cells would be approximately 10%.

We observed that the survival of purine-starved cells was more than 10-fold higher than that of the cell population growing in SD media in all sublethal stresses tested (see Figure 5a).

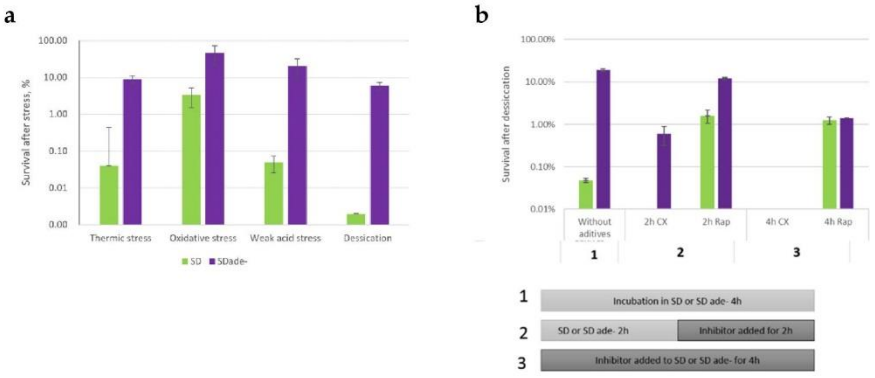


**Figure 5.** *ade8* strain stress resistance after cultivation in SD or SD ade− medium. (**a**) Cell survival after exposure to sublethal stress conditions. *ade8* cells were cultivated in SD and SD ade− for 4 h, then exposed to heat shock (53 °C, 10 min), oxidative shock (10 mM $H_2O_2$, 50 min), acetic acid stress (plated on YPD with 0.1 M acetic acid, pH 4.5), or desiccation (30 °C for 6 h) after stress treatment; cfu·mL$^{-1}$ OD$^{-1}$ was assessed by plating on YPD plates. (**b**) Cell survival after desiccation and addition of cycloheximide (35 µg·mL$^{-1}$) or rapamycin (250 µg·L$^{-1}$) during starvation (top panel); setup of experiment (bottom panel). Cells were treated with cycloheximide or rapamycin at various time points during adenine starvation and then desiccated. CFU plating was done as in other stress assays.

We wanted to understand whether desiccation tolerance is a phenotype that evolves immediately after the shift to starvation media, or if some "adaptive reactions" occur while cells are starving for purine. To test this development of the stress resistance phenotype, we used a desiccation assay, since this treatment gave the most distinct signal for cells cultivated in SD ade− and SD media. To determine that, we added cycloheximide translation inhibitor to cells either at the beginning of starvation or 2 h after, when active cell proliferation had ceased. The results are depicted in Figure 5b. Translation inhibition during auxotrophic starvation lowered desiccation tolerance after starvation. Therefore, we conclude that the stress resistant phenotype indeed develops during purine starvation via new protein production, and signaling of the lack of purine also starts while cells are performing their final division.

Our results demonstrate that purine starvation preconditions cells to become stress resistant. However, it is not known how cells coordinate the lack of purine with these massive phenotypical changes. Other authors have shown that desiccation tolerance can be TOR pathway dependent [28]. This fact, together with the decreased RNA content, led us to inquire whether the TOR signaling system mediates purine starvation toward the development of a specific phenotype. We used desiccation tolerance as a marker of the purine starvation phenotype and compared the desiccation tolerance of purine-starved cells and cells grown in SD media, adding rapamycin during incubation, in an experimental setup similar to the cycloheximide assay. Rapamycin inhibits downstream signaling from target of rapamycin (TOR) proteins. The addition of rapamycin did affect desiccation tolerance, but to a lesser extent than purine starvation (see Figure 5b). For purine-starved cells, 19% of cells were desiccation tolerant if no rapamycin was added, compared to 12% after 2 h of rapamycin treatment and 1% after 4 h of rapamycin treatment. The addition of rapamycin to SD grown cells increased their desiccation tolerance, but not to the degree of purine-starved cells: 0.05% with no rapamycin, 1% with 2 h rapamycin, and 1% with 4 h rapamycin (see Figure 5b). Since purine-starved cells exhibit 10–20 times higher desiccation tolerance than rapamycin-treated cells, we conclude that the TOR system might be involved in purine starvation signaling, but there are additional systems in play.

### *3.5. Purine-Starved Cells Exhibit a Distinct Transcriptome Resembling Stationary Phase Cells*

The results from translation inhibition (cycloheximide assay, Figure 5b) show that stress resistance is driven by gene expression. Therefore, to assess gene expression changes over purine starvation, we performed transcriptome analysis via RNAseq.

RNA was extracted from flash-frozen yeast biomass from *ade8* cells that spent 4 h in either SD or adenine-deficient media. The samples were harvested in biological triplicate and all data represent the average of the triplicates. The significance criterion for gene up- or downregulation was chosen as the logarithm of fold change less than −2 (downregulated) or greater than 2 (upregulated). When comparing expression data of *ade8* cells cultivated in SD ade− or SD, we found 455 significantly upregulated genes (more expressed in SD ade− conditions) and 244 downregulated genes (more expressed in SD). The transcription of the rest of the genes was not significantly affected by the presence or absence of purine. Among the top 20 most upregulated transcripts, we found genes coding for stress resistance proteins (*SIP18* and its paralogue *GRE1*, *DDR2*, *HSP12*, *HSP26*), stationary phase response proteins (*SPG4*, *SPG1*) and carbohydrate metabolism (*HXT5*, *HXT6*, *TKL2*, *GND2*). Interestingly, expression of several spore-related genes was also upregulated, for example *SPS100*. For a full gene list, see File S3.

The most prominent downregulation was observed in the expression of various tRNA and protein genes related to the transcription process. This was also demonstrated by a GO term enrichment search for all genes that were affected by purine starvation ($-2 > \log FC > 2$). The results are depicted in Figure 6. Metabolic process enrichment terms were grouped in two clusters corresponding to up- and downregulated genes: one cluster including genes of redox processes, and various catabolic genes, which were upregulated, and the other cluster consisting of genes mainly connected with translation, which were

downregulated (Figure 6a). The metabolic processes that were the most significantly enriched in our dataset were connected with downregulated genes and the translation process. From the upregulated genes, redox processes and carbohydrate metabolism were the GO terms that were most enriched in purine-starved cells. A table with all GO terms and their expression statistics can be found in File S2.

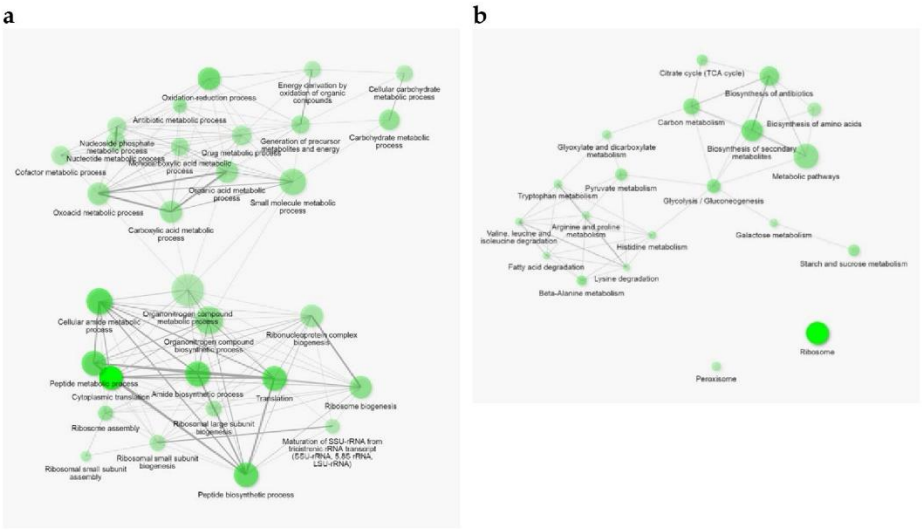


**Figure 6.** Gene pathway enrichment analysis, in which transcripts significantly up- or downregulated ($-2 > \log_2 FC > 2$) when compared to *ade8* cells cultivated in synthetic complete media were selected. For those genes, enrichment analysis was done and plotted using ShinyGO v0.61 [29]. Plot also shows relationships between enriched pathways. Two pathways (nodes) are connected if they share 20% (default) or more genes. Darker nodes are more significantly enriched gene sets, bigger nodes represent larger gene sets, and thicker edges represent more overlapped genes. (**a**) Enrichment in GO metabolic process terms; (**b**) enrichment in KEGG pathway terms.

When analyzing GO terms for enriched genes, we saw several terms connected to metabolism, so we looked into the metabolic genes with regard to which pathways were affected and whether the results coincided with our metabolite analysis. By plotting all gene expression changes on the yeast metabolic pathway (also below $\log_2 FC$ 2/−2) (https://pathway.yeastgenome.org/), we could observe that genes of energetic metabolism were mainly upregulated in glycolysis (*GXK1*, *HXK1*, *TDH1*, *ENO1*, *PYK2*); also, Krebs cycle and glyoxylate cycle genes and their isoforms were more highly expressed compared to fast-growing cells, with the exception of *ACO2* and *MAE1*, which were less expressed. At the same time, it can be seen that genes involved in the use of alternative carbon sources were also upregulated: galactose (*GAL10*, *GAL1*) and xylose (*GRE3*, *XYL2*). In the electron transport chain, we see that genes for NADH dehydrogenase (*NDI1*), all genes for both isoforms of succinate dehydrogenase (*SDH4*, *SDH3*, *SDH2*, *SDH1*, *YJL045W*), and some genes of ubiquinol cytochrome c reductase complex (*QCR9*, *QCR10*) were also upregulated.

It appears that although the ATP charge is not changed, a considerable drop in ATP concentration induces energy production pathways to gain more ATP. This also applies to less commonly used AcetylCoA sources; we can see genes of fatty acid oxidation being upregulated (*POX1*, *FOX2*, *POT1*), along with acetate utilization (*ACS1*) and ethanol degradation (*ADH2*, *ALD2*, *ACS1*). Although all of these genes are expressed in higher amounts, which points to increased flux through energetic metabolism and Krebs cycle, cells do not produce more $CO_2$ or have increased glucose consumption. It looks like purine starvation causes dysregulation of carbon energetic metabolism, where cells are trying to

achieve a higher concentration of ATP, by using all available carbon sources and removing glucose repression during metabolism.

At the same time, we see an accumulation of reserve carbohydrates that is not reflected in gene expression. We do observe an accumulation of trehalose and glycogen, but gene expression levels in the pathways for degradation and synthesis are upregulated for trehalose and glycogen in a similar manner. Some gluconeogenesis genes are also upregulated (*MDH2*, *PCK1*).

Several amino acid related processes are affected. Besides purine auxotrophy, *ade8* strain is an auxotroph for tryptophan, histidine, leucine, and uracil. Indeed, amino acid Trp, Leu, and His synthesis pathways are downregulated due to the adequate supply of respective amino acids from the medium. None of these amino acids get depleted during purine starvation (see Figure 1c). However, we see also downregulation of other amino acid synthesis: Tyr, Phe, Met, Ser, Pro, Asn, Arg, Val, Ile, and Lys pathways, which agrees with our observations on growth cessation and the downregulation of translation processes. At the same time, production of glutamate from -oxoglutarate and glycine from glyoxylate are upregulated, which would explain the observed upregulation of Krebs cycle and glyoxylate cycle enzymes.

Several cofactor metabolism pathways were affected with oxidative part of PPP and 1C metabolism most prominently. In PPP, *SOL4* and *GND2* genes are highly upregulated, and in 1C metabolism most of the genes are downregulated with the exception of glycine cleavage complex, which provides 5,10 methylenetetrahydrofolate.

Our strain was defective in *ade8* gene, which is the third step of purine biosynthesis that starts with PRPP, and these three steps require glutamine, glycine, ATP, and 10-formyltetrahydrofolate as cofactors. We can say that we noted genetic upregulation of processes providing these substances. However, as purines and the cofactors that are produced for synthesis are involved in a variety of cellular processes, metabolic dysregulation happens on several levels.

We compared the transcriptome of *ade8* during purine starvation with that of stationary phase cells (Geodataset GSE111056 [30]). We chose to compare our dataset with the data acquired using the Illumina sequencing platform (similar to ours) with at least n = 2 replicates for each condition from GEO. We used the same data handling procedure as with our expression data (as described in File S4). When comparing the two datasets, we found that similar genes were simultaneously upregulated and downregulated in both conditions (see Figure 7 and a full list of gene expression analyses in File S3). When plotted on a cell map, downregulation of genes associated with translation is most obvious (Figure 7b). On the other hand, upregulated genes do not show such clear clustering. Genes related to peroxisome function seem to be upregulated in both datasets. We performed an analysis of GO term enrichment within genes that were upregulated in purine-starved and stationary phase cells to see if there were some common functions (Figure 7c). GO terms that were enriched in genes that were upregulated in both conditions were similar to the GO terms of purine-starved cells; mainly genes connected to catabolism, reserve carbohydrates, and redox processes were found to be enriched in this dataset.

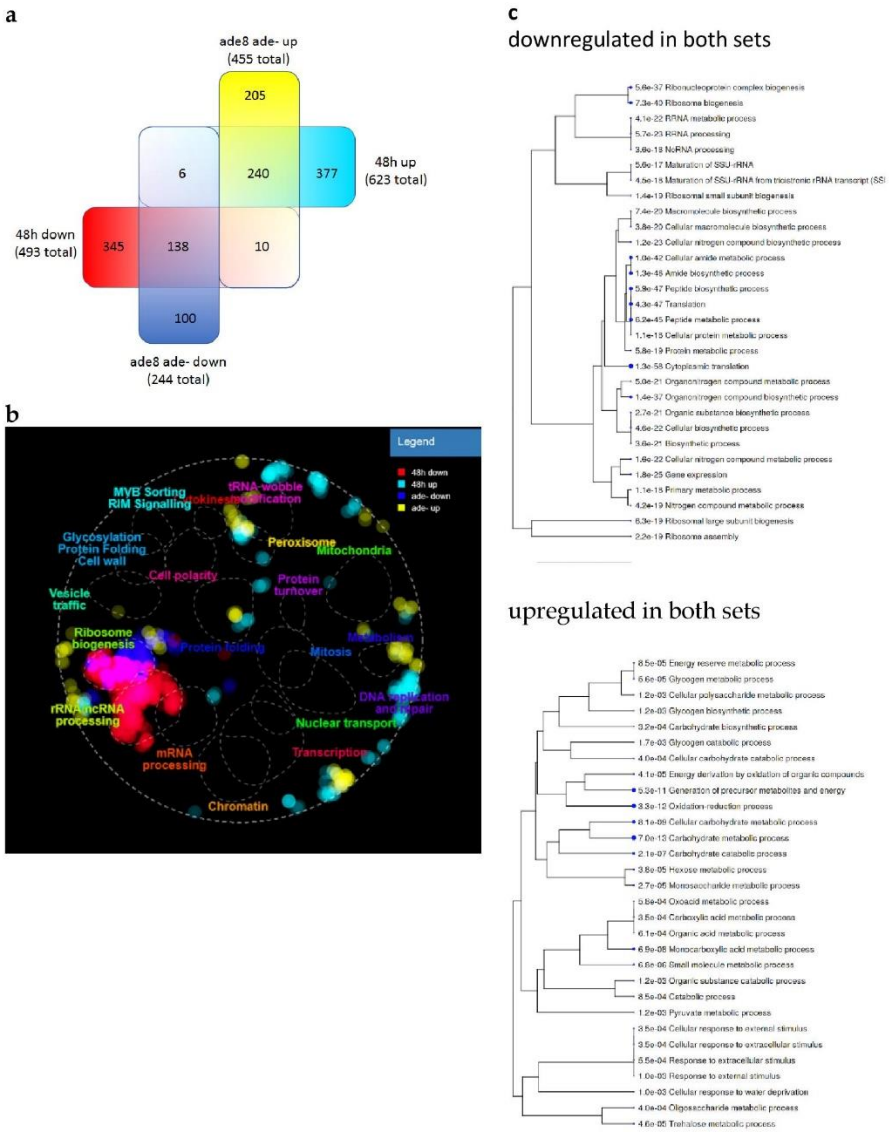


**Figure 7.** Comparison of gene expression of *ade8* strain in SD ade− media after 48 h and JPY10I strain (*MATa/α ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 lys2Δ0/lys2Δ0 ADE2/ade2Δ::hisG HIS3/his3Δ200*) after 48 h growth in full media (Geodata set GSE111056). Gene sets were obtained by comparing gene expression during starvation and stationary phase with expression of respective strain during exponential phase. Significantly ($-2 > \log_2 FC > 2$) up- or downregulated genes were further analyzed. (**a**) Number of common genes that changed their expression when comparing purine-starved and stationary phase cells. (**b**) Same genes plotted with TheCellMap.org. (**c**) GO term enrichment analysis of upregulated genes in purine-starved and stationary phase cells.

## 4. Discussion

Purine auxotrophic starvation induces a phenotype distinctive from exponential cells. Yeast cells halt proliferation, stop cell cycle in G1/0, and accumulate reserve carbohydrates (glycogen and trehalose) to become resilient to multiple stresses. In addition, purine starvation elicited a transcription pattern distinct from exponential cells, sharing many traits with stationary phase cells. Currently, it is not known whether these remarkable phenotypic changes are induced by purine depletion per se, or lack of purine metabolites initiates

effects similar to "natural" starvation (nitrogen or carbon), because purine depletion might be signaled through the same pathways as natural starvation. We will discuss potential scenarios showing how purine depletion can lead to the specific purine starvation phenotype we observed.

### 4.1. Intracellular Adenylate Pool Is Not Sufficient to Sustain Cell Proliferation

Purines are essential metabolites of the cell metabolism. Growing cells metabolize purines (guanine and adenine) to fuel the synthesis of new DNA and RNA nucleotides. Additionally, purines are used as cofactors and energy-carrying substances in myriad catabolic and anabolic reactions. For example, ATP and GTP are necessary for translation to occur; two ATP molecules are invested to activate each glucose molecule within glycolysis, etc.

The specific ATP consumption of 1 g of growing CEN.PK strain cell biomass is approximately 5.7 mM $g^{-1}DW\ h^{-1}$. A significant portion of that (70%, or at least 4.5 mM ATP $g^{-1}DW^{-1}\ h^{-1}$) is devoted to protein synthesis and turnover [31]. Approximately 0.63 mM ATP $g^{-1}DW^{-1}$ h is used for maintenance functions during aerobic cultivation [32]. Thus, it is possible to keep cells alive in the nonproliferative state with a handful of ATP, as we have seen in purine-starved cells.

Purine concentration within the cytoplasm of budding yeast is about 7 mM, most of which is ATP (5 mM) and GTP (1.5 mM) [33]. Meanwhile, the purine content of the haploid genome of budding yeast is $4.65 \times 10^6$ of guanine and $7.45 \times 10^6$ adenine nucleotides, which is approximately 1/10 of the molar amount of purines in the cytoplasm. Exponentially growing yeast cells contain approximately 50 times more RNA than DNA [34]. Therefore, when the external supply of purine is stopped, cytoplasmic purine resources alone cannot ensure the needs of new daughter cells in purine auxotrophic cells. However, purine auxotrophs tend to accumulate purine moieties in the form of inosine or hypoxanthine [17,35] within vacuoles of their cells. The existence of purine reserves accumulated within the cells of purine auxotrophs can explain the increased cell number during the first hours of cultivation in purine-deficient medium (Figure 1b). We observed that cells had a lower amount of rRNA after 4 h of starvation. After the beginning of purine starvation, most yeast cells finished the DNA synthesis phase and halted budding; putative arrest in G1 was observed (see Figures 1b and 2b). Most of a cell's RNA is ribosomal RNA (rRNA), therefore, by degrading rRNA it would be possible to free nucleotides required for DNA synthesis. Analyzing the expression data, we see that the expression of ribonucleotide reductase *RNR2* ($\log_2$FC = 2.04) and RNR4 ($\log_2$FC = 1.64) was upregulated after 4 h in ade− media. The observed replication cessation indicates that auxotrophic cell growth strongly depends on an external purine supply. If purine supply stops, then the internal purine reserves of auxotrophic cells cannot sustain further proliferation and cell doubling ceases; internal reserves may sustain one doubling, but no more.

### 4.2. Purine Starvation Induces Accumulation of Metabolites Capable of Increasing Stress Tolerance

If mild stress is applied, cells adapt to it and become ready for stronger challenges in the future. For example, NaCl pre-treatment increases yeast cell tolerance to $H_2O_2$ stress [36]. Additionally, it was demonstrated that methionine starvation might also prepare cells to be resilient to peroxide stress [8]. Previously, we showed that desiccation tolerance of W303*ade2* cells was indeed significantly higher if cells were starved for purine [17]. Here we explored whether purine starvation "prepares" cells for multiple sublethal stresses (not only desiccation), and, indeed, we found that a strong, resilient phenotype is generated during purine starvation. Moreover, if protein expression is blocked by cycloheximide during purine starvation, the formation of a resilient phenotype is abolished (Figure 5b, cycloheximide treatment). This, in turn, points out that the resilience phenotype is established due to the expression of specific genes or induction of a genome-wide transcriptional program.

When cultivating *ade8* strain in completely synthetic media, the main fermentation products were ethanol, biomass, and $CO_2$, but in the purine-deficient media acetate and glycerol accumulated, while the carbon proportion devoted to biomass decreased (see

Figure 3b). Reserve carbohydrates (trehalose and glycogen) also accumulated in the purine-starved cells (Figure 4b). Interestingly, the accumulation of glycerol and/or trehalose per se is attributed to increased stress tolerance and can help in survival after strong environmental perturbations (desiccation, extreme heat, etc.) [37,38]. Besides small carbon metabolites (e.g., trehalose), other factors can ensure viability after desiccation. Heat shock proteins (Hsp12p, Hsp26) are the next most important factors after trehalose that can ensure tolerance to multiple stresses, including desiccation [39]. Indeed, the expression of *HSP12* during purine starvation was highly upregulated (see File S3). In fact, it was among the top 10 most upregulated transcripts. Therefore, accumulation of trehalose and Hsp12p can explain the high stress tolerance of purine-starved cells.

### *4.3. Msn2/4p Are Master Regulators of Purine-Starved Cell Transcriptome*

Cells use nutrient-dependent intracellular signaling, such as PKA and TOR, to coordinate their metabolism and cell growth with available resources, such as carbon and nitrogen. These signaling cascades converge to several transcription factors, which are then translocated into the nucleus and induce a set of growth or stress response genes. Thus, the specific transcriptional makeup of growing or non-growing cells is set.

The environmental stress response (ESR) is a specific transcriptional program induced by many environmental stresses (heat, oxidation, starvation, etc.). During ESR response in *S. cerevisiae*, upregulated genes for all stresses are genes involved in oxidation proceses and stress signaling. The ESR transcriptional program is mediated via protein kinase A (PKA) and stress responsive Msn2/4p. Genes that are negatively regulated during ESR are connected to the ribosome biogenesis and fermentation. Therefore, when induced, this "transcriptional program" ensures cell survival in multiple environmental stresses [27,40].

As we also see gene expression changes similar to the ESR, we explored whether purine starvation initiates a specific transcriptional pattern. To do that, we analyzed which transcription factors were most probably responsible for the gene transcription pattern induced by purine starvation when compared to exponentially grown cells. When analyzing our expression data with the YEASTRACT tool [41], a set of transcription factors was found most likely to be associated with the upregulated and downregulated genes. Several transcription factors were proposed to be involved in upregulated gene set ($p < 0.05$) Msn2p and Msn4p were transcription factors capable of upregulating 75% and 68%, respectively, of our gene set (see full list with transcription factors in File S2). Moreover, our previous research showed that, indeed, Msn2p and Msn4p are involved in purine starvation elicited desiccation and tolerance to thermal shock. When truncating the DNA binding domain of these proteins, cell desiccation tolerance decreases. The decrease in desiccation tolerance is more significant if Rim15p, an upstream regulator of Msn2p and Msn4p, is truncated [42].

Other proposed transcription factors can regulate smaller portion of our gene set, but still are in the agreement of stress resistance phenotype induction. We found stress response related transcription factors Gis1p, Hsf1p, Crz1p, carbon source influenced Mig1p, Hap4p, Cat8p, Adr1p and cell cycle, meiosis dependent Rlm1p, Gat4p, Rme1p, and Yph1p in the list of proposed transcription factors. Effects that would be induced by transcription factors predicted by YEASTRACT correspond to the observed phenotype cells have halted cell cycle, rerouted carbon fluxes, and became resistant to multiple stresses. This allows us to speculate that purine starvation is perceived within the cell, and a coordinated response is launched.

We also compared our data from SD ade− media with publicly available datasets of stationary phase yeasts. Stationary phase cells are an example of where the ESR transcription program is on [30]. We saw similar patterns of gene expression. In both cases, purine-starved *ade8* and stationary phase cells, there was upregulation of heat shock protein genes (*HSP12*, *HSP26*), oxidative markers (cytoplasmic catalase *CTT1*), and hydrophilins essential to overcoming the desiccation–rehydration process (*SIP18* and its paralogue *GRE1*). Therefore, we can now place purine starvation among other stresses capable of inducing ESR.

### 4.4. Intracellular Signalling of Purine Starvation

Pelletier and colleagues used carcinoma cell lines to see how nucleotide depletion affects the cell cycle [43]. The results showed that when purine levels dropped, ribosome assembly was delayed, as nucleotides are needed for RNA. This caused failure of cell cycle checkpoint and p21 accumulation, arresting cells in G1. Overexpression of the *S. cerevisiae* p21 analogue *CIP1* (Ccr4-Not complex inhibitor) also caused arrest in the G1 phase [44]. Yeast cells respond to other environmental stresses, such as hyperosmotic stress, by activating *CIP1* in an Msn2/4p dependent fashion, thus delaying cell cycle [45]. Although *CIP1* was not significantly overexpressed in purine-depleted cells, this does not rule out the involvement of this inhibitor linking purine depletion to G1 arrest.

Hoxhaj and colleagues explored how HeLa cells react to purine synthesis inhibitors [46]. They noticed similar patterns as we did: intracellular concentration of AMP, ADP, and ATP decreased, but cellular adenylate charge stayed constant. They proposed that purines are sensed with the help of the mTOR signaling system, where TSC complex would be responsible for sensing the lack of adenine nucleotides and inhibiting the mTOR pathway further on. Our expression analysis shows downregulation of translation machinery consistent with the involvement of TOR signaling; at the same time, it is necessary to note that *S. cerevisiae* lacks TSC complex [47]. We compared the effect of TOR inhibition by rapamycin with purine starvation on yeast desiccation tolerance (see Figure 5b). We observed that purine starvation induced increased stress desiccation resistance, higher than rapamycin alone. Moreover, purine starvation with simultaneous rapamycin treatment did not increase desiccation tolerance when compared to starvation alone. Therefore, if the TOR system is involved in purine sensing in *S. cerevisiae*, purine depletion signaling is received by TOR somewhere downstream of the canonical rapamycin-sensitive TOR protein Frp1p [48].

## 5. Conclusions

Auxotrophic starvation induces a stress tolerance phenotype in the case of methionine starvation [8] but leads to a stress susceptible phenotype in uracil or leucine starvation [9]. Our results show that purine starvation enriches G1/G0 cells in the culture and "prepares" yeast cells to become multiple-stress tolerant.

We think these distinct phenotypic changes can be explained by one or a combination of several mechanisms acting during purine starvation: a drop in nucleotide content leads to cessation of the cell cycle, accumulation of general stress resistance metabolites trehalose and small heat shock proteins Hsp12p and Hsp26, and firing of Msn2/4-dependent transcriptional program, similar to ESR. The similarity of phenotypes elicited by purine starvation and stationary phase cells by the involvement of similar signaling pathways (Msn2/4p) leads us to conclude that purine starvation indeed elicits ESR phenotype in yeast cells. Moreover, examples from other purine auxotrophic organisms highlight that the ability to survive purine depletion by inducing a stress resistance phenotype might be a universal trait of eukaryotic cells [17].

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/jof8010029/s1, File S1: q values (in mCmols·Gdw$^{-1}$·h$^{-1}$) from *ade8* cultivation in SD+ and ade− media, File S2: Full datasets of GO terms and transcription factors, File S3: Transcription data, File S4: NGD data analysis methods.

**Author Contributions:** Conceptualization, J.L. and A.K.; methodology, K.P. and K.T; investigation, Z.O., I.V., K.S., K.P., K.T. and A.K.; writing—original draft preparation, J.L. and A.K.; writing—review and editing, Z.O.; visualization, A.K.; Funding acquisition and supervision, J.L. All authors have read and agreed to the published version of the manuscript.

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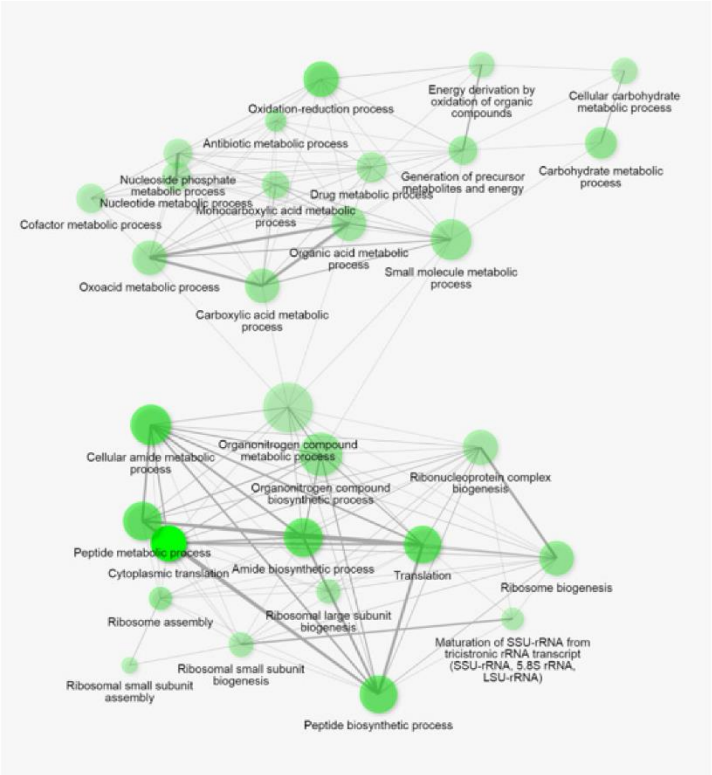
Figure 6 from article in higher resolution

**a**




**b**

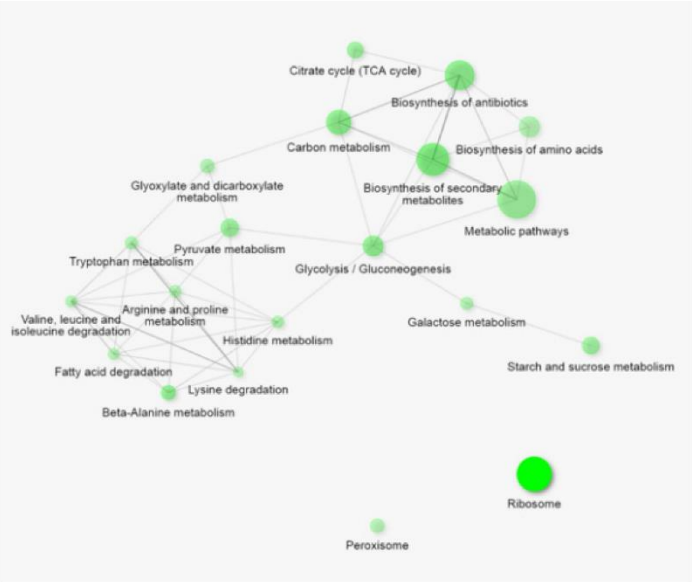
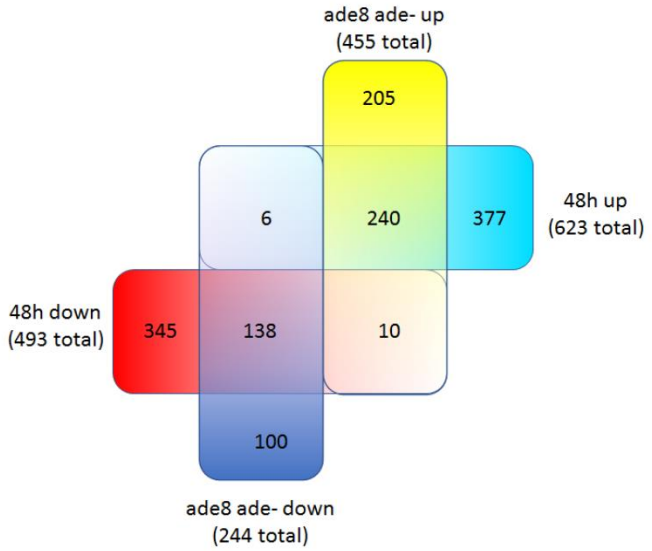


Figure 7 from article in higher resolution

**a**

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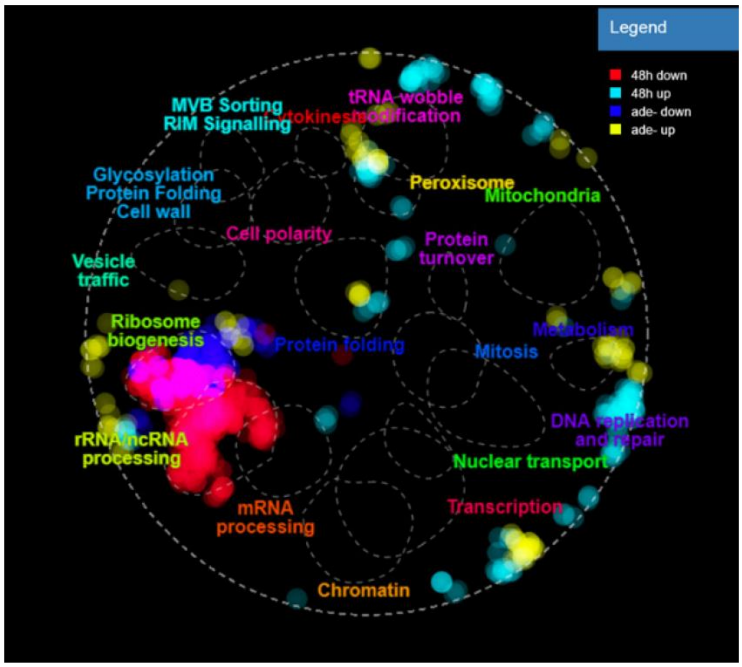
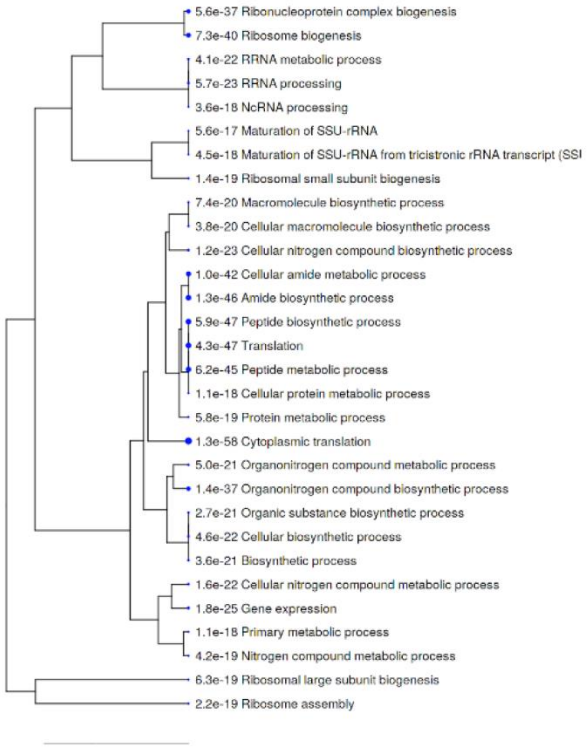
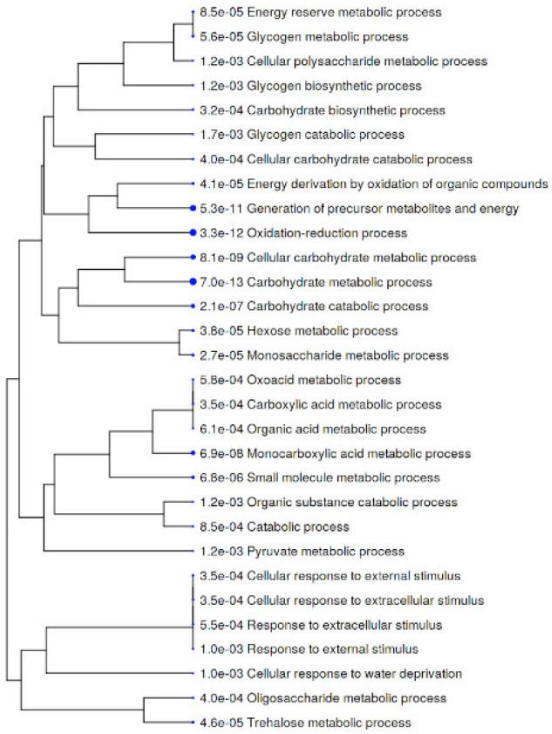



**c**

downregulated in both sets




upregulated in both sets



# 3.3. Adenine starvation is signalled through environmental stress response system in budding yeast Saccharomyces cerevisiae

DOI: 10.22364/eeb.15.29

Key points:

- When transcription factors Msn2p, Msn4p, Rim15p are truncated ( reduced functionality) purine starvation dependent desiccation tolerance is lessened
- Rim15p truncation shows a more prominent response as Msn2p, Msn4p indicating that coordinated response via signalling pathways controlling cell proliferation via nutrient is in play.

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*Original Paper*

# Adenine starvation is signalled through environmental stress response system in budding yeast *Saccharomyces cerevisiae*

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**Abstract**

In the wild, budding yeast *Saccharomyces cerevisiae* often undergoes periods of nutrient abundance and absolute scarcity. It is capable of effectively halting its cell cycle in G1, in the case of lack of any basic nutrients. However, when lacking metabolic intermediates, the yeast behaves differently. Purine and not pyrimidine auxotrophic starvation in *S. cerevisiae* elicits rapid cell cycle arrest and increase of several stress (oxidative, acid, heat) resistances. Until now, molecular mechanisms governing formation of phenotype during auxotrophic adenine starvation in *S. cerevisiae* are not understood. The aim of the current research was to determine if the elements of environmental stress response system play a role during phenotype formation in adenine starvation in budding yeast. We tested if *MSN2/4* or *RIM15* C-end truncation affects desiccation tolerance in full media and after adenine starvation. We found that functional defects of each element of environmental stress response systems affected desiccation tolerance, however, C-end truncation of *RIM15* lowered desiccation tolerance by several orders of magnitude, while *MSN2/4* C-end truncation only by 2 to 4 times. Therefore, we hypothesize that there are other elements of the environmental stress response system except MSN4 and MSN2, responsible for adenine starvation specific, stress tolerant phenotype formulation.

**Key words:** adenine starvation, desiccation tolerance, MSN2, MSN4, RIM15, *Saccharomyces cerevisiae*.
**Abbreviations:** ESR, Environmental Stress Response; PKA, Protein Kinase A; SD, synthetic dextrose; RTG, Retrograde Signalling; TOR, Target of Rapamycin.

## Introduction

Starvation is a typical event in the microbial lifetime, and it is estimated that most microorganisms on Earth are in the resting state initiated by lack of carbon/nitrogen/any other source necessary for biomass increase (Gray et al. 2004). In natural settings yeast often undergoes periods of nutrient abundance and absolute scarcity and is capable of effectively reacting to decrease of availability of carbon, sulphur and nitrogen sources. When starving for the aforementioned "basic" nutrients, yeast cells enter a stress resistant phenotype that aids survival till the external nutrient supply is restored (Boer et al. 2010; Petti et al. 2011). For genetic manipulation purposes various auxotrophies (interruptions of amino acid or nucleotide metabolism) have been introduced in yeast. Interestingly, when cells are starved for external supply of those auxotrophic metabolic intermediates (sometimes called "synthetic limitation"), for example, nucleotides or amino acids, yeast cells do not finish their cell cycle and stop division, and they continue to actively metabolize sugars and do not gain stress resistance. This phenotype has been described for uracil and leucine auxotrophic starvation and has earned the description "glucose wasting phenotype" (Boer et al. 2008).

Adenine auxotrophic starvation in many aspects induces a phenotype similar to cells in G0 arrest or quiescence. *Saccharomyces cerevisiae* cells with adenine auxotrophy (*ade8* or *ade2* mutants) when starving for adenine became desiccation tolerant, their budding index declines, and their chronological life span is increased (Kokina et al. 2014). The mechanism how and why cells translate lack of adenine into a desiccation and starvation resistant phenotype is yet unknown.

Various signaling pathways in the yeast cell link nutrient supply to cell growth rate and stress resistance. Target of Rapamycin, (TOR, nitrogen supply), Protein Kinase A (PKA, carbon supply), Retrograde Signalling (RTG) are the prominent examples of such pathways (see review by Broach 2012).

In the case of adenine starvation, we have observed more than a 200-fold increase in desiccation tolerance in *S. cerevsiae* cells (Kokina et al. 2014). Other authors have linked such a huge increase in desiccation tolerance to various genetic factors, including activity of targets of PKA and TOR pathways (Calahan et al. 2011; Welch et al. 2013). Msn2p, Msn4p and Rim15p are components of a system ensuring universal "Environment Stress Response" (ESR). The transcription factors Msn2p and Msn4p upregulate

genes containing STRE sequences in their promoters (Berry, Gasch 2008). Rim15p is sensor-effector kinase that integrates nutrient and stress signals into widespread ESR response via activating endosulphines Igo1/2p. Those, in turn, provide stability for newly synthesised mRNA (Sarkar et al. 2014). Previously, Msn2/4p together with Rim15p, have been identified as crucial elements ensuring desiccation tolerance in *S. cerevisiae* (Welch et al. 2013).

Our aim was to test if ESR is involved in cell phenotype formation during adenine auxotrophic starvation. To test this, we determined if stable nuclear translocation of Msn2/4p is necessary to ensure adenine starvation mediated desiccation tolerance. We constructed C-end truncations of *MSN2/4* or *RIM15*, checked their identity and effect on growth. We found the cultivation time necessary for complete halt of cell proliferation (adenine starvation), thus giving us a timescale for adenine starvation specific effects. Finally we tested C-end truncation effects on desiccation tolerance of WT and each of newly constructed strains in fast growing and adenine starved cells.

## Materials and methods

### *Yeast strains and genetic constructions*

All experiments were carried out using *Δade8 S. cerevisiae* strains of CEN.PK2 background (Kokina et al. 2014). Transcription factors were truncated by transforming yeast with linear PCR fragment containing flanking homologous sequences of the respective transcription factor and 6xHis tag and G418 marker in between (Janke et al. 2004). After transformation strain identity was confirmed by colony PCR using gene specific test primers and internal primer (Nat_ctrl_Hb) from the insert (Lõoke et al. 2011). All the details regarding strains, plasmids and primers are given in the Table 1. All primers were synthesised by Sigma Aldrich.

### *Growth conditions and media*

All starvation experiments were conducted in synthetic dextrose (SD) media (Verduyn et al. 1992) with all necessary auxotrophic agents added in surplus (Pronk 2002). For purine starvation, synthetic media without adenine was used.

Growth rate analyses were performed in a 96 well multimode reader TECAN Infinite M200 PRO. *S. cerevisiae* cells were grown in synthetic media containing glucose, washed three times with distilled water and resuspended in the synthetic media with all combinations of amonia/proline and glucose/galactose as N and C sources.

To assess adenine starvation effect on desiccation tolerance, cells were grown in full SD media up to exponential phase ($OD_{600}$ less than 1), washed with distilled water, and re-suspended to $OD_{600}$ = 0.5 in SD media without adenine, but with all other broth components added. The yeasts were incubated for 4 h in a rotary shaker (140 rpm, 30 °C) and then desiccated.

### *Desiccation tolerance assay*

Desiccation tolerance was assayed by estimating CFU $mL^{-1}$, before desiccation and after dehydration. One millilitre of culture at $OD_{600}$ = 1 was washed with distilled water twice, diluted serially and spotted on YPD (yeast extract 1%, bactopeptone 2%, glucose 2%, agar 2%) plates. The

**Table 1.** Yeast strain, plasmids and primers used in the present study

| | Name | Description/sequence/ | Source |
|---|---|---|---|
| Yeast strain | WT | CEN.PK2-D *MATα leu2-3/112 ura3-52 trp1-289 his3Δ1 MAL2-8c SUC2 Δade8* | Kokina et al. 2014 |
| Plasmid | pYM46 | PCR template for C-terminal myc tag plus 7 His residues: marker pAgTEF-kanMX-tAgTEF, selectable phenotype: G418 resistance | Janke et al. 2004, EUROSCARF |
| Primers | MSN4_S2 | CTTGTCTTGCTTTTATTTGCTTTTGACCTTATTTT TTTCAATCGATGAATTCGAGCTCG | This study |
| | MSN4_S3 | GCATTCAGACGCAGTGAGCACTTGAAAAGGCATA TAAGATCGTACGCTGCAGGTCGA | This study |
| | MSN2_S3 | GAAATTTAGTAGAAGCGATAATTTGTCGCAACAC ATCAAGCGTACGCTGCAGGTCGA | This study |
| | MSN2_S2 | TGAAGAAAGATCTATCGAATTAAAAAAATGGGGT CTATTAATCGATGAATTCGAGCT | This study |
| | RIM15_S2 | CAGTTATTTTTTTTAATTATCTTTATCTTAAAATT TATCAATCGATGAATTCGAGTCCG | This study |
| | RIM15_S3 | CAGGAGGCGGCAACCAGTAGAGTCTTTGACGAT GTTTTAGCGTACGTCGCAGGTCGA | This study |
| | MSN4_test_F01 | AGAAGGCATTCAGACGCAGT | This study |
| | RIM15_test_F01 | CCAATTGTGGCCATAACAAA | This study |
| | Jli_MSN2-t01 | CCATTATCGCCTGCATCATCAT | This study |
| | NAT-HgB_ctrl | ACGAGGCAAGCTAAACAGATCT | This study |

remaining cell suspension was centrifugated, and the pellet was left to desiccate for 6 h at 30 °C in a desiccator, then diluted in distilled water in room temperature. Rehydrated cell solution was thoroughly mixed, serially diluted and spotted on YPD plates. The viability (%) was calculated by dividing the number of CFU $mL^{-1}$ after and before desiccation, similarly to Calahan et al. (2011).

*Statistics*

All experiments were performed in triplicates, error bars represent standard deviations. Asterix is used where $p < 0.05$.

## Results and discussion

*Adenine starvation halts cell proliferation*

At first we determined effect of adenine starvation on the wildtype cells growth dynamics. Results are shown in the Fig. 1.

*S. cerevisiae* wildtype strain used in this study has multiple auxotrophies. It is auxotroph for histidine, adenine, tryptophan, leucine and uracil. As a well characterised control for yeast cell auxotrophic starvation, we added uracil deficient media and observed the growth pattern in this medium. When cultivated in adenine or uracil deficient media, auxotrophic starvation led to halt of cell proliferation, as cells lack building blocks for further growth. Previously we have observed that optical density alone is not a reliable measurement of actual cell number in the case of adenine-starved cells. While starving for adenine, *S. cerevisiae* cells tend to increase in volume and therefore cell number per mL in unit of absorbance

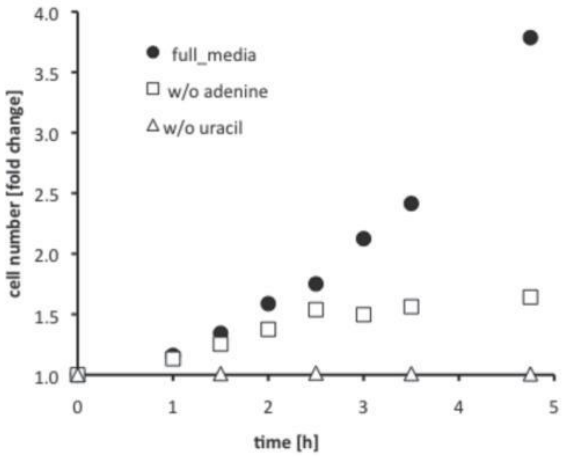


**Fig. 1.** *S. cerevisiae* wildtype growth dynamics in full SD media and in the SD media without adenine or uracil supplement (w/o adenine or w/o uracil). Growth dynamics were registered with a Z™ Coulter counter® (Beckman Coulter). It is depicted as fold change (cell number of the each time point normalised to initial cell number). Data points are average from independent triplicates; standard deviation is less than 4%, error bars not visible.

value is affected (Kokina et al. 2014). To be sure that we observed only halt of cell proliferation rather than increase in cell volume, we used direct cell counts via particle counter, not spectrophotometrical OD measurements. To compare cultivations with different initial cell numbers, we normalised all cell number per mL against initial cell number (see Fig. 1).

Cells cultivated in adenine free, synthetic dextrose media cease to multiply later than in the case of uracil starvation. Based on our data (Fig.1), we assumd that adenine starvation sets in 2 to 3 h after a shift to starvation media, while in the case of uracil starvation, it set in immediately after media shift. Therefore, to induce a "adenine starvation phenotype", we cultivated wildtype cells in adenine deficient media for 4 h.

*Msn2/4p and Rim15p C-end truncation affects cell growth*

Transcription factors were truncated by transforming yeast with the linear PCR fragment containing flanking homologous sequences of the respective transcription factor and 6xHis tag and G418 marker in between (Janke et al. 2004). When inserting fragments, the last 100 bp of coding sequence were removed. The last 50 amino acids of MSN2/4p code for DNA binding Zn finger domain and are responsible for binding to target STRE sequences (Estruch, Carlson 1993). Rim15p contains REC domain in it's C-end; it seems to be involved in autophosphorylation and dimerisation activities (Wanke et al. 2005).

We tested growth of truncated strains in synthetic media with varying carbon and nitrogen sources. We chose glucose and galactose as repressive and derepressive examples of carbon sources and ammonium sulphate as example of repressive and proline as non-repressive nitrogen source (Gancedo 1998; Cooper 2002). As a result, we observed different growth patterns when strains where grown under different C or N sources. This reflects divergent functions that these factors have in adopting cell growth in the presence of different (repressive or derepressive) carbon or nitrogen sources, see Fig. 2. Previously we had confirmed identity of our constructs by colony PCR (Lõoke et. al. 2011); these media shift studies served as functional confirmation of successful gene truncation and effect on the cell signalling system.

In synthetic media with glucose and ammonia as carbon and nitrogen sources, the Msn2p C-end truncated strain exhibited similar specific growth rate (μ) as the wildtype. Strains with Msn4p, and Rim15p C-end truncated grew 25% slower (see Fig. 2), 0.4 $h^{-1}$ for the wildtype and *msn2*, 0.28 $h^{-1}$ and 0.26 $h^{-1}$ for *msn4* and *rim15*, respectively. Similar effects of Msn2 functional impairments on strain growth have been demonstrated previously by Estruch and Carlson (1993). In both cases, C-end specific truncation (our results) and *msn2/4* partial knockouts (Estruch, Carlson 1993) resulted in hampered growth of the strains on galactose as the main carbon source.

In all carbon-nitrogen source combinations, the

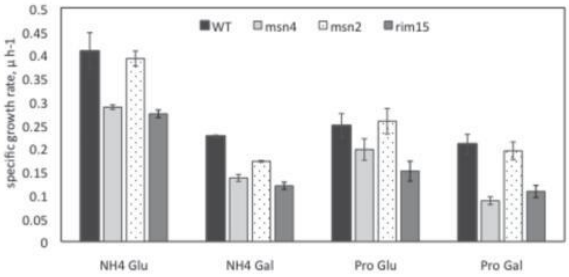


**Fig. 2.** Growth rate (μ $h^{-1}$) of the wildtype (WT) strain and it's corresponding strains with C-end truncated *msn2, msn4, rim15* mutants cultivated in SD+ media with different carbon (Glu, glucose; Gal, galactose) and nitrogen ($NH_4$, ammonium; Pro, proline) sources. Each bar depicts the mean of four independent cultivations. Error bars represent standard errors.

Rim15p C-end truncated strain grew slower and exhibited a comparatively long lag phase. Interestingly, low growth speed and extended lag phase have been observed with sake yeast possessing a frame shift mutation in the Rim15p C-end and lacking 75 amino acids of the C-end (Watanabe et al. 2012).

The nitrogen source shift affected Msn2/4p C-end truncated strains in the same way as the wildtype. Derepressing the nitrogen source together with derepressing the carbon source decreased growth of all strains, since carbon and nitrogen metaboslims share common metabolites, like α ketoglutarate (Cox et al. 2002; Tate, Cooper 2013). The results showed that the introduced changes are more responsive to changes in the carbon, not nitrogen source.

### *Msn2/4 and Rim15 C-end truncation affects* S. cerevisiae *cell desiccation tolerance after adenine starvation*

Desiccation tolerance is a typical feature of *S. cerevisiae*, which is often used for biotechnological purposes (like preparation of dried yeast for bread, beer and wine production). To survive desiccation, cells should be ready for a number of stresses (oxidative, salt, heat, etc.; review in Dupont et al. 2014). It is also known that condition of a cell before desiccation directly correlates to survival of desiccation tolerance. (Welsh et al. 2013). Here we use desiccation "stress" as a "environmental stress" to determine the activity state of ESR.

We attempted to determine if transcription factors Msn2/4p or their upstream kinase Rim15p have any role in adenine starvation induced desiccation tolerance in *S. cerevisiae*. We grew wildtype and derived strains with C-end truncated Msn2/4p or Rim15p till $OD_{600}$ approx 1 or less, harvested the cells, washed them, and resuspended them in SD complete broth or SD lacking adenine with final $OD_{600}$ = 0.5. After cultivation in shake flasks on rotary shaker for 4 h, 30 °C, we harvested cells from all the shake flasks, desiccated and spotted them on solid YPD agar and counted colonies. Viability was calculated as proportion of culture CFU after desiccation against CFU before desiccation. Desiccation tolerance results are shown in Fig. 3.

After adenine starvation, we obtained similar desiccation tolerance results for wildtype as published previously (Kokina et al. 2014). We observed a huge (more than 1000 times) increase in desiccation tolerance in the adenine starved cells when compared to desiccation tolerance of rapidly growing wildtype cells.

Desiccation tolerance of all C-end truncated strains were statistically significantly different from the wildtype. In the case of strains with C-end truncated Msn2/4p, desiccation tolerance after adenine starvation was lower than in wildtype cells. Desiccation is rather complex treatment, which includes elements of oxidative, heat shock and osmotic stresses (Franca et al. 2005). Therefore, functional impairment of transcription factors responding to all those stresses (Msn2p and Msn4p) will inevitably lead to lowered tolerance. However, drop of the viability in these strains was within the same order of magnitude. Since our spot test allowed us to estimate population viability from 100% down to 0.00001%, therefore a "significant signal" when using such a rough metric indicated changes in orders of magnitudes and not changes within the same order of magnitude. Calahan et al. (2011) observed a drop in desiccation tolerance in *msn2* and *msn4* knockouts within the same order of magnitude in saturated cultures, similarly to our C-end *MSN2/4* truncations.

Interestingly, Rim15p C-end truncation led to increase in desiccation tolerance in exponentially growing culture (SD media with all necessary supplements). It reached 0.03%, which is approximately 50 times more than typical desiccation tolerance of the wildtype and other strains when grown in full media. In comparison, the Rim15p

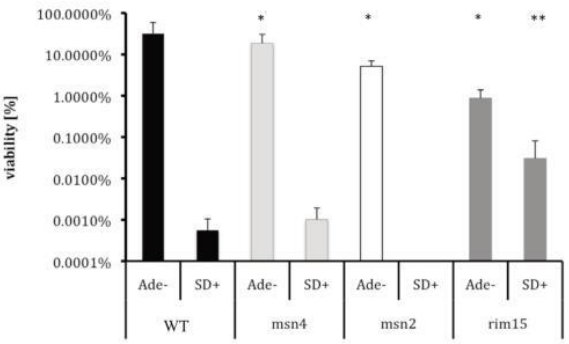


**Fig. 3.** Desiccation tolerance of wikdtype (WT) and it's derivative strains with C-end truncated *msn2, msn4* or *rim15*, after 4 h cultivation in full SD media (SD+) or SD media without adenine (Ade-). Desiccation was performed at 30 °C in a desiccator. Each bar represents the mean of three independent samples, error bars represent standard deviations. Stars depict significant differences ($p < 0.05$) between wildtype and engineered strain adenine starvation treatment (*) and full media (**).

C-end truncated strain desiccation tolerance after adenine starvation increased just 30, not 1000 or more times, when compared to wildtype.

Truncation of Rim15p led to a more severe desiccation tolerance decline than in the *msn2/4* double knockout (Calahan, et al. 2011). This indicates that there are other elements of Environmental Stress Response system responsible for adenine starvation specific, stress tolerant phenotype formulation, except besides *MSN4* and *MSN2*.

Sake yeasts form a compact *S. cerevisiae* strain subgroup, where at least seven strains have C-end truncated versions of Rim15p (Liti et al. 2009; Wang 2012; Watanabe 2012). We observed that the strain with Rim15p C-end truncation had increased desiccation tolerance in it's exponential phase. While several sake yeast strains ferment glucose to ethanol rapidly, they are characterised for low ethanol and heat stress tolerance. This has been linked to defects in Msn2/4p signalling (Watanabe 2011). Also, these strains enter quiescent state with low efficiency (Urbanzcyk, 2011). Recently, genetic defects in the Msn2/Msn4 upstream regulator *RIM15* have been found. When transforming cells with the "correct" version of *RIM15*, growth, heat and ethanol tolerance defects were complemented (Watanabe 2012).

Our results allow us to speculate that the Rim15p C-end truncated version might have phenotypic advantage during sake production. We presume that improved ethanol production together with comparatively "increased" desiccation tolerance in the exponential phase could be a phenotype being unintentionally selected throughout the rich history of sake production (Kitigaki, Kitogoto 2013).

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# 3.4. Purine auxotrophy: Possible applications beyond genetic marker

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Key points:

- Purine synthesis is evolutionary conserved.
- In S. cerevisiae ribosomal nucleotides may serve as nucleotide reserves in case of lack of deoxyribonucleotides.
- Purine auxotrophy is a common phenomenon among parasitic monera, protozoans, and metazoans.
- Parasites rely on scavenging of purines from their environment thus making that a potential therapeutic target.
- Auxotrophic yeast cells are suitable as models for such parasites.

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BUDDING TOPIC

WILEY Yeast 2019

# Purine auxotrophy: Possible applications beyond genetic marker

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## Abstract

Exploring new drug candidates or drug targets against many illnesses is necessary as "traditional" treatments lose their effectivity. Cancer and sicknesses caused by protozoan parasites are among these diseases. Cell purine metabolism is an important drug target. Theoretically, inhibiting purine metabolism could stop the proliferation of unwanted cells. Purine metabolism is similar across all eukaryotes. However, some medically important organisms or cell lines rely on their host purine metabolism. Protozoans causing malaria, leishmaniasis, or toxoplasmosis are purine auxotrophs. Some cancer forms have also lost the ability to synthesize purines *de novo*. Budding yeast can serve as an effective model for eukaryotic purine metabolism, and thus, purine auxotrophic strains could be an important tool. In this review, we present the common principles of purine metabolism in eukaryotes, effects of purine starvation in eukaryotic cells, and purine-starved *Saccharomyces cerevisiae* as a model for purine depletion-elicited metabolic states with applications in evolution studies and pharmacology. Purine auxotrophic yeast strains behave differently when growing in media with sufficient supplementation with adenine or in media depleted of adenine (starvation). In the latter, they undergo cell cycle arrest at G1/G0 and become stress resistant. Importantly, similar effects have also been observed among parasitic protozoans or cancer cells. We consider that studies on metabolic changes caused by purine auxotrophy could reveal new options for parasite or cancer therapy. Further, knowledge on phenotypic changes will improve the use of auxotrophic strains in high-throughput screening for primary drug candidates.

**KEYWORDS**

budding yeast, cancer, evolution model, malaria, purine auxotrophy, *Saccharomyces cerevisiae*

## 1 | INTRODUCTION

Every organism needs a permanent supply of basic elements—nitrogen, carbon, sulphur, potassium, and so on. Usually, organisms are capable of synthesizing all the necessary building blocks for their biomass by themselves. However, for some organisms or tissues, supplementation with additional building blocks such as amino acids, nucleotides, or their intermediates is necessary to sustain uninterrupted growth. In their absence, starvation for a particular amino acid or nucleotide compound might set in. Organisms starving for basic elements stop growing and "switch on" the stress resistance phenotype, whereas in starving for amino acids or nucleotides, they rapidly lose viability and are stress intolerant (Boer, Amini, & Botstein, 2008; Petti, Crutchfield, Rabinowitz, & Botstein, 2011).

Purines are essential metabolites in every organism. They are involved in many cellular processes—storage and expression of genetic

  

information, energy metabolism, redox metabolism, cell signalling, and others. Therefore, disturbances in their metabolism have many physiological outcomes (Chapman & Atkinson, 1977).

Interestingly, not all organisms synthesize their own purines. A number of organisms or cell lines scavenge their purines from the environment or their hosts. Prototrophic organisms can lose their purine synthesis ability due to mutation and become purine auxotroph. These organisms would face purine starvation if there is no purine in their close surroundings. This seems to be the typical situation for many parasites that are purine auxotrophs. Interestingly, purine auxotrophic starvation is physiologically distinct from pyrimidine or amino acid starvation. In contrast to leucine or uracil starvation, purine starvation causes a rapid increase in stress resistance (Kokina, Kibilds, & Liepins, 2014). This, in turn, points to potential beneficial effects to the organism due to loss of purine synthesis capacity.

In many aspects, budding yeast *Saccharomyces cerevisiae* can serve as a simple, yet powerful eukaryotic model for purine metabolism. By using purine auxotrophic yeast strains, it is possible to explore the evolutionary path from "purine prototrophy" to "purine auxotrophy." Further, exploring purine auxotrophic starvation could help explore new drug targets for tackling cells or organisms with purine auxotrophy, like intracellular parasites or cancer cells.

This review aims to demonstrate the common principles of purine metabolism in eukaryotes and the effects of purine starvation in eukaryotic cells and to discuss possibilities of purine auxotrophic *S. cerevisiae* strains as model organisms for purine starvation elicited metabolic states, with applications in evolution studies and pharmacology.

## 2 | PURINE METABOLISM IN EUKARYOTES

New purine bases in eukaryotic cells are obtained in two ways—salvage and *de novo* synthesis. In the salvage pathway, purine bases are taken up from the environment or recycled within the cell and attached back to the ribose 5-phosphate, thus restoring nucleotides. Most eukaryotes have two purine salvage enzymes—one that can produce AMP (adenine phosphoribosyltransferase, EC 2.4.2.7) and another hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) that can produce GMP or IMP, and sometimes xanthosine monophosphate as well (Berg, Tymoczko, & Stryer, 2002).

When rapidly growing and proceeding through the cell cycle, the cell inevitably needs to take up or synthesize more purines to supply the necessary bricks for new DNA and RNA and to provide cofactors and energy carrying substances for the new cell. In the cell, purines are mostly found within nucleotides: deoxyribose nucleotides (dNTP) or ribose nucleotides (NTP). Ribose nucleotides form RNA, are used as energy sources in biochemical reactions, and are also found within various cofactors (NAD, FAD, CoA, etc.). dNTPs are used for DNA synthesis. dNDP is synthesized from the corresponding NDP by the enzyme rNDP reductase (Mathews, 2014).

The purine synthesis pathway and its regulation are highly conserved in all eukaryotes from fungi to mammals (Agmon et al., 2017). Most probably, the last common ancestor had a pathway with the same structure that diversified into the now known three eukaryotic domains (Armenta-Medina, Segovia, & Perez-Rueda, 2014; Vázquez-Salazar, Becerra, & Lazcano, 2018).

In *S. cerevisiae*, the purine *de novo* synthesis pathway comprises two parts—sequential chain of reactions adding C and N atoms to ribose phosphate until inosine monophosphate (IMP) is formed, which is the common substrate for GMP and AMP synthesis. To synthesize IMP, the glycine backbone is fused with nitrogen provided by the amide groups of two glutamine molecules and one aspartate. Additional carbon atoms are provided by two formate and one $CO_2$ molecules (Pedley & Benkovic, 2017). *De novo* pathway gene expression is regulated by Bas1/2p transcription factors that respond to the concentrations of pathway intermediates phosphoribosylaminoimidazolecarboxamide (AICAR) and phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR), metabolites that are products of reactions catalysed by Ade1p and Ade13p. The total flow of metabolites through the purine *de novo* synthesis pathway is regulated by the first enzyme Ade4p that is sensitive to ATP and ADP concentrations in the cell (Pinson et al., 2009; Rébora, Desmoucelles, Borne, Pinson, & Daignan-Fornier, 2001; Rébora, Laloo, & Daignan-Fornier, 2005).

The purine content of the budding yeast haploid genome is $4.65 \times 10^6$ of guanine and $7.45 \times 10^6$ adenine nucleotides. Simultaneously, purine dNTP (dATP + dGTP) nucleotide concentration in the cytoplasm is 60 μM (Koç, Wheeler, Mathews, & Merrill, 2004). Thus, in the case of a haploid genome, purine dNTP pools of DNA and cytoplasm are roughly the same. The size of the free dNTP pool depends on the cell cycle phase (Koç et al., 2004), and synthesis of new dNTP species is allosterically regulated by the amount of end products. Purine synthesis is regulated by the amount of dATP and dGTP, whereas pyrimidine synthesis is regulated by dATP and dCTP (Mathews, 2014). In their review, Pai and Kearsey showed that the dNTP pool is upregulated at the beginning of the S phase. The influence of the dNTP pool size is studied mainly by interfering with feedback mechanisms of dNTP synthesis. High concentrations of dNTPs decrease the DNA polymerase fidelity and influence the length and start of the S phase. A small dNTP pool leads to cell checkpoint activation, replication fork stalling, and mainly influences hard to replicate sequences, regions of low sequence complexity, and fragile DNA regions. The frequency of ribose nucleotide incorporation into DNA increases and low dGTP concentration in particular leads to telomere shortening (Pai & Kearsey, 2017).

Literature shows that free (not incorporated in RNA molecules) purine ribose nucleotide concentration within the cytoplasm of baker's yeast is about 7 mM, of which ATP forms 5 mM and GTP constitutes 1.5 mM (Ljungdahl & Daignan-Fornier, 2012). The molar amount of RNA in exponentially growing yeast cell is approximately 50 times higher than that of DNA. Around 80% of cells RNA molecules is ribosomal RNA, tRNA (around 15%) and mRNA being the least abundant. Although rRNA and tRNA are relatively stable molecules, half-life of mRNA in yeast cells is around half an hour long (Geisberg, Moqtaderi, Fan, Ozsolak, & Struhl, 2014; von der Haar, 2008). The RNA nucleotide pool size is relatively stable during the cell cycle (Chabes et al.,

2003; Chapman & Atkinson, 1977; Koç et al., 2004). If environmental conditions change, old mRNA will be degraded and will provide source of nucleotides before relatively costly biosynthesis of purines or pyrimidines starts. Also, degradation of rRNA has been described as response during starvation to provide cells with additional energy and nitrogen sources (Pestov & Shcherbik, 2012; Xu et al., 2013). Therefore, we hypothesize that if the supply of new purines is halted (stopping *de novo* synthesis or purine supply from the media), the RNA nucleotide pool could form a source for purines in the cytoplasm.

Of all purine containing moieties in the cell, ATP has the greatest intracellular concentration in yeast, ranging from 1 to 5 mM (Albe, 1990, Ljungdahl & Daignan-Fornier, 2012). Although the ATP amount can change during various stress conditions, the ATP:ADP:AMP ratio or cell energy charge is more crucial than the drop in a single adenosine nucleotide concentration. It is considered that cells tend to maintain a constant energy charge (Chapman & Atkinson, 1977). Active maintenance of stable energy charge could also explain why AMP is converted to IMP upon glucose pulse (Walther et al., 2010). Signals regarding nutrient availability including energy charge in eukaryotic cells are integrated by two main signalling pathways: protein kinase A (PKA) and target of rapamycin (TOR) pathways. Orientation towards metabolic direction and proceeding with the cell cycle is in accordance with these systems (reviewed by Broach, 2012).

The amount of available purines might be a critical "checkpoint" for the cell to enter the S phase (if the supply of purines is sufficient for another cell) or to stay in G1/G0 (if the supply is inadequate). Mother/daughter cell separation is impaired in auxotrophic guanine starvation (Sagot, Schaeffer, & Daignan-Fornier, 2005). Further, putative G1 arrest and a massive increase in stress resistance is observed when *S. cerevisiae de novo* synthesis mutants (*ade2* and *ade8)* are starved for purine (Kokina et al., 2014).

## 3 | PURINE AUXOTROPHY AMONG PARASITES

Purine auxotrophy is a common phenomenon among monera, protozoans, and metazoans. For example, all parasitic protozoans and many intracellular bacterial pathogens are purine auxotrophs. Two of the well-known examples are *Toxoplasma gondii* (causes toxoplasmosis) and *Plasmodium falciparum* (causes malaria) (Downie, Kirk, & Mamoun, 2008; Weiss & Kim, 2011). Parasitic worm genome analyses revealed that purine auxotrophy is also common among parasitic platyhelminths and roundworms (nematodes). Parasitic worms lack some or all *de novo* purine pathway enzymes (International Helminth Genomes Consortium, 2019).

These auxotrophic organisms save resources for expensive purine *de novo* synthesis as one nucleotide "costs" ~50 ATP molecules (cost calculated including energy spent for synthesis of all intermediate metabolites) (Lynch & Marinov, 2015). Many apicomplexan parasites lack all the genes encoding enzymes of *de novo* purine synthesis and rely only on purines harvested from their host. Using salvage pathways, parasites collect different species of purines—adenine, xanthine, hypoxanthine, adenosine, and inosine—and transform these into forms necessary for their metabolism (Downie et al., 2008; Hwang & Ullman, 1997). Parasitic organisms absorb purine sources through specific nucleotide transporters (Chaudhary et al., 2004; Major, Embley, & Williams, 2017). However, the inability to synthesize purine is not specific to parasites. For example, *Tetrahymena* is a genus of free-living protozoans that requires exogenous purine and pyrimidine supply to sustain growth. It has not lost its entire purine synthesis pathway, but several steps are missing (Hill, 1972).

As purine moieties are found in a number of important biomolecules, loss of the entire purine *de novo* synthesis pathway is interesting from the evolutionary viewpoint. Researchers are still discussing the origin of parasitism—does the package of adaptations (including loss of purine *de novo* synthesis) precede parasitism in the given taxa or do free-living organisms become parasitic and then lose the ability to synthesize purines (Armenta-Medina et al., 2014; Janouskovec & Keeling, 2016). Loss of purine *de novo* synthesis is probably one of the parasitic characteristics that evolved even before an organism became parasitic (Janouskovec & Keeling, 2016). In apicomplexans and trypanosomatids, loss of the *de novo* pathway appears to have occurred before the start of a parasitic lifestyle (Jackson et al., 2016; Janouškovec et al., 2015). *Tetrahymena spp*. purine auxotrophic, yet free-living organisms can be an excellent model for parasite evolution. Coyne et al. (2011) have already shown the potential of free-living *Tetrahymena thermophila* as a model for evolutionary adaptation to a parasitic lifestyle. This organism may also be a key for modelling loss of the purine *de novo* pathway.

Inability to synthesize purines *de novo* along with lack of purine supply is reported to elicit stress tolerance phenotype. The intracellular parasite and purine auxotroph *Leishmania donovani*, shows G1 arrest and stress tolerance when starved for external adenine. Moreover, purine-starved *L. donovani* cultures remain viable for more than 50 days (Martin et al., 2016). This indicates the particular benefits acquired by purine auxotrophic starvation in some specific environmental contexts. Therefore, loss of purine *de novo* synthesis might confer fitness advantages and contribute to organism evolution.

## 4 | APPLICATIONS OF PURINE AUXOTROPHIC YEAST

Auxotrophy in *S. cerevisiae* is a well-known phenomenon, and several auxotrophic markers (adenine, tryptophan, leucine, uracil, etc.) have been used for gene engineering for more than three decades (Pronk, 2002).

Purine auxotrophy is well known in microbiology due to mutations in two *de novo* synthesis pathway loci (*ade2* or *ade1*) that are common in laboratory budding yeast strains. With an insufficient adenine supply, the colonies develop a characteristic red ochre colour due to accumulation and subsequent oxidation of pathway intermediates (AIR or CAIR); due to pathway conservation, the same colour is observed with analogue mutations in other yeast species as well, for example, in

*Pichia spp*. and *Candida spp*. (Du, Battles, & Nett, 2012; Poulter & Rikkerink, 1983).

Development of the characteristic colony colour is a classical tool in yeast genetics to visually spot *ade2* and *ade1* mutants (Roman, 1956). Purine auxotrophs are used as a visual marker in tetrad dissection and help select the spores of interest (Kumar, Gaur, Gupta, Puri, & Sharma, 2015). Appearance of white colonies in a red population is visually effective to detect revertant mutant colonies or to spot sectors of colonies as mutants (Yuen et al., 2007). As the red colour develops due to oxidation, the presence or absence of colour in *ade1/2* mutants suggests the level of oxidative stress within the cell (Bharathi et al., 2016).

In addition to the traditional use of purine auxotrophs in yeast genetics for the red/white screen, there is a growing interest in their application to various drug screens to identify new drug targets. Here, we describe the application of adenine auxotrophic yeast strains in the search for drugs against parasites, cancer, and evolution studies.

In the 21st century, protozoan parasites are still a serious threat to human health. More than 219 million infections and 400,000 death cases of malaria were estimated in 2017 (World Health Organisation, 2018); *P. falciparum* is responsible for most of the lethal cases. The protozoan *Leishmania spp*., the agent of leishmaniasis, causes approximately 1 million new infections and up to 65,000 death cases annually (World Health Organisation, 2019). Some parasites actively react and adapt to the host immune system and have evolved strains that are insensitive to traditional drug treatments. *Trypanosoma spp*. are one of the typical examples for which an effective drug has not been found yet (Field et al., 2017).

High-throughput screening (HTS) is a typical method to determine drug candidates from huge libraries of potentially active compounds. There is growing interest in exploiting *S. cerevisiae* for new HTS tasks and particularly in antimalaria research (Denny, 2018; Williams et al., 2015). The idea of using purine metabolism as a target for antiparasitic drugs is appealing due to the fact that the parasite relies exclusively on the host for its purine supply and as its purine uptake mechanism is distinct from that of the host (review in el Kouni, 2003, Frame, Deniskin, Arora, & Akabas, 2015). Yeasts can be used as instruments for antiparasitic drug screening using purine metabolism. They are easily made purine auxotrophic, and their purine nucleoside transporters can be substituted with parasite analogs. Frame et al. used a purine auxotrophic (*ade2)* yeast strain, replaced endogenous nucleoside transporter *FUI1* with *P. falciparum* analogue *Pf*ENT1, and tested the inhibition of toxic adenosine analog uptake. The most potent inhibitors of the *Pf*ENT1 found by yeast HTS were tested in *P. falciparum* culture and proved to be highly active (Frame et al., 2015). Thus, yeast-based primary drug HTS screens can be set up for screening drug candidates against a wide array of purine auxotrophic protists, like *Leishmania spp*., *Toxoplasma spp*., and *Trypanosoma spp*.

Parasite DNA itself can be an alternative druggable purine-related drug target, whose structure could be damaged by the imported chemically inactive prodrugs. This concept has been tested in a *Trypanosoma brucei* model where the prodrug, purine analog 2-(3-acetyl-3-methyltriazen-1-yl)-6-hydroxypurine, was specifically taken up by purine transporter and hydrolyzed to purine and a methyldiazonium cation. The latter binds DNA and stops parasite proliferation by cell cycle arrest at the G2-M cell phase. The aforementioned screening was performed using *T. brucei* cells harvested from mammalian hosts. The lead compound was taken up by transporter H2 with higher affinity than its typical substrates—adenine and hypoxanthine (Rodenko et al., 2015). Yeast could thus be a valuable instrument to accelerate primary drug screening. The yeast endogenous purine transporter Fcy2p could be replaced with heterologous from the parasite and used in primary drug screening. Potentially, yeast assays would take less resources and be time saving with no need for cultivating parasites with a comparatively long doubling time (24 hr or more) (Denny & Steel, 2015; Weber, Rodriguez, Chevallier, & Jund, 1990).

Cancer causes every sixth death globally. In 2018, around 18 million new cancer cases were diagnosed and around 10 million deaths were caused by cancer (Global Cancer Observatory). Antimetabolites of purine metabolism (like thioguanine, thiopurine, and fludarabine) are well-known cancer drugs. They are used to treat haematological malignancies including acute lymphocytic lymphoma (ALL) (reviewed by Parker, 2009). However, a subset of ALL cells can survive treatment with these antimetabolites and cause relapse. In these relapsing cancers, mutations in purine metabolism genes have bene identified. Approximately 20% of ALL relapse patients carry a mutation in 5'-nucleotidase (coded by NT5C2) (Tzoneva et al., 2013). Loss of fitness mutations in NT5C2 leads to accumulation of purine nucleotides and promote their export. Thus, cells survive therapy due to decreased accumulation of the drug for the cost of slow growth and decreased overall purine content (Tzoneva et al., 2018).

Because purine metabolism in *S. cerevisiae* and human cells is functionally similar, parts of it can be "humanized" in yeast—replaced by genes coding for analogous human proteins. Humanized yeast strains can then be used as screens for specific drug targets (Agmon et al., 2017). We propose that purine auxotrophic yeast strains expressing both—human mutant nucleotidase and nucleoside transporters—could form a tool for drug screening against a particular subtype of relapsing ALL.

Genome instability is considered one of potential reasons for cancer (Yao & Dai, 2014). Purine auxotrophic yeasts can be used as a tool to explore the genetic basis of genome instability. Yuen et al. estimated the frequency of white revertants in the *ade2* screen. They tested whether a particular knockout induces chromosome aberrations. Thus, they identified genes with an increased frequency of chromosome aberrations and organized these hits into functional groups. Purine *de novo* synthesis genes was one of these; some pathway knockouts showed up to 30 times higher genomic instability than the wild type (Yuen et al., 2007). Duffy et al. used a similar *ade2* revertant screening assay to find yeast genes, which when overexpressed, induced chromosome aberrations. Indeed, some of the hits that they found have human orthologs (e.g., tyrosyl-DNA-phosphdiesterase and RNA polymerase II), which are overexpressed in cancer cells (Duffy et al., 2016).

Purine metabolism is affected in relapsing ALL; there are also other instances of alterations related to purine metabolism leading to

tumourigenesis. Potentially, the reason for increased genomic instability in purine mutants is replication stress due to an insufficient supply of purines and subsequent dNTP. Depletion of the cell dNTP pool leads to replication stress and potentially induces double strand breaks and genome instability and might initiate cancer development (Bester et al., 2011). One carbon-folate metabolism is a pathway linking energy supply with the cell's anabolic (including synthesis of purines) needs. DNA modifications (methylation and acetylation) are folate and purine dependent (reviewed in Locasale, 2013 and Shuvalov et al., 2017). Lack of an adequate folate supply promotes tumourigenesis by lowering the methylation of specific tumour suppressors (Wasson et al., 2006). Interestingly, tumours that accumulate intermediates of the purine *de novo* synthesis pathway can survive periods of nutrient starvation. For instance, cancer cells that accumulate SAICAR retain viability after two days of glucose depletion (Keller, Tan, & Lee, 2012).

Spatial localization of the purine synthesis machinery within the cell is a comparatively new research area. Purine synthesizing enzymes colocalize in complexes—purinosomes, which are formed when there is an increased demand for purines (e.g., during cell proliferation in the G1 phase). In the case of tumour formation, rapid cell growth is observed and purinosome formation thus might become another yet emerging drug target for acute cancer types like leukaemia (Pedley & Benkovic, 2017). Formation of purinosomes has been confirmed in mammalian cell lines as well as in budding yeast (Narayanaswamy et al., 2009; Shen et al., 2016).

Due to the conservation of purine and folate metabolism across eukaryotes and the ease of cultivation and genetic manipulation, budding yeast can be used to model each of the aforementioned aspects of tumourigenesis. In this context, purine auxotrophic strains provide ample options for how to model genetic and metabolic processes in cancer cells and to find novel cancer drug targets. Purine *de novo* synthesis mutants form small subpopulation of cancers; however, these are often relapsing cases. Insensitivity to traditional antimetabolite treatment and poor prognosis are the main reasons for seeking new druggable targets against these cancers specifically (Li et al., 2015; Zaza et al., 2004). Yeast might be practical to explore the potential evolution trajectories of particular purine mutant/s in the presence of antimetabolite drugs. Knowledge of the mutation spectrum leading to relapsing cancer cases and the specific treatment used for each of these cases might serve as starting point for modelling cancer evolution under the selection pressure of antimetabolites in yeast. For example, methotrexate treatment in yeast promotes the survival of *sec21* mutants, a human *COP1* analog (Wong et al., 2017). Results from yeast evolution experiments complement the data from mice and human studies, where mutations in *COP1* cause malignancies (reviewed by Marine, 2012).

It seems that a shift to purine auxotrophy in some environmental contexts could be beneficial rather than debilitating. Increase in stress resistance when starved for purine is an indirect proof for this. Adenine starvation in *S. cerevisiae* purine *de novo* synthesis mutants (*ade2*, *ade8*, and *ade4*) evokes resistance to stress (heat, weak acid, and oxidative), increases desiccation tolerance, arrests the cell in G1 phase, and increases the cell half-life (Kokina et al., 2014; Matecic et al., 2010; Ozolina, Kokina, & Liepins, 2017). These observations together with those of Martin et al. (2016) regarding increased viability of purine-starved *Leishmania donovani*, indicate the existence of some universal response to purine depletion within the cell.

Additionally, if the number of purine auxotrophs is small, a prototrophic population can sustain them just because of cross feeding phenomena from cell debris or metabolite leakage. Cross feeding has already been demonstrated to sustain microbial communities with different substrate and metabolic intermediate preferences (Campbell, Herrera-Dominguez, Correia-Melo, Zelezniak, & Ralser, 2018). Indeed, dead cells in a population can provide purines to sustain the growth of purine auxotrophs in synthetic communities (Shou, Ram, & Vilar, 2007). Yeast populations are already known to accumulate metabolic mutations and retain these mutants in the population for longer times. Accumulation of petite mutations in beer tanks is a practical example of this (Jenkins et al., 2009).

Even in rapidly growing yeast cell populations, there will always be a subgroup of "outliers": cells with small specific growth rates, low intracellular concentrations of cAMP, and decreased PKA pathway activity. The reason that these cells are sustained in the population is coined as a "bet hedging" phenomenon—these cells are potential survivors of many sudden and severe environmental stresses (Li, Giardina, & Siegal, 2018). In sudden and harsh environment stresses (like heat shock), only these few outliers will survive (Levy, Ziv, & Siegal, 2012).

Purine-starved eukaryotic cells are more capable of surviving severe stress compared to rapidly growing prototrophs; therefore, we think that the ecological and evolutionary reason for so many instances of naturally occurring purine auxotrophs might be (at least partly) their increased stress resistance (Kokina et al., 2014; Martin et al., 2016).

## 5 | CONCLUSIONS

Due to the ease of cultivation and accumulated knowledge on budding yeast physiology and genetics, many eukaryotic drug screens can be performed in *S. cerevisiae*. Therefore, the effects of the cultivation broth should be considered. In case of purine mutants, potential changes in cell growth patterns and phenotype in the case of an insufficient adenine supply should be taken into account.

A growing body of research demonstrates the use of *S. cerevisiae* as a model for diseases caused by purine metabolism in humans (reviewed in Daignan-Fornier & Pinson, 2019). Because purine depletion in auxotrophic cells/organisms elicits a similar phenotype, which in many cases is undesired (resilient to some drugs and treatments), budding yeast are a practical instrument for modelling and solving these problems. We think that application of purine auxotrophic budding yeast strains could be used more often in the search for new drug targets against parasites as well as cancer. Simultaneously, the yeast model can provide insights on the evolution of purine auxotrophy

and elucidate how the metabolic state of an organism can affect the survival of a population.

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# 4. Discussion

## 4.1. Care should be taken when using adenine auxotrophs in research

In research published in Kokina et al. 2014 we used strain W303-1A. This strain carries *ade2-1* point mutation that introduces stop codon prematurely. Strains of *ade2* and *ade1* mutants have been used in yeast research quite extensively due to their red pigmentation that develops late phases of growth when adenine is depleted from media. The accumulation or lack of pigment has been the basis not only for selection of the *ade1* and *ade2* mutants, but also has been employed in the research of mitotic and meiotic recombination (Johnston, 1971), amyloid aggregation (Bharathi et al., 2016) and *petite* identification and mtDNA research (Shadel, 1999). The red pigment itself seems to be composed of polymerised ribosylaminoimidazole molecules varying in molecular weight and containing a number of amino acids. (Smirnov et al., 1967). Later research has shown that accumulated intermediate aminoimidazolribotyl (AIR) form cytosol are glutationated and delivered in vacuole via GRX4 transporter, where it is polymerised and modified with amino acids giving rise to the red pigment that is autofluorescent and does not leave vacuole. (Smirnov et al., 1967; Fisher, 1969; Sharma et al., 2003, Jainarayanan et al., 2020). While red pigmentation is fairly noticeable if cells are grown on solid media, in liquid cultures it is less distinguishable. Red pigment gives autofluorescence to cells during imaging and hinders cell visualisation (Weisman et al., 1987), so usually care is taken to use adenine-enriched media when growing cells for visualisation studies. On the other hand, stress physiology research is quite often performed on adenine auxotrophs after an exponential growth phase in rich media, without any additional supplementation (Carrasco et al., 2001, Petrezselyova et al., 2010). Our research points to drastic changes in cell physiology after adenine depletion such as changes in stress resistance, macromolecular composition and optical properties of the cells. Care should be taken when using full media, as this can lead to biased conclusions of causes of the effects observed if that happens after adenine depletion sets in.

## 4.2. Internal purine resources are sufficient to finish the cell cycle

If adenine is present in the media it will be transported into the cells with the help of Fcy2p transporter at fairly consistent speed. Chemostat measurements for growth rate 0.015 - 0.14 $h^{-1}$ show adenine uptake rate 30-40 mg adenine per g dry cell weight (vanDusen et al., 1997). If adenine is provided in excess in the media, only a fourth of uptaken adenine is incorporated in nucleic acids as purine bases. The rest is used for nucleotides and nucleosides needed for cell metabolism and the excess can be converted into hypoxanthine and stored in vacuole (Burrige et al, 1977, Reichert Winter, 1974, Sharma et al., 2003, Jainarayanan et al., 2020). Our measurements also show that growth of W303-1A in synthetic media happens with

the same rate if adenine is present and only after exhaustion of adenine cell growth curves start to differ (Kokina et al., 2014, Fig4), confirming that exponentially growing cells should be the same, irrespective of adenine content in the media.

Yeast extract peptone (YEP) media is the most common media used in cultivation of yeast. Nucleotides and corresponding bases in this media are provided only by yeast extract that is obtained by autolysis of yeast cells. Most of the nucleotides are coming from RNA degradation as it is the most abundant nucleotide source in cells. Zhang and colleagues (2003) have shown that adenine content in yeast extract varies batch by batch due to variability of the autolysis process. Addition of extra adenine did increase biomass yield of adenine auxotrophic strain irrespective of adenine content in the YE batch, proving adenine to be a limiting nutrient in yeast extract. Our research (Kokina et al., 2014) agrees with VanDusen et al. (1997) observation that adenine auxotrophs stop their growth before exhaustion of glucose if grown in YEPD. We also show that additional 100 mg/L adenine is the amount of adenine when growth of adenine auxotrophs in SD is not limited by adenine. Our research published in 2021 shows that most adenine auxotrophs arrest cell cycle in G1 phase already after 1,5-2 h of adenine exclusion from media showing that internal supplies of nucleotides are sufficient for finishing the nucleic acid synthesis for cells that were in the middle of cell cycle when adenine was excluded.

## 4.3. Are purine starved cells quiescent?

In multicellular organisms cells are not dividing all the time. Also unicellular organisms have times when growth is limited. If such a non-dividing cell has lowered metabolic activity, increased stress resistance and is capable of resuming growth - these are called quiescent cells.

Ability to stop the cell cycle is one of the prerequisites for the cell to become quiescent. When depleted of purines, purine auxotrophic cells arrest most of the cells within the first two hours of starvation as can be seen in Kokina et al. (2022) Fig 2a, allowing to speculate that adenine starved cells may be quiescent.

When examining purine starved yeast cells many hallmarks of the quiescence are observed - not only cell cycle is stopped, but also we see accumulation of storage carbohydrates, decrease of cellular concentration of RNA and downregulation of ribosome biosynthesis. In figure 5 different aspects that had been reviewed in quiescent cells by Sun and Gresham 2020 are compared with our observations in adenine starved cells.

| | | Proliferative cell | Quiescent cell | Purine starved cell |
|---|---|---|---|---|
| Morphology changes | Cell size | ↑ | ↓ | ↑ a, b |
| | Cell wall | ↓ | ↑ | NA |
| | Cell density | ↓ | ↑ | ↑ a |
| Molecular changes | Cell cycle arrest | ↓ | ↑ | ↑ a, b |
| | Autophagy | ↓ | ↑ | NA |
| | Metabolism | ↑ | ↓ | ↓ b |
| | RNA content | ↑ | ↓ | ↓ b |
| | Ribosome biosynthesis | ↑ | ↓ | ↓ b |
| | Translational repression | ↓ | ↑ | NA |
| Cellular changes | Vacuole/Lysosome | ↓ | ↑ | NA |
| | Cytoplasm mobility | ↑ | ↓ | NA |

Figure 5. Comparison of fast growing cell and quiescence hallmarks from Sun and Gresham (2020) with properties of cells we observe in our research. NA - not assessed, data in a - Kokina et al., 2014, b - Kokina et al., 2022

Many quiescence hallmarks do correspond directly to our observations in purine starvation. Some others we can extrapolate from our data: for example glucose specific uptake rate and $CO_2$ generation drops when cells are adenine starved, that would correspond to physiological parameter “slow down of metabolism”.

While many hallmarks that we observe during 4 h starvation do correspond with quiescent cells, if we compare long term survival of purine starved cells with cells starved for leucine and carbon, then purine starvation would fall in the middle of natural and artificial starvations (Kokina et al., 2014, Fig6A), similar to the methionine starvation described by Petti et al. (2011). We see that adenine starved cells have lost half of their population by starvation day 1, which corresponds to the observations of Henry (1973). Chronological life span extension is characteristic for quiescent cells. Yeast strain used by us was not only adenine auxotroph but also histidine, leucine, uracil and tryptophan auxotroph, also strain used by Henry harboured several auxotrophies. It may be that these other auxotrophies play a role in long term starvation survival. To elucidate that pure adenine auxotroph long term survival without additional auxotrophies in genetic background would be needed.

Most effects that we observed are pronounced already after 4h cultivation in adenine depleted media and are purely adenine starvation caused as other auxotrophic agents are still present (Kokina et al., 2022, Fig 1c). When starved for purines, yeast consumes less glucose and carbon is rerouted to acetate and glycerol (Kokina et al., 2022, Fig 3b). From biomass FTIR analysis we see an increase in lipid relative content (Kokina et al, 2022, Fig 4a). Glycerol in yeast can be used as osmoprotectant but is also important in lipid synthesis. Another purpose of glycerol synthesis is to act as a NADH sink if oxidation in the respiration chain is not available (Klein et al., 2017). Acetate production on other hand produces NADH thus the production of glycerol and acetate to balance redox cofactors is less probable. Acetate is used

in acetylCoA synthesis that is substrate for fatty acid synthesis. As we observe massive accumulation of trehalose and glycogen, it is possible that additionally carbon is stored in lipid form that causes an increase of glycerol and acetate synthesis. Lipid accumulation is also observed in nitrogen starvation if glucose is present (Albers et al., 2007).

When comparing phenotype of purine starved cells with quiescent cells one observation clearly contradicts quiescent cell hallmarks. Our observations show an increase in the cell size of adenine starved cells. The increase in cell size was observed independent of genotype - W303 with *ade2* (Kokina et al., 2014, Fig3 and S4) and CEN.PK background with *ade8* mutations (Kokina et al., 2022, Fig2c). Cells continue to swell during starvation and will increase their cross section up to three times during 10 days of starvation (Kokina et al., 2014, S4). While we can not definitely say what is the reason for swelling, considering increase of storage carbohydrates in cells by approximately 20% of total dry weight during the first 4 h of starvation (Kokina et al., 2022, Fig 4b), one can speculate adenine starved cells accumulate storage molecules that leads to increase in cell size. Trehalose accumulation is observed during nitrogen starvation not only in *S. cerevisiae* (Klosinska et al, 2011) but also in fission yeast *Schizosaccharomyces pombe* (Sajiki et al., 2013). Trehalose and glycogen are storage carbohydrates, synthesis of which are regulated by several hierarchical systems namely TOR, PKA, Snf1p, Pho85p, and the energy sensor Pas kinase (François et al., 2012). It is known that depletion of sulphate, phosphate, or zinc is not accompanied by storage carbohydrate accumulation (François et al., 2012). As during the purine starvation researched by us there is abundant glucose in the media, we can assume that TOR signalling rather than PKA or Snf1 system is involved in regulation of observed increase of storage carbohydrates.

## 4.4. Purine starvation is similar but not quite the same as nitrogen starvation

Starvation able to induce quiescence is considered a natural starvation. Some auxotrophic starvations fall in the same category as they mimic starvation for some macronutrient: methionine starvation mimics sulphur depletion (Petti et al., 2011). Our observations show that metabolically purine starved cells resemble nitrogen starved cells - accumulation of reserve carbohydrates, shifting carbon flow towards glycerol. We have also compared stress resistance of adenine auxotrophs on otherwise prototrophic background and purine starved cell stress resistance is very similar to the nitrogen starved cells (unpublished data). At the same time some differences to the nitrogen starvation can be observed - purine starved cells also produce considerable amounts of acetic acid, that is not observed in nitrogen starved cells (Albers et al., 2007). Nitrogen starved cells also arrest the cell cycle in G1 and finish the budding process for cells that were in other cell cycle phases, but newly formed daughter cells are smaller than mother cells (Johnston, 1977), purine starved cells do not show appearance of smaller cells (Kokina et al., 2014, Kokina et al., 2022).

During nitrogen starvation a portion of the cytoplasm is non-selectively sequestered into autophagosomes. Consequently, ribosomes are delivered to the vacuole/lysosome for destruction, enzymatically cleaved until nucleosides and delivered into cytosol where they are

degraded further. After the start of the starvation nucleosides peak in the cytosol but they fall to initial or even below prestarvation levels within the first two hours of starvation. This is not observed in case of carbon or phosphate starvation or rapamycin induced growth arrest, where nucleosides stay in cytosol (Huang et al. 2015, Xu et al., 2013). Our results also show that purine starved purine auxotrophic cells will perform RNA degradation as the RNA amount in the cell decreases. We have not measured amounts of all nucleotides in cytosol, but the concentration of AMP, ADP and ATP has decreased. Interestingly decreasing amounts of ATP intracellular concentration is observed in phosphate starved cells, but not in nitrogen starved ones (Xu et al., 2013). Thus in the aspect of intracellular metabolite dynamics purine depletion resembles natural starvations - phosphate and nitrogen ones.

## 4.5. How purine starvation is perceived in cells? We propose that mechanisms additional to the Gcn4p response play a role. Possibly TOR mediated Rim15p governed response.

Phenotypic similarity of the purine starvation to the natural starvations leads to the hypothesis that lack of purines is perceived and signalled in the cell. If cells are placed in adenine deficient media with cycloheximide - translation suppressor – present, stress resistance phenotype fails to develop. Also addition of cycloheximide after 2 h of starvation lessens resistance, proving that transcriptional activity after cell cycle arrest is crucial for stress resistance development (Kokina et al., 2022, Fig 5b). This leads to consideration of what kind of transcriptional programs are active in purine starved cells and what triggers these programs in action.

While it is not known if the low levels of nucleotides would be signalled in the yeast cell *per se*, research on *Arabidopsis* shows that Rnr2 mutants with defective vacuolar rRNase undergo constitutive apoptosis, that can be rescued additional purines, but not pyrimidines in the cytoplasm. Other authors show that autophagy is induced via TOR signalling system (Kazibwe et al., 2020) This would indirectly suggest that cellular response to lowered cytoplasmic purine content is mediated via TOR. Similar research has not been performed on yeast cells, but mutant with overexpressed vacuolar RNase of the T(2) family RNY1, shows decreased oxidative stress resistance and chronological lifespan in stationary phase (MacIntosh et al, 2013), thus proposing that nucleotide balance plays a role in stress resistance also in yeast cells.

*PHO2*, also known as *BAS2* or *GRF10*, encodes a homeodomain transcriptional activator. Pho2p is required to express genes in several different pathways such as purine nucleotide biosynthesis, histidine biosynthesis, and phosphate utilisation. Genes that are known to be regulated by Pho2p include *PHO5, PHO81, HIS4, CYC1, TRP4, HO, ADE1, ADE2, ADE5,7 and ADE8* (Liu et al., 2000, Daignan-Fornier, Fink, 1992). Pho2p activates transcription along with one of at least three distinct partner proteins: Swi5p, Pho4p, and Bas1p. Pho2p and Pho4p cooperatively bind to the promoter site of *PHO5* (which encodes for a secreted acid phosphatase) and are required for PHO5 expression when cells are starved of phosphate (Bhoite et al., 2002). Pho2p and Swi5p together activate HO (Brazas, Stillman,

1993) while Pho2p and Bas1p activate genes in the purine and histidine biosynthesis pathways. Presence of AICAR promotes Pho2p and Pho4p interaction, whereas SAICAR - Pho2p and Bas1p, to upregulate ade *de novo* biosynthesis gene transcription (Pinson et al., 2009). While this shows interconnectedness of purine pathway regulation with phosphate pathway, in our case (*ade2* and *ade8* mutations) synthesis pathway is interrupted above synthesis of either - AICAR and SAICAR, thus, none of these interactions should be promoted and while there is some overlap in metabolomics with phosphate starvation it is not signalled on Pho2p level.

Ability to stop proliferation and acquire stress resistance phenotype is connected to the chronological lifespan of the cell. Mechanisms influencing chronological lifespan frequently overlap with quiescence ensuring ones. Most interventions that extend lifespan are, or induce, limited amounts of stress that have a beneficial effect via the phenomenon of hormesis, because these stresses would be toxic or lethal at higher doses. These lifespan-extending hormetic stresses induce a protective cellular stress response. This conserved stress response in yeast was termed the General Amino Acid Control (GAAC) because it was initially identified as a response to amino acid depletion that upregulates the genes required for amino acid synthesis (Hinnebusch, Fink, 1983). The scheme of the GAAC can be seen in figure 6. This pathway is also induced by a variety of conditions including starvation for purines (Rolfes, Hinnebusch, 1993) and is now frequently called Integrated Stress Response especially in mammalian cell research. When analysing transcriptome and phenotype of purine starved cells we also see that starved cells become stress resistant for variety of stressors (Kokina et al., 2002, Fig 5a) and Gcn4p responsive genes are upregulated - for example, autophagy connected (*ATG33, ATG34, ATG8* and others) for full list see Kokina et al., 2022, S2 and S3. In total around 70% of significantly upregulated or downregulated genes in our data set are connected to regulation by Gcn4p.

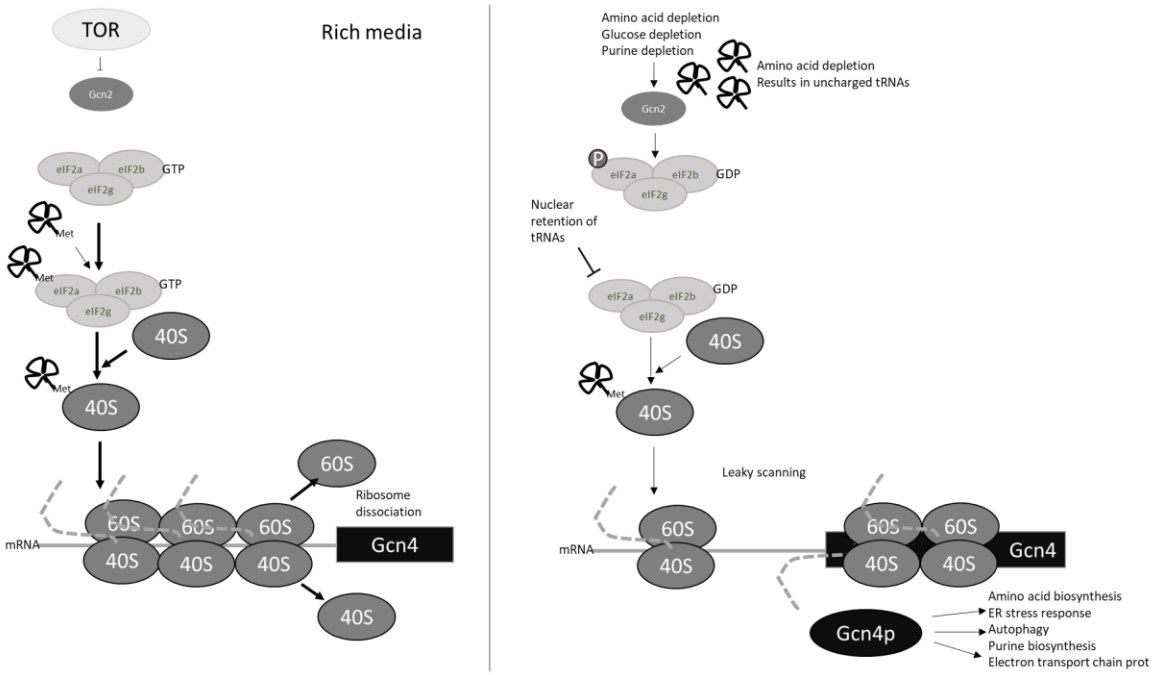


Figure 6. Model of Gcn4 expression regulation adapted from Postnikoff et al., 2017. In rich media conditions TOR suppresses Gcn2p and translation initiation factor eIF2 is able to mediate aminoacylation of 40S ribosome subunit. Several small inhibitory microORFs are located before the Gcn4 gene, thus the ribosome is dissociated before Gcn4 gene translation. In amino acid deplete conditions unloaded tRNA accumulation stimulates Gnc2 preventing functioning of eIF2. Due to leaky scanning of mRNA, Gcn4 protein is translated and induces transcription of a variety of genes.

Research by Rolfes and Hinnebusch (1993) shows that Gcn1p, Gcn2p, Gcn3p (alpha subunit of eIF2b) and Gcn4p are required for response to purine starvation, proposing that purine starvation is sensed with the same mechanisms as amino acid starvation. In severe purine starvation Gcn4 system is responding not only on translational, but also transcriptional level. Researchers note that purine starvation is sensed also in presence of all amino acids. Amino acid starvation is sensed by uncharged tRNAs. When examining *gcn2* mutants with point mutations researchers identified that response to the purines involve the same domains of Gcn2p as amino acid starvation. As for the aminoacylation of tRNAs ATP is needed and we know that during purine starvation absolute ATP concentration decreases, it may be that this causes accumulation of uncharged tRNAs that are sensed by Gcn2p. To elucidate this, additional research on the amount of tRNA and their charge during purine starvation would be needed.

tRNAs have been shown to have additional roles except delivery of amino acids to the ribosomes. TOR complex is directly regulated by presence of tRNA (Kamada, 2017). Nutrient starvation induces transport of tRNA to the nucleus (Whitney et al., 2007), thus there is possible overlay of tRNA control over cell signalling systems besides Gcn2p mediated GAAC that may be involved also in purine starvation perception.

Rolfes and Hinnebusch (1993) also note that expression of purine *de novo* synthesis genes is regulated by additional mechanisms, as Gcn4p mediated response does not fully explain transcriptional answer during purine starvation. Our data (Ozoliņa et al., 2017) shows that other transcription factors, especially Rim15p plays a role in purine starvation phenotype development. Rim15p also have been shown to be the hub connecting various starvations to the quiescence phenomenon (Sun et at., 2020), as purine starved cells do show several quiescent cell hallmarks, Rim15p mediated response could be responsible for at least a part of purine starved cell phenotype.

Transcription analysis of our strain when starved for purines shows a high number of genes that are up or downregulated compared to the fast growing cells (Kokina et al., 2022). When performing analysis with these genes in YEASTRACT to identify possible transcription factors that would cause this expression pattern Gcn4p does correspond to roughly 70% of our upregulated and downregulated genes, but is deemed as statistically insignificant (Kokina et al., 2022, S2) as opposed to Msn2p and Msn4p, that does explain roughly the same amount of genes, but are shown as statistically significant ($p<0.05$) possible regulators of the dataset. This also strengthens our hypothesis that Gcn4p driven response is not the only factor responsible for purine starved phenotype development.

When analysing possible transcription factors governing transcriptional response during purine starvation, a variety of transcriptional factors are proposed as statistically significant. Highest coverage of all significantly regulated genes is shown by transcription factors Msn2p and Msn4p that are shown to upregulate 75% and 68% of upregulated genes in our dataset. Msn2p and Msn4p mediate the so-called environmental stress response (ESR) (Gasch, Verner-Washburne, 2002). ESR is also considered a hormetic response. When we compare genes regulated by these transcription factors, we can see that while there is an overlap each of these transcription factors do regulate different gene sets (Figure 7). For example, Msn2p and Msn4p regulates trehalose accumulation process, that is not regulated by Gcn4p

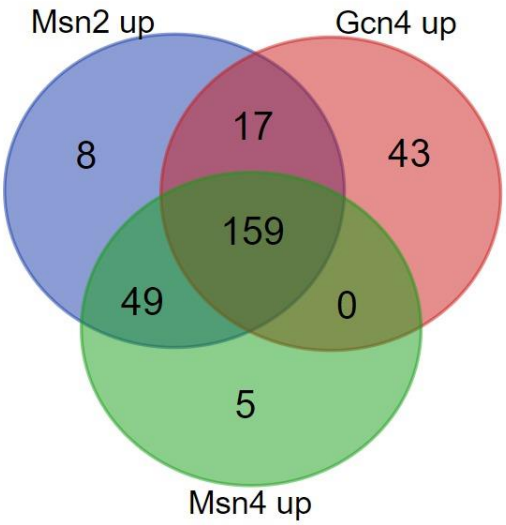


Figure 7. Comparison of purine starvation upregulated genes (logFC>2) proposed to be regulated by Msn2p, Msn4p and Gcn4p by YEASTRACT.

Both Msn2p and Msn4p are regulated by Rim15p (Orzechowski Westholm et al., 2012), that corresponds to our observations of significance of Rim15p in purine starvation phenotype (Ozoliņa, 2017), Rim15p also induces Gis1p, that would explain 40% of our upregulated genes. Suppression of Gis1p has been shown to produce higher glycerol and acetate yields (Orzechowski Westholm et al., 2012). Rim15p transport in the nucleus depends on PKA and TOR signalling systems. Partial TOR involvement in purine starvation phenotype is also confirmed by rapamycin treatment. If fast growing cells are treated with rapamycin, stress resistance grows, but does not reach purine starvation levels. On the other hand, purine starved cells do not increase stress resistance after rapamycin treatment (Kokina et al., 2022). This would confirm that the TOR system is involved, but not the only one, governing purine starvation phenotype.

A link between TOR and GAAC has been demonstrated in *S. cerevisiae*. TOR prevents dephosphorylation of Gcn2p by inhibiting one or more phosphatases. Phosphorylated Gcn2p will have lower ability to bind uncharged tRNA, thus a suppression of TOR system is needed for pronounced GAAC response (Cherkasova, Hinnebusch, 2003; Kubota et al., 2003). Our experiments show that stress resistance when cells are incubated in ade- environment with rapamycin added in the very beginning of starvation is lower than cells that have spent 2 h in environment without purines and then incubated for two more hour in presence of rapamycin (Kokina et al., 2022, Fig 5b) thus the interplay between TOR and GAAC in purine starvation is more complex than TOR repression allowing GAAC to take place.

At the same time several other transcription factors are highlighted by YEASTRACT - Fhl1p, Rme1p, Cat8p, that explain smaller amounts of upregulated genes (56%, 18%, 7%) are involved in stress response that is connected to the DNA replication stress. There is evidence that purine starvation slows down replication forks in purine auxotrophic chinese hamster cell line (Zannis-Hadjopoulos, 1979). We know that purine starved cells finish their cell cycle even if purine deprivation happens in the middle of cell cycle. While we can assume that during

purine starvation there will be purine shortage for DNA building process that may cause replication stress, it is not the main reason for purine starvation phenotype judging from the gene expression data.

## 4.6. Transcription of purine starved cells does not agree with the observed metabolome

While we see strong phenotypic response to the purine starvation on transcriptional level there are several conflicting pathways with observed changes - upregulation of glycolysis and citric acid cycle enzymes while glucose flow is diminished. Upregulation of trehalose and glycogen cleaving enzymes while we see accumulation of trehalose and glycogen. Upregulation of some glycolysis and gluconeogenesis, fatty acid synthesis and degradation enzymes at the same time. We have also seen that translation is crucial for stress resistance phenotype development in purine starvation (Kokina et al., 2022). This leads to the thought that while there may be active transcriptional response to the purine starvation, endeffect of the purine starvation phenotype on the metabolic level is governed mostly by posttranscriptional regulation.

Flux through the metabolic pathways is governed not only by the amount of enzymes but also availability of the substrate and activity of the enzymes. Activity of the enzymes can be influenced by posttranslational modifications, of which phosphorylation is the most common one (Oliveira, Sauer, 2012). For example key enzymes governing equilibrium between glycogen and trehalose accumulation or cleavage are regulated by phosphorylation. Glycogen synthase (Gsy2p) is more active in a non phosphorylated state, while glycogen phosphorylase is active in its phosphorylated state. Phosphorylation of Gsy2p is governed by cyclin dependent regulators, most notably Pho85. Similarly, the trehalose cleavage enzyme neutral trehalase Nth1p is active when phosphorylated by the PKA system (François et al., 2012). While ATP concentration in purine starved cells falls to 0.3 of initial amount (Kokina et al., 2022) it is unlikely that lack of ATP *per se* is the cause of changes in protein phosphorylation levels, as the common cofactors such as NAD, ATP are usually found in concentrations that are one or two fold higher that Km of the enzymes (Nelson, Cox, 2017).

Pinson et al. (2019) shows that the amount of NAD+ in the cell depends on the amounts of ATP, thus, we may assume that in purine starved cells NAD+ may be in lower concentration. Database (http://growthrate.princeton.edu/metabolome), that was created from chemostat data produced by (Boer et al., 2010) allows to assess cellular concentrations of various intracellular metabolites during growth limited by C, N, P or uracil or leucine. Of the available metabolites I selected all containing adenine - AcetylCoA, adenosine, ADP, ATP, SAM, NADP+, NAD+, FAD, cAMP - ATP, ADP and NAD+ are deemed as growth limiting in phosphate limitations, whereas adenosine shows overflow metabolism in phosphate limitation and NAD+ overflows in C limitation. Rest of metabolites are not shown as growth limiting or showing overflow metabolism in any of the mentioned limitations. It must be kept in mind that in chemostats cells are still growing albeit at different speeds - a situation that is different from starvation, where no growth would be observed.

Methionine starvation is shown to influence mTOR via the concentration of SAM in mammalian cells (Gu et al., 2017). In yeast, methionine levels are monitored and specific Met4p acts as a transcription factor that is activated during lack of methionine. But also in the yeast cells methionine influences TOR activity via SAM concentration and Ppa2p methylation. In low SAM concentrations TORC1 is inactive, tRNAs are not thiolated, growth stops and autophagy is promoted (Lauinger, Keiser, 2021). As for synthesis of SAM adenine is needed and we see similarities in purine starvation and methionine starvation, intracellular SAM concentration measurements during purine starvation would be a valuable tool to assess the impact of this TOR system branch on yeast cells. Data from chemostats indicate that SAM concentrations rise in cells as growth rate increases in C, N and P limitations and overall SAM concentration is higher in P limited cells, but SAM concentration and growth speed shows negative correlation in leucine and uracil limitations. It must be also considered that we see downregulation of SAM synthesis (*MET6, SAM1*) and THF metabolism connected *(MIS1, MET13, ADE3*) genes in purine starved cells (Kokina et al., 2022, Sup3). If methylation processes are affected, we could also see a regulation of posttranslational modifications on this level.

## 4.7. What is the place of purine starvation in evolutionary landscape

The most widely accepted model of the origin of life states that organic molecules arose via simple chemical reactions. The Miller-Urey experiment is one of the most famous examples of such a process. It has been shown that purine nucleotides also could form under conditions likely present on primitive Earth (Oro, 1961, Nam et al., 2018). ATP - the main energy carrier in the cell is also shown to be able to form in prebiotic conditions, and accept phosphoryl group more readily as other nucleotide diphosphates (Pinna et al., 2022). As purines form DNA and RNA required for maintenance and expression of genetic information and also are included in a variety of cofactors purine synthesis are under strong positive selective pressure.

Purine synthesis pathway is conserved across all domains of life. While enzymes and their cofactors may differ in eukaryotes, prokaryotes and archaea, the intermediates of the pathway will always be the same with the exception of $N^5$-CAIR ($N^5$-carboxyaminoimidazole ribonucleotide), which is bypassed in eukaryotes (Chua, Fraser, 2020). Notable exceptions are intracellular parasites, especially parasitic protozoans, who have lost their *de novo* synthesis pathway completely. Parasitic protozoa are an evolutionarily divergent group of unicellular eukaryotes that are responsible for a wide range of human and veterinary diseases. The most clinically relevant protozoan parasites are the apicomplexans *Plasmodium spp*. and *Toxoplasma gondii* (the causative agents of malaria and toxoplasmosis, respectively) and the trypanosomatids *Trypanosoma brucei, Trypanosoma cruzi* and *Leishmania spp.*, which cause African sleeping sickness, Chagas' disease, and leishmaniasis, respectively (Gazanion, Verges, 2018). These parasites are usually intracellular parasites and during the evolution have obtained various auxotrophies thus they must rely on a host for these metabolites. Although many of these parasites have complicated life cycles with two or more hosts, loss of the purine *de novo* pathway has been evolutionarily advantageous for them. Loss of biochemical synthesis

pathways (asparagine, phenylalanine, biotine and others) in the nutrient rich environment has been shown with *E. coli* (D'Souza et al., 2016, D'Souza, Kost, 2014). This loss leads to the fitness increase in the new evolved strains. Overall, in long term evolutionary laboratory experiments with fixed media, an increase of fitness with the cost of adaptability has been observed (Couce, Tenaillon, 2015). Interestingly, genetic analysis points to the auxotrophic lifestyle may have evolved before these protozoans became parasites. For example *Bodo saltans* - free living relative of *Trypanosoma* and *Leishmania* is already a purine auxotroph (Jackson et al., 2016, Januskovec, Keeling, 2016).

Most of parasitic protozoans have lost purine *de novo* biosynthesis genes and rely on productive salvage reactions for their intracellular purine pools, interestingly, pyrimidine auxotrophs are less common (Gazanion, Verges, 2018). Purine auxotrophy was also more prominently found in the genetic screen for auxotrophies in Gram negative bacterias (Seif et al., 2020). Frequently loss of function is driven by higher fitness of the resulting strain, while it is known that amino acid auxotrophies identified by the Seif et al., are connected with host–pathogen interactions, suggesting that these auxotrophies may give selective advantage during host–pathogen interactions, the reason why purine auxotrophies are common is not yet well established.

Our data and data from *Leishmania* research (Carter et al., 2010) point to gain of stress resistance phenotype in the absence of purines. Gcn4p is proven to react to purine limitation in yeast (Rolfes, Hinnebusch, 1993) and plants (Lageix, et al., 2008). In bacteria nutrient limitation or starvation induces the stringent response. Stringent response depends on a transient increase in the level of (p)ppGpp that causes reduced accumulation of stable RNAs (rRNA and tRNA), transcriptional downregulation of genes linked to growth (e.g., ribosome biogenesis) and upregulation of genes required for survival (e.g., nutrient acquisition and stress responses), and it also directly binds a number of proteins to regulate their activity. While specific binding targets may differ between Gram-negative and Gram-positive species, (p)ppGpp has been shown to regulate replication, transcription, translation and GTP biosynthesis by binding to proteins that participate in these processes, including DNA primase, RNA polymerase, small GTPases, enzymes involved in purine biosynthesis, and transcriptional regulators. Considerable differences exist between bacterial species with regard to both the mode of action and metabolism of (p)ppGpp. In Gram-negative species such as *E. coli*, ribosomes sense the uncharged tRNAs at the ribosomal A site during amino acid starvation, causing protein synthesis to stall. This leads to synthesis of (p)ppGpp, which acts as an allosteric regulator of RNA polymerase (Sivapragasam, Grove, 2019). Similarities in stringent response and Gcn4 mediated GAAC point to a widespread purine sensing mechanism that is observed in various lineages of life.

# 5. Conclusions and direction of following research

- Yeast extract may be insufficient purine source for adenine auxotrophic strains and adenine is exhausted before glucose.
- After adenine exhaustion apparent increase in optical density of the culture is due to the cell swelling.
- Long term survival places adenine starvation between carbon and leucine starvations.
- Purine starved yeast cells arrest their cell cycle at G1/G0 phase in the first two hours of purine starvation.
- Purine starved yeast cells increase thermal, oxidative, weak acid and desiccation stress resistance by several orders of magnitude compared to fast growing cells.
- During the first four hours of purine starvation cells accumulate reserve carbohydrates and reduce glucose flow, reorienting part of carbon flux towards production of glycerol and acetate.
- Transcription analysis shows that 4 h purine starved cells downregulate transcription and translation processes similarly to other environmental stresses.
- Purine starved cells acquire stress resistance that depends on translation and is higher than rapamycin elicited resistance indicating additional cell signalling sistem activity.
- Pattern of gene regulation of purine starved cells highly overlaps with cells entering stationary phase.
- Transcription analysis indicates involvement of environmental stress response programm, that is signalled through Msn2p and Msn4p via Rim15p.
- Purine auxotrophic yeast cells are suitable as models for intracellular parasites as purine auxotrophy is a common phenomenon among parasitic monera, protozoans and metazoans.

After agglomeration of the results and the knowledge from literature following model of development of purine starvation phenotype can be made Fig 8

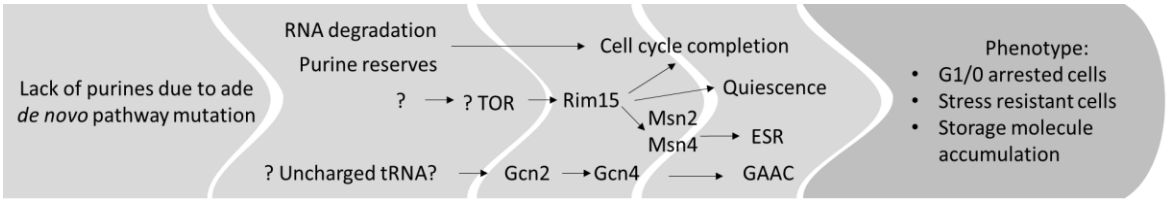


Figure 8. Summary of main characteristics of observed purine depletion phenotype and possible causes and signalling events leading to the phenotype discussed in the thesis.

Achieved results allows us to describe purine starved cell yeast cell phenotype that has not yet been described before. Results show that care should be taken if working with adenine auxotrophs to avoid purine depletion as that results in a pronounced stress resistance phenotype. While we have described the phenotype and some hypothesis on the signal for this phenotype are being made, to elucidate mechanism of cell signalling of purine starvation additional experiments are needed. Firstly a long term starvation without additional auxotrophies in strain background would allow us to better describe chronological life span of adenine synthesis mutants during purine starvation.

To better elucidate changes happening in cells during purine starvation tRNA dynamics and loading should be assessed during purine starvation. This would better describe the involvement of GAAC. For TOR system involvement, mutants defective in different parts of the TOR signalling system may be used to understand which parts are involved in purine depletion response.

To help with understanding if some intracellular adenosine metabolite such as SAM, cAMP is influencing phenotype development, the amount of all adenine containing metabolites could be measured. If some of these metabolites are indeed in significantly smaller amounts, it would be worthwhile to see if the changes in the metabolites are the cause or consequence of the purine starvation phenotype.

# 6.Theses for defence

Traditional compositions of rich media may be purine limiting for adenine auxotroph cells.

Internal purine reserves are sufficient for yeast cells to finish DNA synthesis after onset of purine starvation.

Yeast cells effectively react to the purine depletion by reorienting metabolism and arrest cell cycle via transcriptional response that is at least partially coordinated via Rim15p.

Purine starved cells exhibit quiescence like phenotype.

Purine auxotrophic starvation might be beneficial to parasitic organisms as preconditioning for other environmental stressors.

# Approbation of the research

Conferences:

Poster presentation Ozoliņa Z., Zīle A., Kokina A., Pleiko K., Kristjuhan A., Liepiņš J. Mutation location in the eukaryotic purine synthesis pathway determines response to nitrogen or purine starvation. "Levures, Modèles et Outils" meeting (LMO14), Strasbourg, France, 27. - 29.10.2021.

Poster presentation Ozoliņa Z., Zīle A., Kokina A., Pleiko K., Liepiņš J. Purine deprivation resembles nitrogen starvation in budding yeast. ICY15 meets ICYGMB30 congress, Vienna, virtual, 23.-27.08.2021

Oral presentation Agnese Kokina, Kārlis Pleiko, Zane Ozoliņa, Jānis Liepiņš Purīna trūkuma izraisītā globālā transkriptomikas atbilde maizes raugā. Global transcriptomic response to purine starvation in budding yeast. LU konference, Latvija, 19.02.2021

Poster presentation Kokina A., Pleiko K., Ozoliņa Z. and Liepiņš J. Role of mutations in purine de novo synthesis pathway in cell cycle arrest and stress resistance phenotype during purine starvation. ICYGMB 2019, Gothenburg, Sweden,18.08.22.08.2019.

Poster presentation Ozoliņa Z., Kokina A., Martynova J., Liepins J. 2017. How purine starvation is communicated inside the baker's yeast cell. 28th International Conference on Yeast Genetics and Molecular Biology (ICYGMB 2017), Prague, Czech Republic, 27.08. - 1.09.2017.

Oral presentation A.Kokina; I. Vamža, D. Lubenets, J. Liepins. General stress resistance phenotype is no cell cycle dependent in budding yeast Saccharomyces cerevisiae, 3rd Congress of Baltic Microbiologists 18-21.10. 2016 Vilnius, Lithuania

Oral presentation Liepins J, Kokina A, Nutritional effects on baker's yeast desiccation tolerance, 2nd Congress of Baltic Microbiologists 16.-18.10. 2014, Tartu, Estonia

Poster presentation A. Kokina, J. Kibilds, J. Liepins "Adenine auxotrophy effects on general and stress physiology in bakers yeast S. cerevisiae", Yeast 2013 26th International Conference on Yeast Genetics and Molecular Biology, Frankfurt am Main, German, September, 2013.

Oral presentation A. Kokina; J. Liepins "Trehalose metabolism in yeast S. cerevisiae - effects of common auxotrophies" 1st Congress of Baltic Microbiologists, Riga, Latvia, November 2012.

Poster presentation A. Kokina; J. Liepins "Role of ade8 mutation in stress resistance of yeast Saccharomyces cerevisiae CEN.PK strain background" 22nd IUBMB and 37th FEBS congress, Spain, Sevilla, September, 2012

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LZP-2021/1-0522 - From purine starvation to a stress-tolerant phenotype - uncovering the mechanisms.

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