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Changes in spermidine biosynthesis during chronological aging under methionine restriction in *Saccharomyces cerevisiae*

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„Education is not the learning of facts, but the training of the mind to think."
Albert Einstein (1879-1955)

Abstract

Aging is a process including the consecutive loss of function in cells and tissues over the course of time. It causes this functional decline and eventual the loss of viability, even in the absence of extrinsic causes of death. It is known that caloric restriction, without malnutrition, has beneficial effects for longevity and moreover the nutritional composition of the diet plays a role in lifespan extension. Methionine restriction (short: MetR), for example, leads to a prolonged lifespan by regulation of vacuolar acidity in autophagy dependent manner. Another life-prolonging determinant is spermidine, a polyamine found in all eukaryotes and most prokaryotes, whose level declines during the course of aging. Supplementation of spermidine leads to an increase in lifespan, up to four times longer than that of untreated cells. Since spermidine and MetR are interconnected in living organisms by the main substrate of polyamine biosynthesis S-adenosylmethionine (short: SAM), this project investigated the connection between MetR and spermidine biosynthesis, regarding lifespan extension in *Saccharomyces cerevisiae*. Mass spectrometric (short: MS) analysis could show that under MetR conditions SAM levels decreased, whereas spermidine levels stayed relatively high. Using Northern blot and qPCR analysis as analytical tools, this research showed that MetR, on the one hand, leads to an increase of spermidine biosynthesis enzymes namely upregulation of ornithine decarboxylase and spermidine synthase. On the other hand, a possible decrease of spermidine degradation and interconversion was found, indicated by downregulation of spermine synthase, polyamine acetyltransferase and polyamine oxidase, suggesting that the cell might try to retain a certain level of spermidine during aging under MetR conditions. This led to the conclusion that maintaining a certain level of spermidine (and thus its beneficial effects) may also be involved in the observed lifespan extension of yeast cells under MetR conditions in chronological aging.

Kurzfassung

Altern ist ein Prozess mit fortlaufendem Verlust der Funktionen in Zellen und Geweben. Ein Prozess der eine funktionelle Einschränkung und schließlich auch den Verlust der Lebensfähigkeit herbeiführt, auch in Abwesenheit von äußeren Todesursachen. Es ist bekannt, dass Kalorienrestriktion, ohne Mangelernährung, einen positiven Effekt auf die Langlebigkeit hat und darüber hinaus die Zusammensetzung der Ernährung eine Rolle bei der Verlängerung der Lebenserwartung spielt. Methioninrestriktion beispielsweise führt zu einer verlängerten Lebensdauer durch autophagieabhängige Regulierung der vakuolären Azidität. Eine andere, in der Literatur genannte, lebensverlängernde Determinante ist Spermidin. Ein Polyamin welches in allen Eukaryonten sowie den meisten Prokaryoten gefunden wird und dessen Level während des natürlichen Alterungsprozesses laufend sinkt. Supplementation von Spermidin in Hefezellen führt zu einer Erhöhung der Lebensdauer, um bis zu viermal länger als die von unbehandelten Zellen. Spermidin und MetR sind in lebenden Organismen durch das Hauptsubstrat der Polyaminbiosynthese S-Adenosylmethionin miteinander verbunden. Die, dieser Arbeit zugrunde liegende Forschungsarbeit, untersuchte die Verbindung zwischen MetR und Spermidinbiosynthese, in Bezug auf Lebensverlängerung in *Saccharomyces cerevisiae.* Eine massenspektroskopische Analyse der, für diese Forschung verwendeten, Hefestämme konnte zeigen, dass unter MetR Bedingungen die SAM-Level stark sinken, wohingegen Spermidin relativ hoch gehalten wird. Unter Verwendung von Northern-Blot und qPCR als analytische Werkzeuge, zeigte diese Arbeit, dass MetR einerseits zu einer Erhöhung der Spermidinbiosynthese über eine Hochregulation der Enzyme Ornithin-Decarboxylase sowie der Spermidin-Synthase führte. Andererseits wurde eine mögliche Verringerung des Spermidinabbaus und der Spermidininterkonversion gefunden, dargestellt durch eine Runterregulation von Spermin-Synthase, Polyamin-Acetyltransferase und Polyamin-Oxidase, was darauf hindeutet, dass die Zelle versuchen könnte, während ihres Alterungsprozesses unter MetR Bedingungen ein gewisses Level an Spermidin zu erhalten. Dies führte zu dem Schluss, dass der Erhalt einer bestimmten Menge an Spermidin in der Zelle auch in der beobachteten Lebensverlängerung von Hefezellen unter MetR Bedingungen im chronologischen Altern eine Rolle spielen könnte.

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1 Introduction

1.1 Budding yeast *Saccharomyces cerevisiae* as a model for aging research

When we talk about aging, we talk about a stochastic process including the consecutive loss of function in cells and tissues over the course of time. We talk about processes that cause this functional decline and eventual loss of viability, even in the absence of extrinsic causes of death (Piper et al., 2006).

To study the molecular processes of cells, including aging, different model organisms are used as important tools of biological research. The most common model organisms are fungi, bacteria, plants, flies, worms and mice; the list of all available model organisms is even longer (Entfellner, 2014). It turned out that *Saccharomyces cerevisiae* and *Caenorhabditis elegans* are useful systems in aging researches, as they are responsive in genetic and molecular analysis and manipulation. They show an increasing mortality rate over time, the same way it appears in human aging (Tissenbaum et al., 2002).

In yeast, both types of lifespan which are relevant for human aging can be observed and investigated: the replicative lifespan (ability to produce new cells, important for tissue regeneration and immune response) and the chronological lifespan (important for non-dividing cells, e.g., neurons). The replicative lifespan is measured by the number of daughters produced by each dividing mother cell. In contrast, chronological aging describes the capacity of stationary cultures to maintain viability over time (Piper et al., 2006). Chronological aging consists of three different stages of growth: after a short lag phase, during which the cells get “accustomed” to the media and its available nutrients, the first is the logarithmic growth - the cells divide rapidly and the metabolism is primarily glycolytic (on respective media). Secondly, a diauxic shift occurs and the culture switches to respiratory growth, which is followed by the final stage, the stationary stage. In this stage the cells stop dividing and the yeast can become resistant to many stressors, including heat and oxidative stress, and is able to survive in this state for a certain period of time before it starts to die (Tissenbaum et al., 2002; Apel, 2011).

In the last years, the examination of molecular determinants of aging has advanced rapidly and a lot of this progress can be attributed to studies in eukaryotic model organisms, such as *S. cerevisiae*. Regarding the use of yeast for aging research, there is increasing evidence that some of the pathways influencing longevity in yeast, are conserved in other eukaryotes, including mammals (Kaeberlein et al., 2007; Longo et al., 2012).

1.2 Yeast and longevity

In recent years, new insights on aging and longevity in yeast have been found. Although aging and lifespan extension is a very complex research field, already simple genetic mutations can cause a prolonged lifespan in model organisms. Many of these mutations diminish the activity of nutrient-signaling pathways, which indicates that they develop a physiological state similar to that experienced during periods of food scarcity in nature (Fontana et al., 2013). It is known that caloric restriction, without malnutrition, has beneficial effects for aging and moreover the nutritional composition of the diet plays a role in lifespan extension. These beneficial effects include lifespan extension and a decelerated aging process by regulation of nutrient signaling pathways like TOR, Akt and Ras (Entfellner, 2014).

MetR is a particular case of dietary restriction and promotes longevity by regulation of vacuolar acidity in autophagy dependent manner. It induces autophagy, restricts necrotic cell death, and mediates longevity of chronologically aging cells in *S. cerevisiae* (Ruckenstuhl et al., 2014). Furthermore, it diminishes the level of mitochondrial reactive oxygen species (short: ROS) and hence might lead to less oxidative damage to mitochondrial DNA and proteins.

Autophagy is a control and survival mechanism, and also an important regulator of energy homeostasis in a cell during nutrient stress (Singh, Cuervo et al., 2011). It is a response to nutrient depletion in which cellular macromolecules (for example aggregated proteins or damaged organelles) are recycled through vesicular transport and degradation in lysosomal or vacuolar compartments. Recent studies have shown that autophagy is related to age-associated diseases, such as neurodegenerative diseases, infectious diseases and cancers (Kaeberlein et al, 2007; Huang et al., 2007). As autophagy decreases during aging, it makes sense that the induction of autophagy

helps the cell to extend its lifespan, which could be shown in several studies. In nutrient rich conditions Tor, Ras, Sch9 pathways and Tap42-associated PP2A are the main negative key regulators of autophagy, whereas starvation or rapamycin treatment inhibit TOR pathway and induce autophagy. There is also evidence that polyamines, especially spermidine, can induce autophagy and lead to lifespan extension (Eisenberg et al., 2009; Morselli, Marino et al., 2011).

It is, for example, reported that supplementation with spermidine extends the lifespan of yeast, flies, worms and human immune cells and furthermore inhibits oxidative stress in ageing mice. The intracellular spermidine concentration and the total of intracellular polyamine levels decrease in ageing mammalian cell culture and during human aging. Until now, it has remained uncertain if the decrease of polyamines is a cause or a consequence of aging (Eisenberg et al., 2009). What we do know at this stage is that the lifespan extension in various aging models caused by spermidine administration (see Figure 1) is related to the suppression of necrosis, epigenetic modifications and an induction of autophagy (Eisenberg et al., 2009).

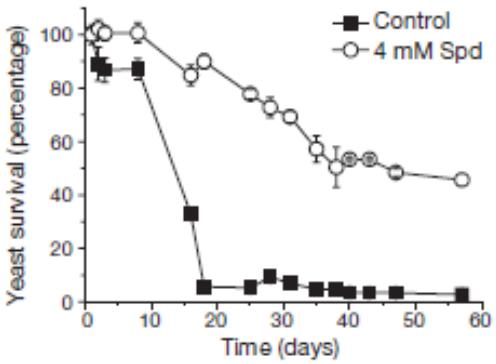

Figure 1. Lifespan extension in *S. cerevisiae* caused by spermidine administration. Survival determined by clonogenicity during chronological ageing of wildtype yeast (BY4741) cells with and without addition of spermidine (4 mM) at day 1. Data represent means ± s.e.m. (n = 5) (Eisenberg et al., 2009).

Autophagy can be induced by many different stressors and factors, in a process divided into two stages. The first stage of autophagy induction, the “rapid phase“, which occurs on a timescale of minutes to hours, depends on the one hand on posttranscriptional regulators and on the other hand on more conserved autophagic responses that rely on the execution of transcriptional programs. The main regulation

of autophagy at posttranscriptional levels is related to multiple (de-)phosphorylation reactions and a change in acetylation levels, as numerous components of the core-machinery are either kinases or kinase substrates and several of them have been shown to undergo changes in their acetylation status (Marino, Kroemer, 2014).

The mechanism of autophagy (see Figure 2) starts with the formation of a double-membraned vesicle, called the “autophagosome” in which cytoplasmic cargo is delivered to the lysosome. After that, the loaded autophagosome merges with the lysosome and the degradation of aggregated proteins, damaged organelles etc. starts. The components, obtained through degradation (amino acids, fatty acids etc.), can be re-used for several functions in the metabolism. Autophagy is a cellular ‘recycling factory’ able to advance energy efficiency through ATP generation and efficient damage control by elimination of non-functional proteins and organelles (Glick et al., 2010).

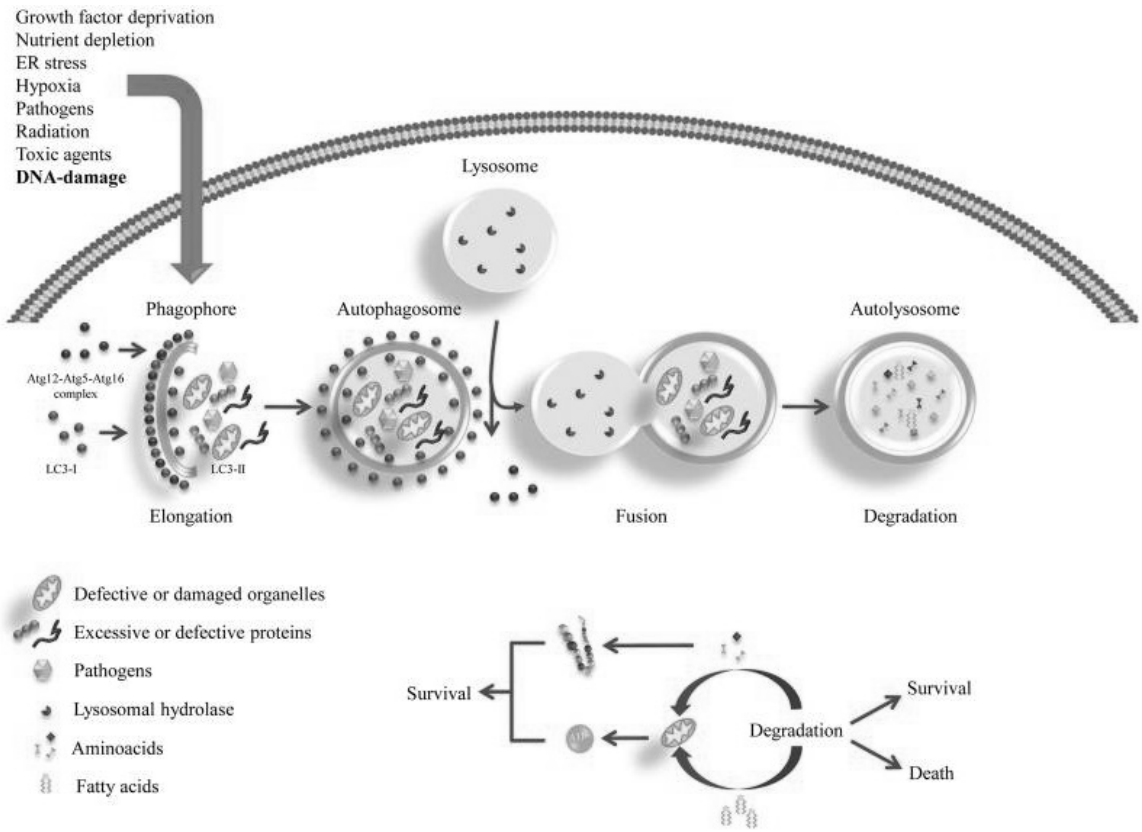

Figure 2. Mechanism of macroautophagy (Rodriguez-Rocha et al., 2012)

The dysfunction of autophagy plays a role in the development of cancer and neurodegenerative disorders such as Alzheimer's, Parkinson's, Huntington's etc. Therefore, autophagy is assumed as a potential therapeutic target for treatment of diverse diseases (Ravikumar B, 2004; Rubinsztein DC et al., 2007; Levine B, Kroemer G 2008; Madeo et al., 2009).

1.3 Polyamines and spermidine biosynthesis

Polyamines are polycations, found in all eukaryotes and most prokaryotes. Their main feature is to interact with negatively charged molecules, such as DNA, RNA or proteins. Polyamines are involved in many functions, which are mostly linked with cell growth, survival and proliferation. This makes them the subject of intensive research, in order to elucidate their functions in physiology. It was found that the polyamine levels decrease during aging, and related to that, that spermidine or a polyamine-high diet is able to extend lifespan in model organisms (Minois, Carmona-Gutierrez, Madeo, 2011).

Polyamine levels are regulated by three different components: catabolism, synthesis and transport. The synthesis of polyamines (see Figure 3), starts with the production of ornithine from arginine by an enzyme called arganase. The obtained ornithine is decarboxylated to putrescine, by ornithine decarboxylase (*SPE1*) (Minois, Carmona-Gutierrez, Madeo, 2011). Next, putrescine is converted into spermidine by spermidine synthase (*SPE3*) – needing decarboxylated S-adenosylmethionine (short: AdoMetDC) as an aminopropyl group donor. S-adenosylmethionine (short: AdoMet or SAM) is produced from L-methionine, and decarboxylated to AdoMetDC by S-adenosylmethionine decarboxylase (*SPE2*) (SGD, Saccharomyces Genome Database). It was found that intracellular levels of polyamines regulate the expression of S-adenosylmethionine decarboxylase, putrescine increases the levels of enzyme activity, and spermidine is able to reduce it (Valdés-Santiago et al., 2012). S-adenosylmethionine decarboxylase is a key enzyme in the synthesis of spermidine and spermine, having a massive influence on growth and sporulation of *S. cerevisiae,* as these are not possible without these two polyamines (Kashiwagi et al., 1990).

After spermidine synthesis, spermine synthase (*SPE4*) is able to convert spermidine into spermine using AdoMetDC as an aminopropyl group donor again. From spermidine there is a degradation step possible in this polyamine pathway, followed by an attainable rebuilding step of the degraded polyamine. The degradation catalyzed by polyamine acetyltransferase (*PAA1*), leading to N-acetylspermidine – and the possibility of a "rebuild" of spermidine via polyamine oxidase (*FMS1*). The same applies to spermine (SGD, Seiler et al., 2005 J. Cell. Mol. Med.).

Previous studies indicate that spermidine is crucial for differentiation and vegetative growth, while spermine is not essential but is involved in stress response, apparently through the modulation of cation channel activities (Takahashi et al., 2010). Putrescine seems to play a minor role in stress response (Valdés-Santiago et al., 2012).

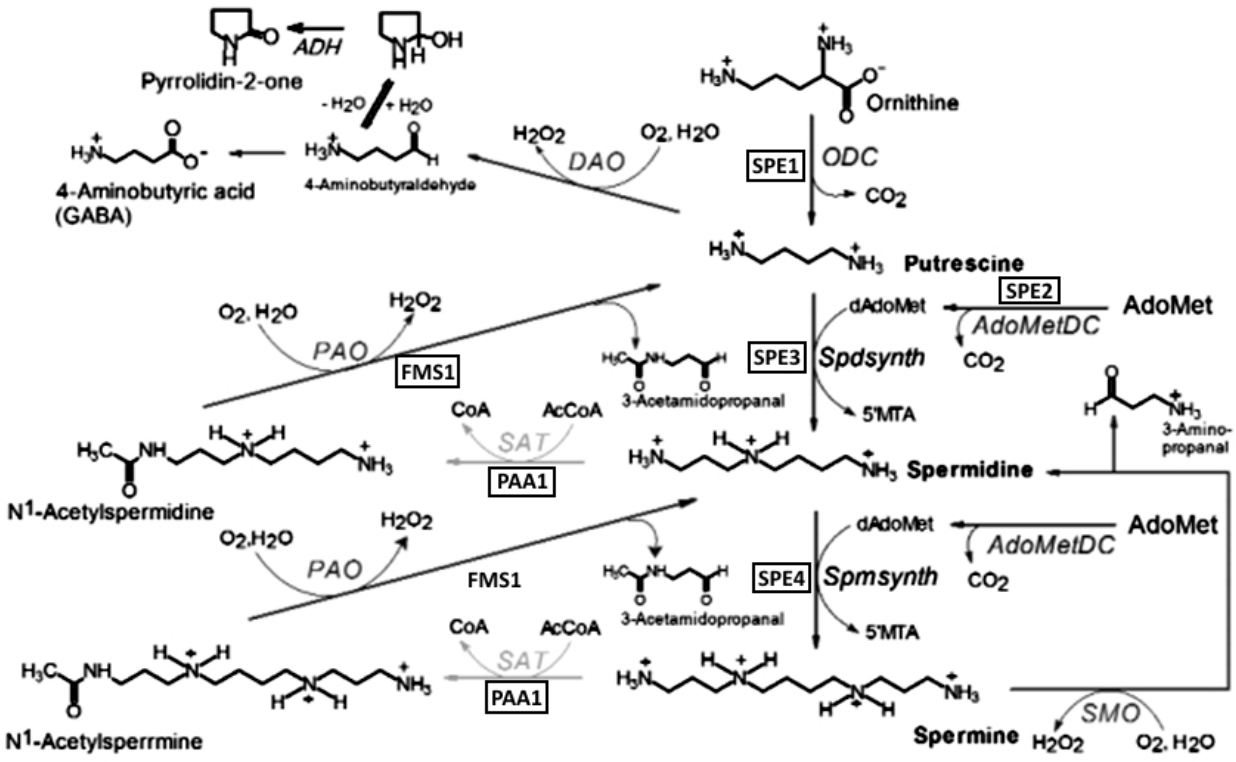

Figure 3. Spermidine biosynthesis (Sabrina Schröder; „Polyamines and apoptosis", Seiler et al., 2005 J. Cell. Mol. Med.)

Studies have shown that polyamine catabolism is a dominant factor in apoptosis, drug response and stress response and has an influence on several diseases, including cancer (Casero et al., 2010). Polyamine catabolism is limited by the cytosolic

polyamine N1-acetyltransferase (*PAA1*), which acetylates spermidine and spermine. After the conversion, the acetylated polyamines are either exported or they move to the peroxisome, where the polyamine oxidase (*FMS1*) oxidizes them (Minois, Carmona-Gutierrez, Madeo, 2011). This oxidation results in the production of H_2O_2, 3-acetoaminopropanal and putrescine or spermidine, depending on the initial substrate (Soda et al., 2011). Under normal conditions the polyamine acetyltransferase is present in small quantities, but it can be quickly and massively induced by high polyamine conditions, high amounts of polyamine analogous and by a diversity of pathophysiological stimuli (Casero et al., 2010).

2 Objectives of this thesis

The aim of this research was to get a better understanding of the recent findings on MetR inducing longevity in *S. cerevisiae*. Besides the regulation of vacuolar acidity in autophagy dependent manner (Ruckenstuhl et al., 2014), several other influencing factors might play a role in this process and we wanted to focus on the role of spermidine and spermidine biosynthesis regarding MetR strains.

Methionine is the precursor of S-adenosylmethionine. Recent data (see Figure 4) could show that MetR conditions lead to a strong decrease in S-adenosylmethionine levels especially on day one of aging. As SAM, the aminopropyl group donor in spermidine biosynthesis, is decreased in MetR strains, it is conspicuous that although SAM levels go down, the polyamine levels of putrescine and spermidine remain relatively high (see Figure 5 & Figure 6). This led to our hypothesis that there might be an up- or deregulation of various enzymes in spermidine biosynthesis to retain high spermidine levels with the purpose of retaining the beneficial effects of spermidine in living organisms and without undermining the positive effects of MetR on longevity. Since SAM, as the main methyl-group donor, may also have an influence on histone methylation and thereby affect transcriptional regulation, we decided to additionally investigate the methylation status of histone H3 via western blot analysis.

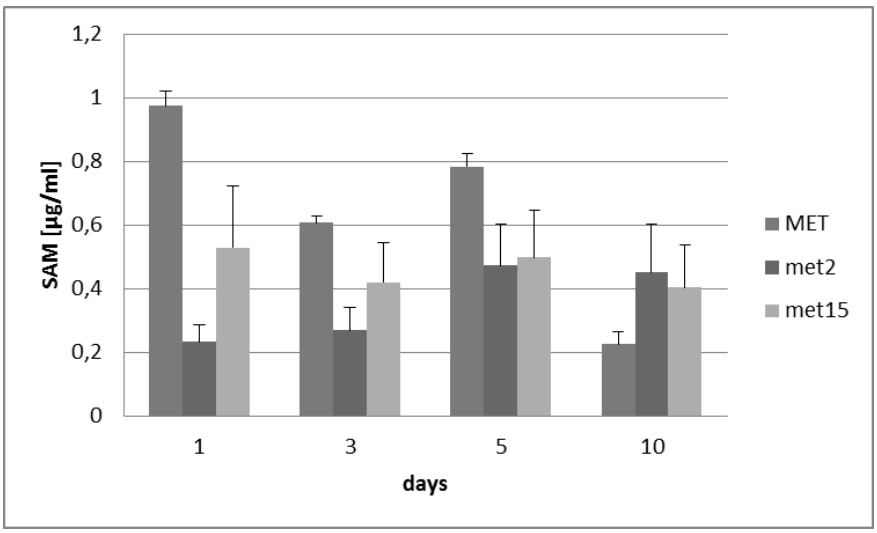

Figure 4. MetR leads to a strong decrease in S-adenosylmethionine levels. MS measurement of SAM levels in aging MetR strains Δ*met2* and Δ*met15* vs. *MET*+ wildtype. Agings conducted in SMD with all amino acids (short: +AA) (Christoph Ruckenstuhl, unpublished data).

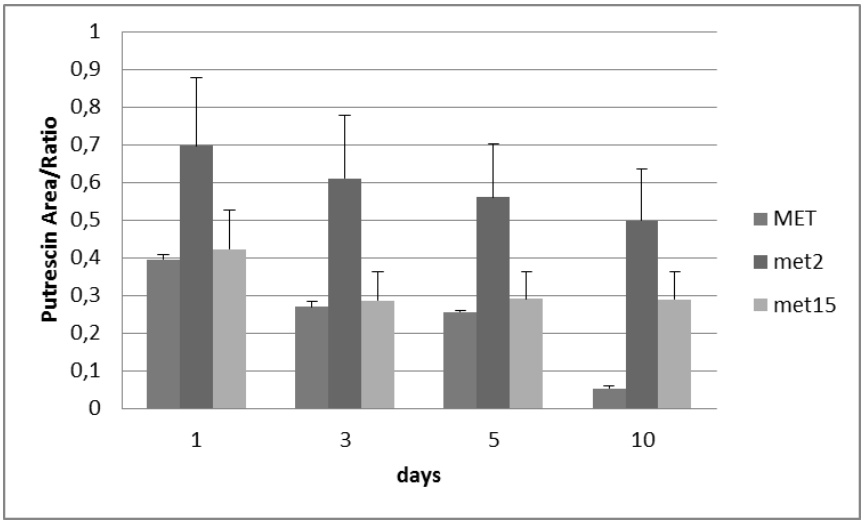

Figure 5. MS analysis showed an increase of putrescine levels under MetR conditions. MS measurement of putrescine levels in aging MetR strains Δ*met2* and Δ*met15* vs. *MET+* wildtype. Agings conducted in SMD+AA (Christoph Ruckenstuhl, unpublished data).

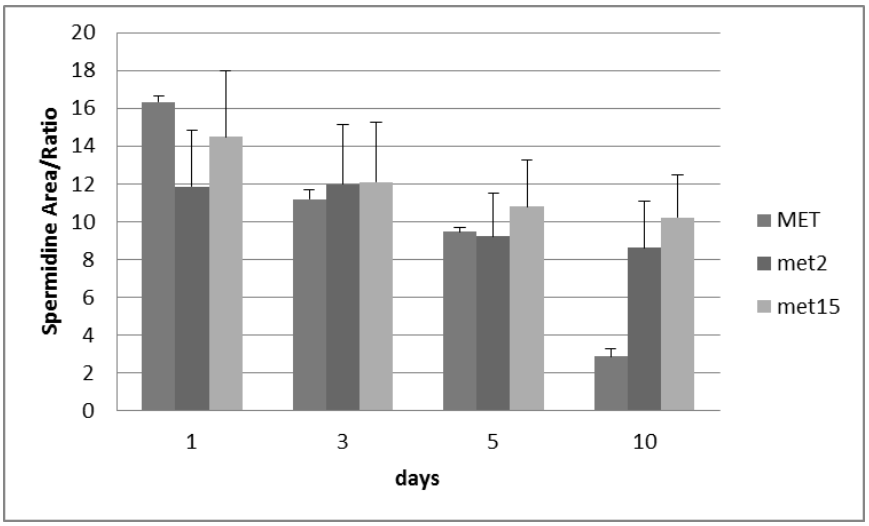

Figure 6. Spermidine levels stayed relatively high under MetR conditions. MS measurement of spermidine levels in aging MetR strains Δ*met2* and Δ*met15* vs. *MET+* wildtype. Agings conducted in SMD+AA (Christoph Ruckenstuhl, unpublished data).

The genes encoding for the enzymes in spermidine biosynthesis, and therefore our "genes of interest" are listed in Table 1.

Table 1 Genes of interest; Data: Saccharomyces Genome Database (SGD)

Gene of interest	Function
SPE1	Ornithine decarboxylase; catalyzes the first step in polyamine biosynthesis; degraded in a proteasome-dependent manner in the presence of excess polyamines; deletion decreases lifespan, and increases necrotic cell death and ROS generation
SPE2	S-adenosylmethionine decarboxylase; required for the biosynthesis of spermidine and spermine; cells lacking Spe2p require spermine or spermidine for growth in the presence of oxygen but not when grown anaerobically
SPE3	Spermidine synthase; involved in biosynthesis of spermidine and also in biosynthesis of pantothenic acid; spermidine is required for growth of wild-type cells
SPE4	Spermine synthase; required for the biosynthesis of spermine and also involved in biosynthesis of pantothenic acid
FMS1	Polyamine oxidase; converts spermine to spermidine, which is required for the essential hypusination modification of translation factor eIF-5A; also involved in pantothenic acid biosynthesis
PAA1	Polyamine acetyltransferase; acetylates polyamines (e.g. putrescine, spermidine, spermine) and also aralkylamines (e.g. tryptamine, phenylethylamine); may be involved in transcription and/or DNA replication

We have also considered the consequences of decreased media acetate levels (and therefore probably AcetylCoA levels) on histone acetylation in the MetR strains *Δmet15* and *Δmet2* (see Figure 7). In mice, heart and muscle tissues responded to nutrient depletion by mounting a strong autophagic response, which were found to be commensurate with a reduction in AcetylCoA (short: AcCoA) levels and a decrease in cytoplasmic protein acetylation. Studies in yeast showed similar results. Through nuclear pores, cytosolic AcCoA is able to freely diffuse into the nucleus, change the acetylation status of histones and other chromatin-binding factors and affect gene transcription programs which in turn might impinge on autophagy. This led to the hypothesis that there might be an epigenetic regulation via histone acetylation under MetR conditions (Marino, Eisenberg, 2014).

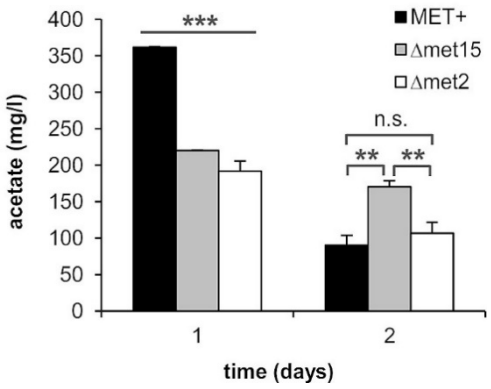

Figure 7. Decreased media acetate levels in Δ*met15* and Δ*met2* were found. Media acetate levels of methionine prototroph (*MET+*), semi-auxotroph (*Δmet15*) and auxotroph (*Δmet2*) on days one and two during chronological aging experiments (n = 8) in Saccharomyces cerevisiae (Ruckenstuhl et. al, 2014).

3 Methods and materials

3.1 Strains

Table 2 *S. cerevisiae* strains used in this work

Name	Genotype	Origin
BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	Lab. Fröhlich
BY4741MET15 (MET+)	MATa his3Δ1 leu2Δ0 MET15 ura3Δ0	Lab. Fröhlich
BY4741MET15 ∆met2	MATa his3Δ1 leu2Δ0 MET15 met2::kanMX4 ura3Δ0	Lab. Fröhlich
BY4741∆gcr1	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gcr1::HIS	Lab. Fröhlich

For this research three different strains were used, a MET+ strain, fully competent in synthesizing methionine, with a chronological lifespan from 10 to 14 days. A ∆*met2* strain, in which a deletion of homoserine transacetylase, leads to a strain unable to perform *de novo* methionine synthesis. This strain has a prolonged chronological lifespan of up to 25 days. In the ∆*met15* strain, the homocysteine synthase was knocked out, the enzyme needed for the conversion of sulfide to homocysteine. This knockout produces a semi-auxotroph strain, ∆*met15* is still able to produce methionine, but in decreased amounts or/and with decreased kinetics. This strain survives up to 22 days in chronological aging experiments (see Figure 8).

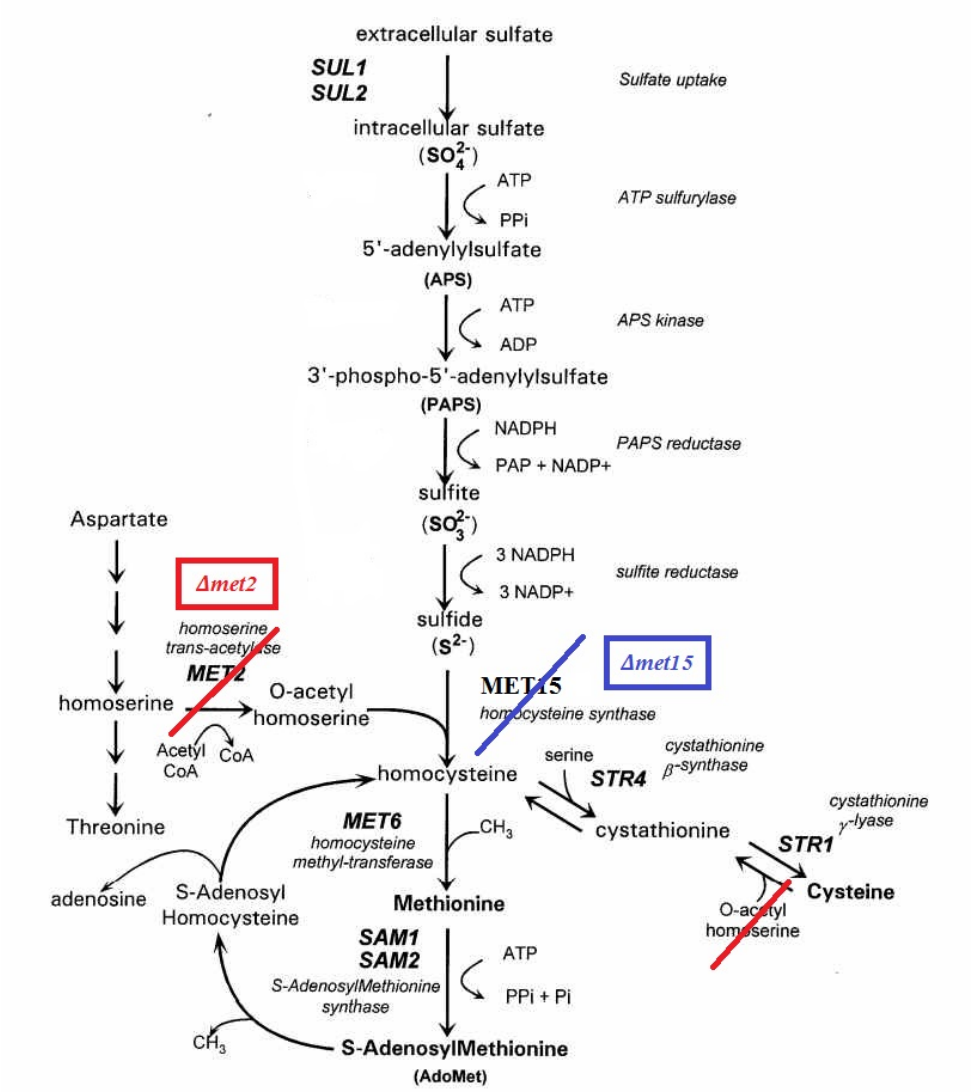

Figure 8. *S. cerevisiae* strains used in this work. Modified from „Metabolism of Sulfur Amino Acids in *S. cerevisiae*“ (Thomas, Surdin-Kerjan et al., 1997)

3.2 Chronological aging experiments and media conditions

S. cerevisiae cells were grown on SMD medium (2% glucose) with all amino acids, inoculated with ONC to an OD_{600} of 0,05 and then aged at 28°C under shaking. Samples at defined time points were harvested and used for method-dependent sample preparation. To determine the transcriptional levels of SPE3 in Δ*gcr* knockout mutants, experiments in YPGal and YPD were conducted. For used growth media see Table 3.

Table 3 Compositions of used growth media

Medium	Components
SMD with all amino acids	0,17 % Yeast Nitrogen Base 0,5 % ammonium sulphate 2% D-glucose
YPGal	1 % yeast extract 2 % bacto peptone 2 % D-galactose
YPD	1 % yeast extract 2 % bacto peptone 2 % D-glucose

3.3 Western Blot

The western blot analysis was performed in order to show epigenetic changes between the examined MetR strain Δ*met2* and the control *MET+* wildtype strain. Changes in histone H3 acetylation under MetR conditions were determined. Another experimental setting was the comparative investigation of dimethyl levels on lysine K36.

3.3.1 Histone extraction

Histone extraction was performed by Glass Bead Method with 20% TCA. Therefore, 12,5 OD units of the sample were collected by centrifugation at 10 000 rpm for 1 minute, washed with 500 µL 20% TCA and immediately frozen in liquid nitrogen. For the extraction the samples were defrosted on ice, 0,2 mL of 20% TCA added and then transferred to Eppendorf Tubes, containing approximately 0,4 g of acid-washed glass beads. The samples were vortexed 2 minutes at 4ºC, afterwards washed with 2x 500 µL 5% TCA and incubated for 10 minutes on ice. TCA was completely removed by centrifugation at 14 000 rpm for 15 minutes and the pellet finally resuspended in 250 µL of 1xFSB (Kao, Osley et al., 2003).

3.3.2 Chemical lysis

3 OD units of the samples were harvested and washed with 1 mL dH20. The cells were resuspended in 150 µL lysis buffer (1,85 M NaOH, 7.5% mercaptoethanol) and incubated on ice for 10 minutes. Then 150 µL of 55% tricholoroacetic acid were added and incubated for additional 10 minutes for protein precipitation. After centrifugation at 10 000 g for 10 minutes (4°C), the supernatant was removed and the cells were

resuspended in 100 µL 1x FSB. The samples were neutralized with 1-5 µL of 1 M tris-solution (colour changes from yellow to blue) and afterwards used or stored at -80°C.

3.3.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Histone samples were run on 15% SDS-polyacrylamide gels with 16 mA, using 1x Tris-glycine-buffer. Membranes were blotted by standard techniques and probed with antibodies at the following dilutions: dimethyl-H3 antibody (1:1000; in 1x TBS, 1 % BSA), H3 α-Rabbit antibody (1:5000; in 1x TBS, 1% BSA), specific acetylated lysine antibodies: H3K9 (1:5000; in 1x TBS, 1% BSA), H3K14 (1:5000; in 1x TBS, 1% BSA) and H3K18 (1: 1000; in 1x TBS, 1% BSA). Detection was performed by chemiluminescence reaction with "Amersham ECL" on film (X-ray films from AGFA enterprise) with signal-intensity-dependant incubation times from 3 minutes up to 1 hour (for detailed workflow see Table 4).

Table 4 Western blot working protocol

Step	Action
1	Run samples on 15% SDS-polyacrylamide gels (16 mA), using 1x Tris-glycine running buffer
2	Blot about 60 min to nitrocellulose membrane (in CAPS buffer, 220 mA)
3	Block overnight with 1% milk powder in 1x TBS (4°C)
4	Incubate 60 min with 1st antibody (room temperature)
5	Wash 3 x 15 min with H_2O
6	Incubate 60 min with 2nd antibody (room temperature)
7	Wash 2 x 5 min with H_2O
8	Wash 1 x 5 min with histone wash buffer
9	Incubate membrane ~ 1 min with ECL
10	Detection on X-ray film

3.4 Generation of Δ*gcr1* (BY4741) knockout strains

To generate Δ*gcr* knockout mutants, a standard protocol for yeast transformation with SORB cells was used. To obtain this strain, a knockout PCR was executed, using pUG27 as a template. The product was then transformed into BY4741 yeast cells. Previous studies indicate that *gcr1* and *gcr2* mutations have massive effects on glycolytic genes and glucose repression is a characteristic of this knockout (Sasaki,

Uemura et al., 2005). As a consequence of this, it was necessary to use a protocol working with YPGal or YPD SORB cells to get positive mutants. Series of tests were previously performed using different experiment conditions: YPGal as well as YPD media for growth of competent cells, SORB cells as well as normal cells. The best results, in this particular case the most positive mutants, were obtained with growth of competent SORB cells in YPGal media.

3.4.1 Knockout PCR

The PCR reaction required the following components (see Table 5).

Table 5 PCR reaction mix.

Component	**V [µL]**
Taq DNA polymerase	0,15
10x Thermopol reaction buffer	3
dNTPs	3
GCR1 knockout primer forward (diluted 1:20)	1,5
GCR1 knockout primer reverse (diluted 1:20)	1,5
pUG 27	6
ddH_2O	15

3.4.2 Growth of competent yeast cells & transformation

50 mL of YPGal or YPD media were inoculated with ONC to an OD_{600} of 0,1 (approximately 1*10^7 cells/mL). The cells were harvested by centrifugation (~ 10 000 rpm, 5 min at room temperature), washed once with 10 mL sterile water and once with 10 mL SORB. SORB was removed by aspiration and the cells were resuspended in 360 µL SORB + 40 µL of carrier DNA (0°C). 50 µL of these competent cells were used for transformation of the ∆gcr1 knockout PCR product. The DNA was placed into a sterile 1,5 mL Eppendorf tube, the competent cells added and mixed well before a six fold volume of PEG was added. Cells were mixed and incubated for 30 minutes at room temperature, mixed by inverting every 10 minutes (for used solutions see Table 6).

After that, DMSO was added to a final concentration of 10% and the maintained suspension heated for 15 minutes at 42°C. The cells were sedimented by centrifugation (2-3 min, 2000 rpm), the supernatant removed and afterwards the cells were resuspended in 100 µL sterile water and plated on YPGal-His.

Table 6 Solutions used for yeast transformation

Solution	Components
SORB	100 mM LioaC 10 mM Tris-HCl, pH 8,1 1 mM EDTA/NaOH, pH 8,0 1 M sorbitol pH 8,0 steril filtered
PEG	100 mM LiOAc 20 mM Tris-HCl, pH 8,0 1 mM EDTA/NaOH, pH 8,0 40 % PEG3350 steril filtered
Carrier DNA	Salmon sperm DNA (10 mg/mL)

3.5 Northern Blot

Northern blot analysis was performed in order to show changes in transcription levels of important genes, involved in polyamine biosynthesis, under MetR conditions.

3.5.1 RNA preparation for Northern blots

Agings were executed in SMD medium with all amino acids (or YPGal medium for ∆gcr1 knockout strains. See Table **7**), inoculated with ONC to an OD of 0,05 and then aged at 28°C. Samples at defined time points were harvested (50 ml of 8h samples, 40 mL of all others) and used for Northern blot analysis. Pay attention that in this great quantity of harvested cells, a precipitate appeared throughout the RNA preparations. This might impair RNA quality, column overload should be avoided. For RNA preparation RNeasy Mini Kit from Quiagen and the attached standard protocol for mechanical disruption of cells was used (RNeasy Mini Handbook, 2012).

Table 7 Compositions of used growth media

Medium	Components
SMD with all amino acids	0,17 % Yeast Nitrogen Base 0,5 % ammonium sulphate 2% D-glucose
YPGal	1 % yeast extract 2 % bacto peptone 2 % D-galactose
YPD	1 % yeast extract 2 % bacto peptone 2 % D-glucose

3.5.2 DIG probes

The used Northern blot detection system is based on digoxigenin (DIG)-marked probes. Therefore DIG-marked dUTP was used in the probe PCRs (for probe PCR composition see Table 8; for the PCR program see Figure 9).

Table 8 Probe PCR composition

Component	V [µL]
Taq polymerase	0,1
10x Thermopol buffer	2,0
(10x DIG DNA labeling mix(Roche Life Science)	2,0
Forward primer (diluted 1:20 in H_2O)	1,0
Reverse primer (diluted 1:20 in H_2O)	1,0
Chromosomal DNA	1,0
Fresenius water	13

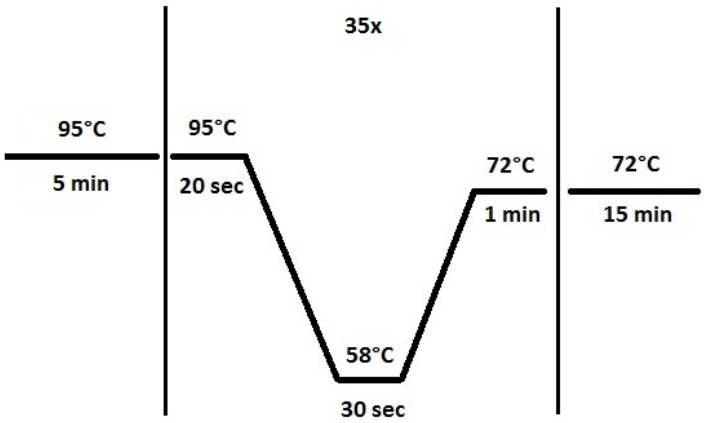

Figure 9. PCR program used for preparation of DIG marked probes

3.5.3 Blot

According to quality and yield of RNA, 3 to 5 µg of RNA were mixed with 15-20 µL sample buffer and 2-5 µL loading buffer, mixed well and denatured 10 minutes at 65°C. After denaturation the samples were cooled on ice and immediately applied on agarose gel for separation.

Northern blot analysis was performed using standard protocols (for detailed workflow see Table 9), for used solutions see Table 10. Samples were separated on 1,2 % agarose gels (diluted in 1x MOPS) with 75 V for approximately two hours, using 1x MOPS running buffer. RNA was transferred to a nylon membrane (Amersham Hybond^{TM}-N hybridization transfer membrane) via capillary blot in 20x SSC overnight (remark: best results were observed with blotting about 16 hours or longer). Afterwards the RNA was UV-cross-linked to the membrane, prehybridized with DIG Easy Hyb and subsequently the digoxigenin-marked probe was applied overnight. Membranes were probed with Anti-DIG-alkaline phosphatase antibody (1:10 000 in 1x blocking solution), followed by detection with CSPD (a chemiluminescence system by Roche) on X-ray films from AGFA enterprise.

Table 9 Northern blot washing and detection procedure

Step	Action
1	Prehybridization, 1 hour (50°C)
2	Hybridization with DIG-marked probe overnight (50°C)
3	Wash 2 x 5 min with washing solution 1 (room temperature)
4	Wash 2 x 15 min with washing solution 2 (50°C)
5	Wash 1 x 5 min with 1x TST (room temperature)
6	Incubate 30 min with blocking solution
7	Incubate 30 min with anti-DIG alkaline phosphatase
8	Wash 2 x 15 min with 1x TST
9	Incubate 5 min with detection buffer
10	Incubate membrane with CSPD 10 minutes at 37°C
11	Detection via Chemidoc or X-ray film

Table 10 Solutions needed for Northern blot

Solution	Components
10x MOPS	0,2 M MOPS, pH 7,0 0,05 M sodium acetate, pH 7,0 0,01 M EDTA pH 8,0
20x SSC buffer	3 M NaCl 0,3 M sodium acetate pH 7,0
Sample buffer (for RNA agarose gel without formaldehyde)	50 % glycerin 1 mM EDTA 0,2 % bromphenol blue 1 mg/mL ethidium bromide
Loading buffer (for RNA agarose gel without formaldehyde)	10 mL deionized formamide 3,5 mL formaldehyde 37 % 2 mL 10x MOPS buffer
Washing solution 1	2x SSC buffer 0,1 % SDS
Washing solution 2	0,1x SSC 0,1 % SDS
10x TST	1,5 M NaCl 1% Tween 20 0,5 M Tris-HCl, pH 7,5
Detection buffer	100 mM Tris-HCl, pH 9,5 100 mM NaCl
Detection	CSPD, chemiluminescence substrate for alkaline phosphatase
Prehybridization & probe solution	DIG Easy Hyb (Roche)
RNA preparation	RNeasy Mini Kit (Quiagen)
Antibody	Anti-DIG alkaline phosphatase, 1:10 000 in 1x blocking solution, blocking solution diluted in 1x TST

3.5.4 Densitometric analysis via Image LabTM

To get a comparative analysis of the Northern blot results a software called Image Lab™ was used. This software allows a direct comparing of different samples, using the density of the specific bands as the basic of calculation for a relative quantification. Furthermore, it is possible to respond to differences in the applied sample quantities via loading control. In this analysis 25 S rRNA was used as loading control. The presented results are each normalized to the respective initial MET+ value.

3.6 Real time PCR

Quantitative real time PCR, short qPCR, was performed with StepOnePlus™ System using SYBR Select Master Mix as a fluorescent detection system. Detection of qPCR

product is performed by measurement of the increase in fluorescence caused by the binding of SYBR® Green dye to double-stranded DNA.

3.6.1 RNA preparation for qPCR

Agings were executed in SMD medium with all amino acids, inoculated with ONC to an OD_{600} of 0,05 and then aged at 28°C. Samples at defined time points were harvested and used for preparation of qPCR samples. 40-50 mL yeast culture were harvested for the RNA preparation, but unfortunately the RNA quality, which should be absolutely high, was not appropriate for qPCR analysis throughout this research (indicated by high background signals). The column may under no circumstances be overloaded. Others report that quantities of approximately 8 OD units showed the best results.

For RNA preparation, RNeasy Mini Kit (Quiagen) and the attached standard protocol for mechanical disruption of cells was used (RNeasy Mini Handbook, 2012). After that, residuals of genomic DNA were removed from RNA preparations with DNAse digestion via Thermo scientific standard protocol. To convert the RNA samples into cDNA for qPCR measurement, a treatment including a reverse transcriptase followed. Finally, the samples were prepared in 96 well plates with SYBR Select Master Mix and appropriate qPCR primers and measured with qPCR. ACT1 was used as endogenous control (for used components see Table 11 & Table 12; for the used qPCR program see Figure 10).

Table 11 Reaction mix for DNAse digestion. Removal of genomic DNA from RNA preparations

Component	V [µL]
RNA	1 µg
RNAse OUT	1
DNAse I, RNase-free	1
10x reaction buffer with $MgCl_2$	1
Fresenius water	6

Table 12 Reaction mix for reverse transcription of RNA preparations

Component	V [µL]
DNAse digestion mix	10
Fresenius water	1
Random hexamer primer	1
dNTPS (10 mM each)	1
0,1 M DTT	1
RNAse OUT	1
5x First strand buffer	4
Superscript III reverse transcriptase	1

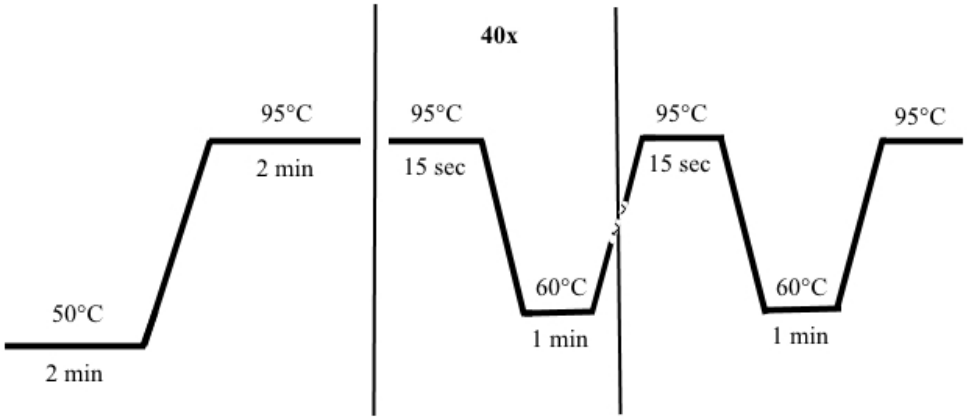

Figure 10. Used qPCR program. qPCR program used for sample analysis with StepOnePlus™ System using SYBR Select Master Mix as a fluorescent detection system

4 Results

4.1 Western blot analysis of differences in histone H3 acetylation and methylation regarding possible epigenetic regulation in *MET+* vs. ∆*met2* strains

Histone modifications, DNA methylation and non-coding RNAs are the main epigenetic mechanisms that may act alone or in cooperation to control the gene expression programme over the lifetime of an organism (Herceg et al., 2013). S-adenosylmethionine (short SAM) is the main biological methyl donor of various transmethylation reactions. Its methyl group can be transferred to different acceptor molecules such as DNA, phospholipids and proteins (Teodoro Bottiglieri, 2014). As a link between S-adenosylmethionine levels and DNA-methylation was found (Valdés-Santiago, 2012), we decided to conduct a H3 dimethyl western blot. H3 is one of the five main histone proteins involved in the structure of chromatin in eukaryotic cells and can undergo several different types of post-translational modifications, for example histone acetylation and methylation, that influence cellular processes.

In this experiment we worked with the *MET+* wildtype strain in comparison to the MetR strain ∆*met2*, incapable of *de novo* methionine synthesis. We performed western blot analysis with samples taken at several points in time of aging (12 hours, day 1, day 5) in 4-fold measurement and used an H3-dimethyl (K36) antibody, which showed no differences in dimethylation levels on H3 at each point in time (see Figure 11).

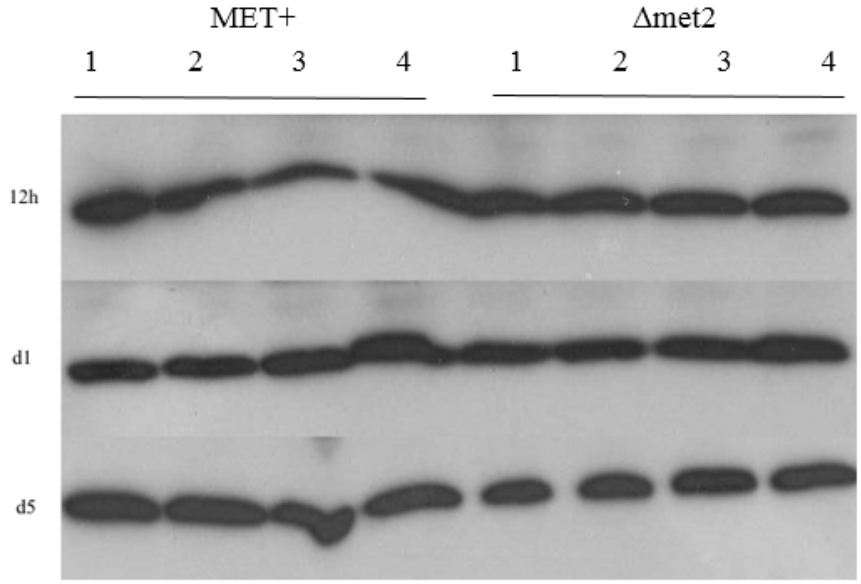

Figure 11. No detectable differences of H3 dimethyl levels (K36) in MET+ wildtype vs. Δmet2 strain. Western blot analysis of samples obtained in aging experiments. Yeast culture grown in SMD with all amino acids (short: +AA) at 28°C. Samples at several points in time harvested, prepared and used for western blot analysis in 4 fold measurement.

Furthermore, the attention was paid to the second main epigenetic modification acetylation, especially focused on histone acetylation. At first, the effects of decreased media acetate on histone acetylation in the MetR strains *Δmet15* and *Δmet2* (see Figure **7)** were considered. A change of the acetylation status of histones and other chromatin-binding factors affects gene transcription programs which in turn might impinge on autophagy. This led to the hypothesis that there might be an epigenetic regulation via decreased histone acetylation under MetR conditions. (Marino, Eisenberg, 2014)

Secondly, another influencing factor of histone acetylation was considered: spermidine. Recent studies have linked the beneficial effects of spermidine on aging and lifespan to histone acetylation. It reduced acetylation on all researched lysine residues (Lys 9, 14, 18) on histone H3 in yeast, and the generation of a Δ*spe1* mutant, unable to synthesize polyamines, led to premature death associated with hyperacetylation (Minois, Carmona-Gutierrez, Madeo, 2011). Based on the MS measurement of spermidine levels (see Figure 6) in the used strains Δ*met2*, Δ*met15* and *MET*+, we were interested if there is an apparent effect of decreased spermidine levels on histone acetylation. For the determination of acetylation levels in the Δ*met2*

strain compared to *MET*+ wildtype, different H3 acetylation antibodies were used and western blots with samples, harvested at several points in time, in 4 fold measurement were performed. At first, the H3 levels in both strains were measured, in one (day one, d1) and five day aged (day 5, d5) samples. The anti H3 α-Rabbit antibody showed no differences in H3 levels in the two different strains *MET*+ and Δ*met2*. In addition, no distinction between the two strains *MET*+ and Δ*met15* regarding the H3 histone acetylation on all determined lysine residues (H3K9, H3K14, H3K18) could be found (see Figure 12).

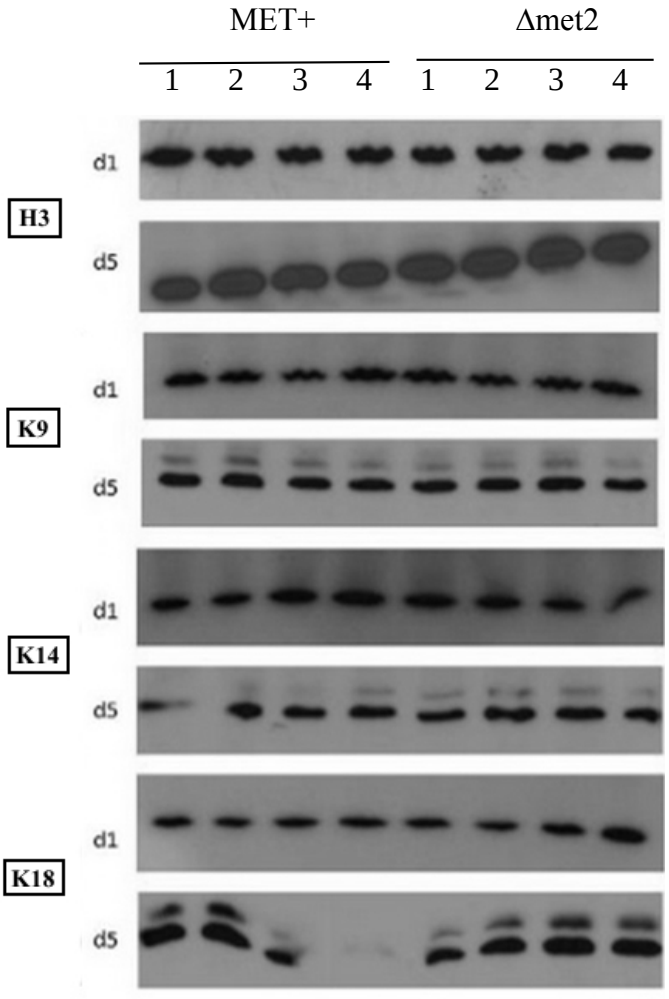

Figure 12. No detectable distinction in the western blot analysis of acetylation levels on specific H3 lysine residues H3K9, H3K14, H3K18 in MET+ wildtype vs. Δmet2 strain. Western blot analysis of samples obtained in aging experiments. Yeast culture grown in SMD with all amino acids (short: +AA) at 28°C. Samples at several points in time harvested, prepared and used for western blot analysis in 4 fold measurement.

In addition, a western blot with an overall acetylation antibody was conducted, in order to see changes in total acetylation levels under MetR conditions. This blot (see Figure 13) could neither show any differences in acetylation levels between *MET+* and ∆*met2*.

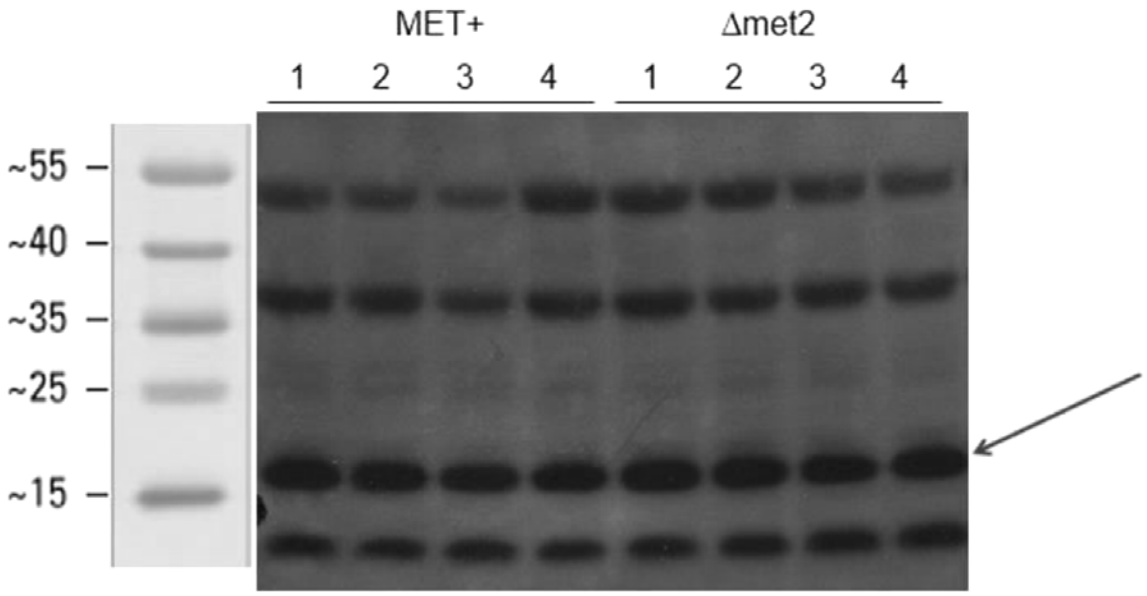

Figure 13. Analysis of overall acetylation levels showed no differences in acetylation levels between *MET+* and ∆*met2*. Western blot analysis of samples obtained in aging experiments. Yeast culture grown in SMD with all amino acids (short: +AA) at 28°C. Samples at several points in time harvested, prepared by chemical lysis and used for western blot analysis in 4 fold measurement. Histone H3 is denoted by the arrow at approximately 17 kDa.

4.2 Northern Blot analysis of genes of interest, encoding important enzymes in spermidine biosynthesis (*SPE1*, *SPE2*, *SPE3*, *SPE4*, *PAA1*)

Recent studies in *S. cerevisiae* have demonstrated that although S-adenosylmethionine levels are decreased in methionine restriction strains, the spermidine and putrescine levels remained relatively high (Christoph Ruckenstuhl, unpublished data). This led to the hypothesis that there might be an upregulation in certain enzymes of spermidine biosynthesis. *SPE3*, the gene encoding spermidine synthase (see Figure 14), was found to be upregulated under MetR conditions in ∆*met15* (Jay E. Johnson, F. Brad Johnson et al., 2014), which gave a first hint of transcriptional changes in spermidine biosynthesis under methionine restriction conditions.

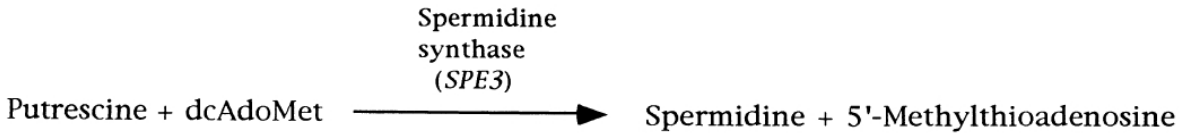

Figure 14. *SPE3* in spermidine biosynthesis (Tabor et al., 1996)

The next step of this research was to take a look at other important genes regarding spermidine biosynthesis. Northern Blots with probes for the genes of interest (see Table 1) were executed.

The *SPE3* probed Northern blot (see Figure 15) could confirm a massive upregulation of *SPE3* levels in MetR strains Δ*met2* and Δ*met15*, especially after eight hours and on day six. Thereby we could reproduce the results of Johnson & Johnson, mentioned above. As *SPE3* is the gene encoding for spermidine synthase, this suggests an upregulation in the synthesis of spermidine in MetR strains.

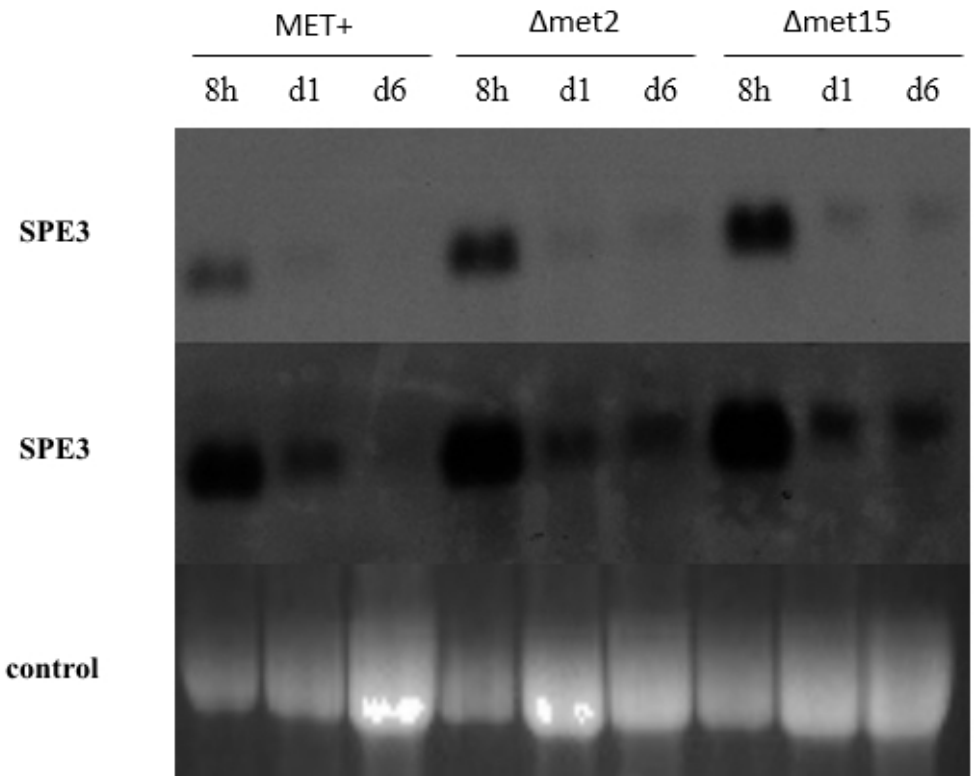

Figure 15. Massive upregulation of *SPE3* levels in MetR strains Δ*met2* and Δ*met15*. Northern blot analysis of *MET+,* Δ*met2* and Δ*met15* strains aged in SMD with all amino acids. Samples were harvested at defined time points (t = 8 hours, day 1, day 6), prepared and used for analysis of *SPE3* levels. 5 µg RNA applied. 25S rRNA loading control.

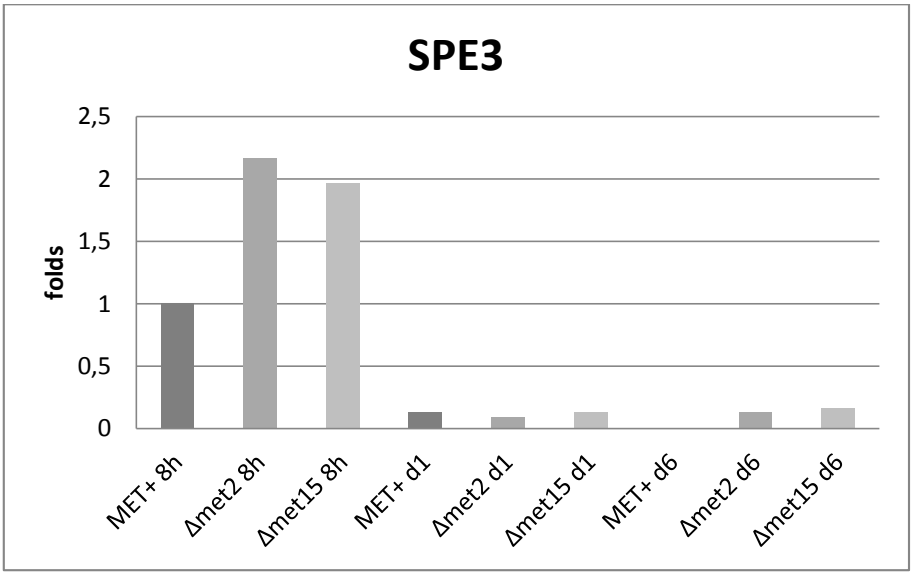

Figure 16. Densitometric analysis of *SPE3* Northern blot (Figure 15) via Image Image Lab™

In Figure 18 a Northern blot with RNA preparations of *MET+* and MetR strains Δ*met2* and Δ*met15* was probed with *SPE1*. A strong decrease of *SPE1* levels on day six in all samples was detectable. Compared to loading control, it becomes apparent that there is an upregulation of *SPE1* levels in the methionine restriction strain Δ*met2* after 8 hours and on day 6. This would correlate with the seen increase in the synthesis of putrescine in Δ*met2*.

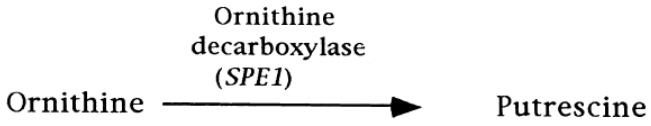

Figure 17. *SPE1* in spermidine biosynthesis (Tabor et al., 1996)

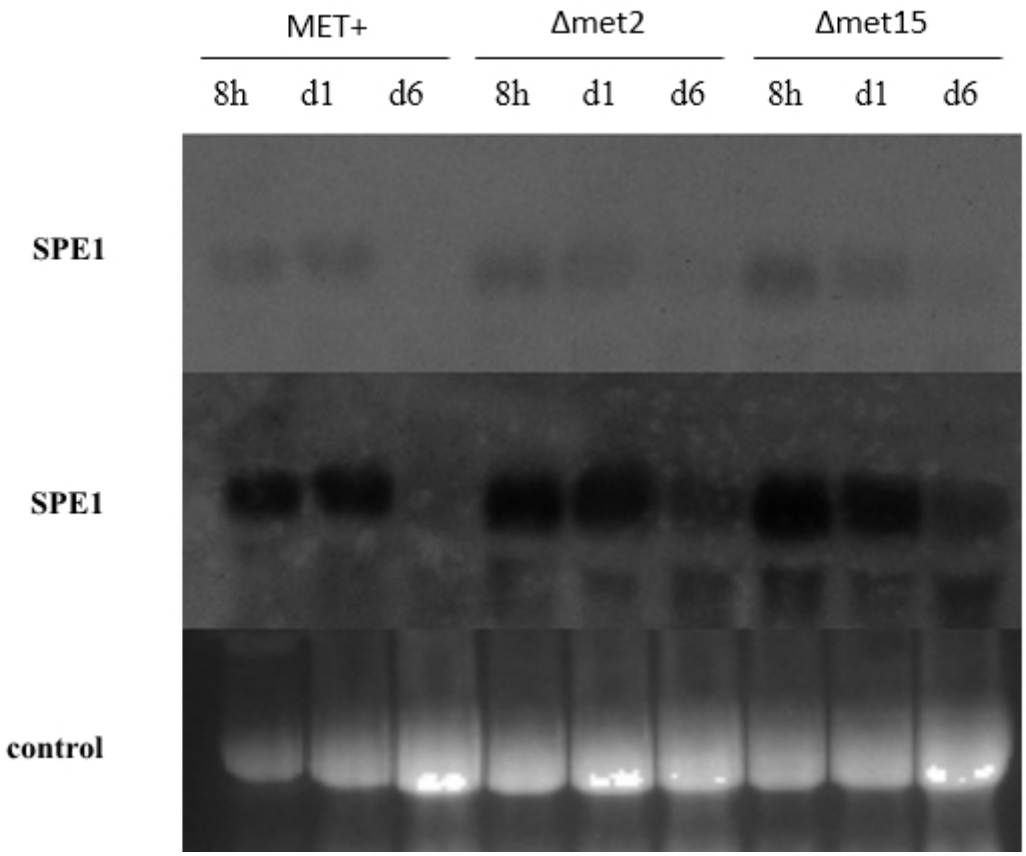

Figure 18. Northern blot analysis could show a slight increase in *SPE1* levels in Δ*met2* strain in contrast to *MET*+ wildtype. Northern blot with RNA preparations of *MET*+, Δ*met2* and Δ*met15* strains aged in SMD with all amino acids. Samples were harvested at defined time points (t = 8 hours, day 1, day 6), prepared and used for analysis of SPE1 levels. 5 µg RNA applied. 25S rRNA loading control.

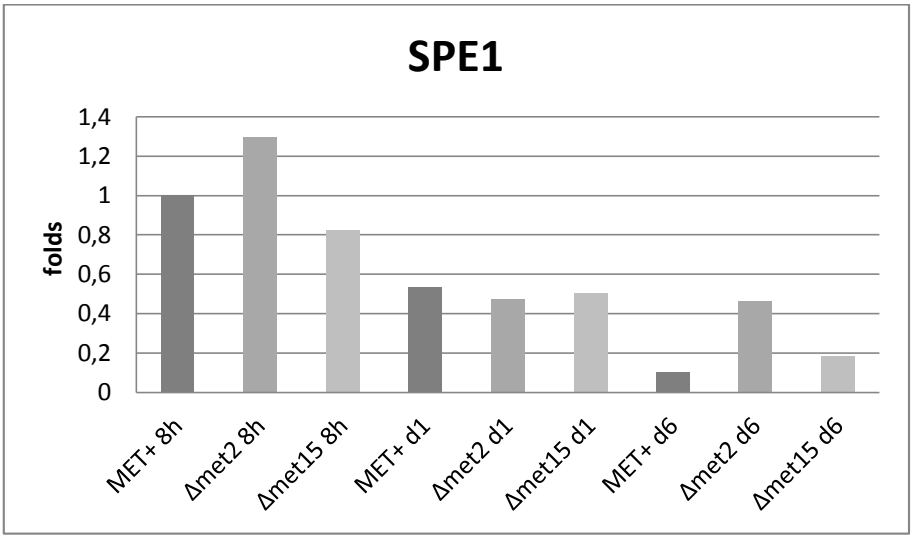

Figure 19. Densitometric analysis of the *SPE1* Northern blot (Figure 18) via Image Image Lab™

Figure 21 shows the same samples probed with SPE2, the gene encoding S-adenosylmethionine decarboxylase, the enzyme converting SAM to decarboxylated SAM (see Figure 20), which is crucial for spermidine synthesis. A decrease in SPE2 levels is visible, starting on day one. Regarding 25S loading control**,** there is no detectable distinction between *MET*+ and MetR strains Δ*met2* & Δ*met15*.

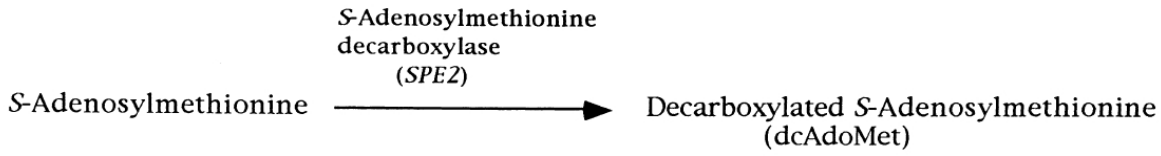

Figure 20. *SPE2* in spermidine biosynthesis (Tabor et al., 1996)

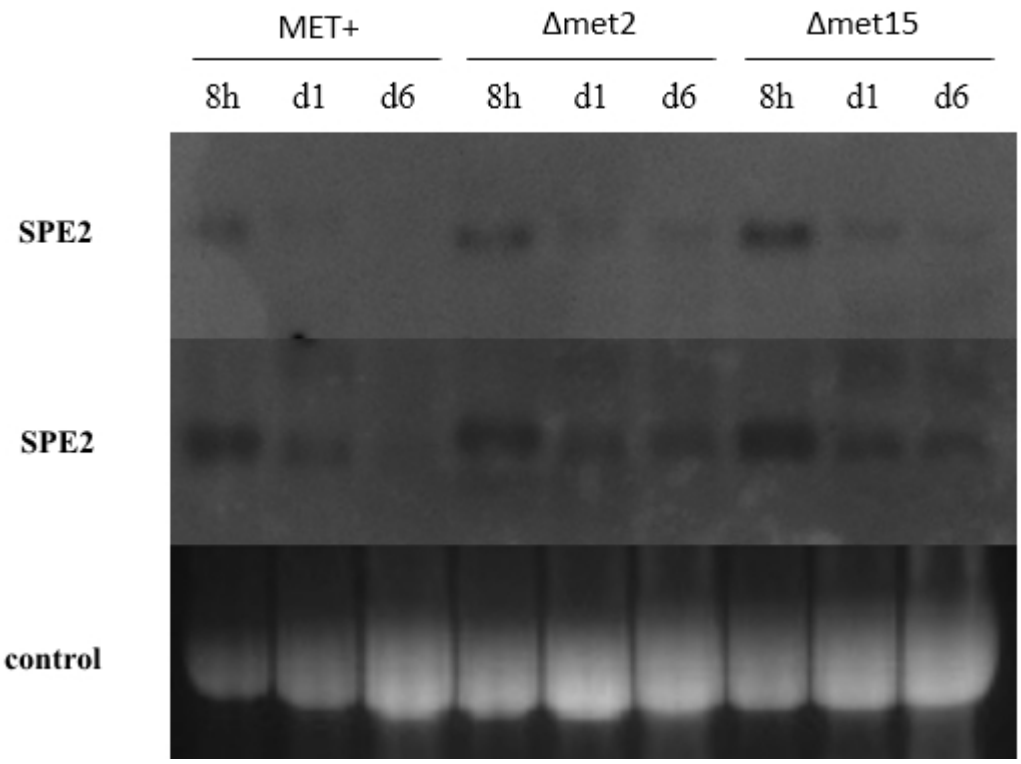

Figure 21. No alteration in transcriptional levels of *SPE2* between *MET*+ and the MetR strains Δ*met2* and Δ*met15*. Northern blot analysis with RNA preparations of *MET+,* Δ*met2* and Δ*met15* strains aged in SMD with all amino acids. Samples were harvested at defined time points (t = 8 hours, day 1, day 6), separated on 1,2% agarose gel and subsequently probed with *SPE2*. 5 µg RNA applied. 25S rRNA loading control.

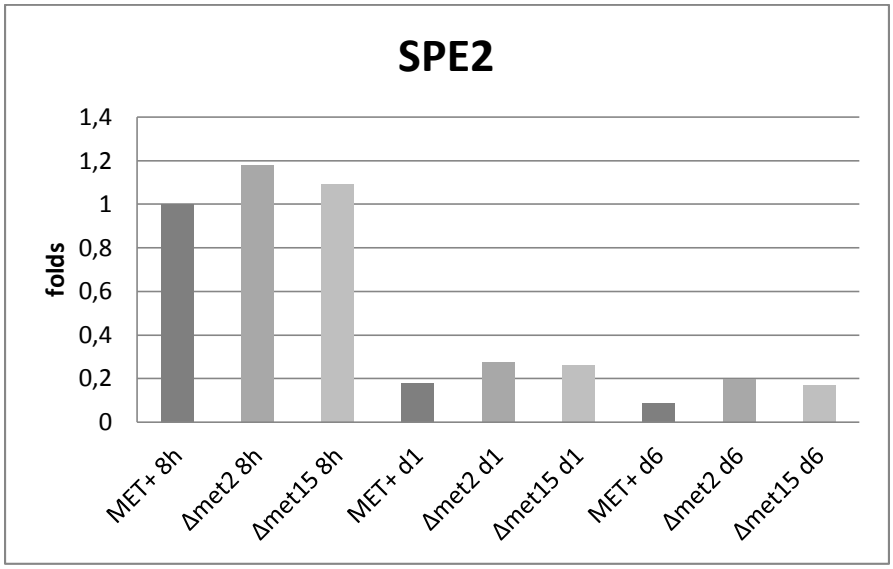

Figure 22. Densitometric analysis of the *SPE2* Northern blot (Figure 21) via Image Image Lab™

Spermine synthase (*SPE4*) is required for the conversion of spermidine and SAM to spermine (see Figure 23). In Figure 24 the *SPE4* Northern blot shows a strong decrease of *SPE4* levels in Δ*met15* at all points in time. In addition, *SPE4* levels are also lowered in Δ*met2* on day 6.

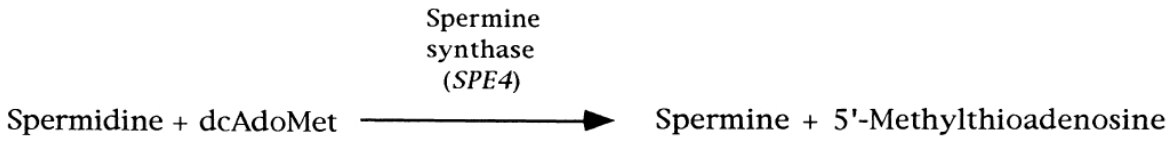

Figure 23. *SPE4* in spermidine biosynthesis (Tabor et al., 1996)

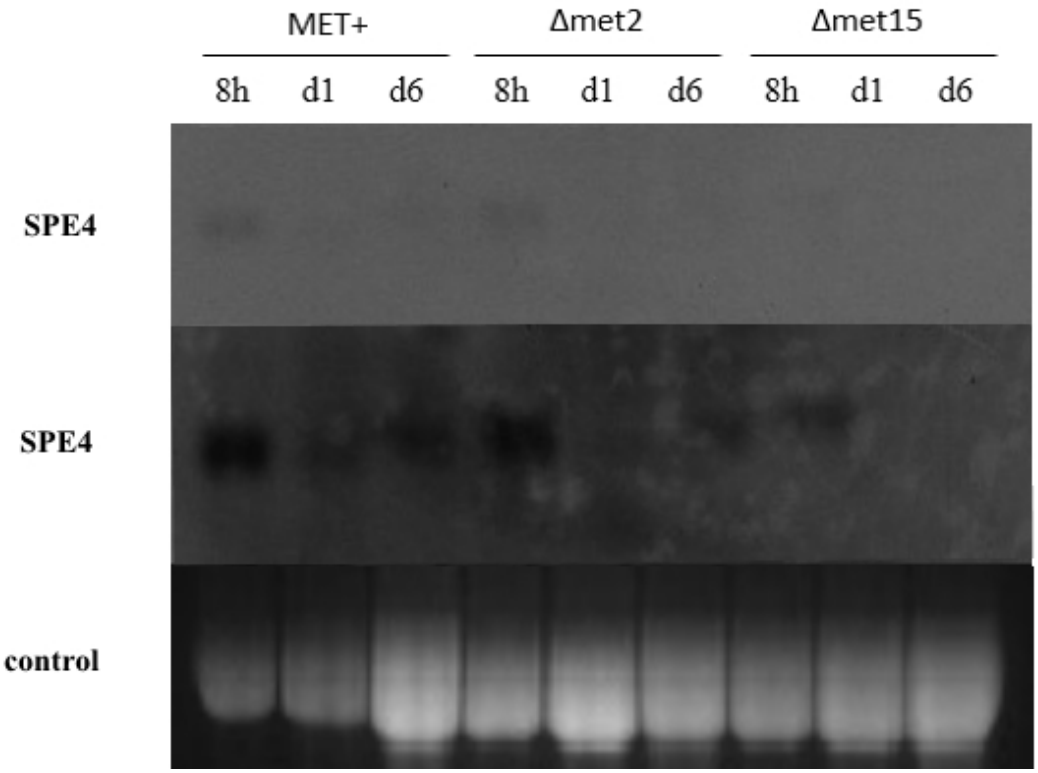

Figure 24. Observable decrease of *SPE4* levels in Δ*met15* and in Δ*met2*, in contrast to wildtype *MET*+, determined by Northern blot analysis. Northern blot analysis of RNA preparations of *MET+,* Δ*met2* and Δ*met15* strains aged in SMD with all amino acids. Samples harvested at defined time points (t = 8 hours, day 1, day 6), prepared for analysis and after all probed with *SPE4*. 5 µg RNA applied. 25S rRNA loading control.

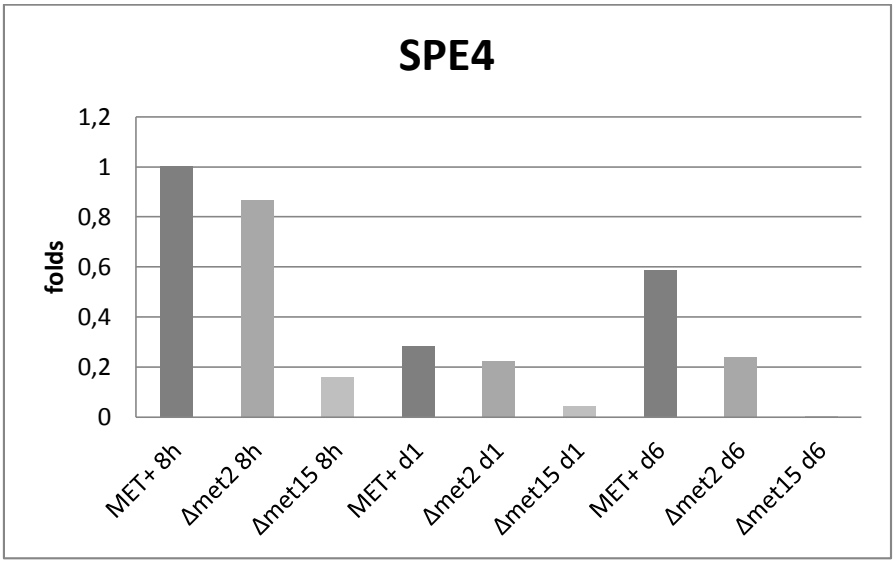

Figure 25. Densitometric analysis of the *SPE4* Northern blot (Figure 24) via Image Lab™

PAA1, the polyamine acetyltransferase, acetylates polyamines, which means, according to the polyamine biosynthesis route (Figure 3), a conversion of spermidine to N-acetylspermidine and spermine to N-acetylspermine. The Northern blot pictured in Figure 26, shows a general decrease of *PAA1* levels from the early six hour sample to day two and a slight, not distinct, upregulation of *PAA1* in methionine restriction strain Δ*met15* after twelve hours.

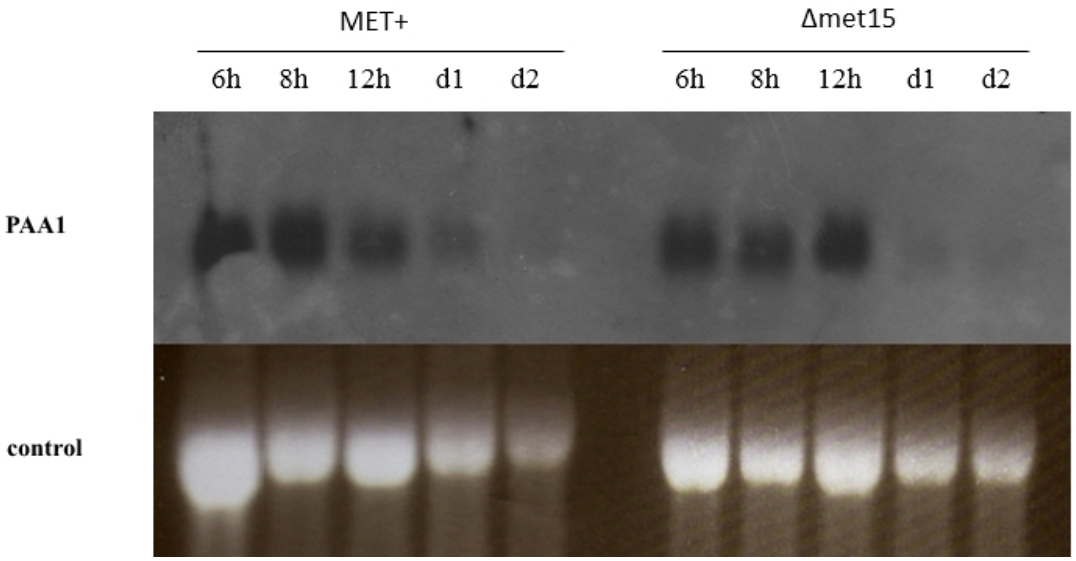

Figure 26. Decrease of *PAA1* levels in course over time in *MET+* and Δ*met15* strain. Increase of *PAA1* levels in *MET+* after 8h and on day one. Northern blot analysis of RNA preparations of MET+ and Δmet15 strains, aged in SMD with all amino acids. Samples harvested at defined time points (t = 6 hours, 8 hours, 12 hours, day 1, day 2), separated on 1,2% agarose gel and subsequently probed with *PAA1*. 5µg RNA applied. 25S rRNA loading control.

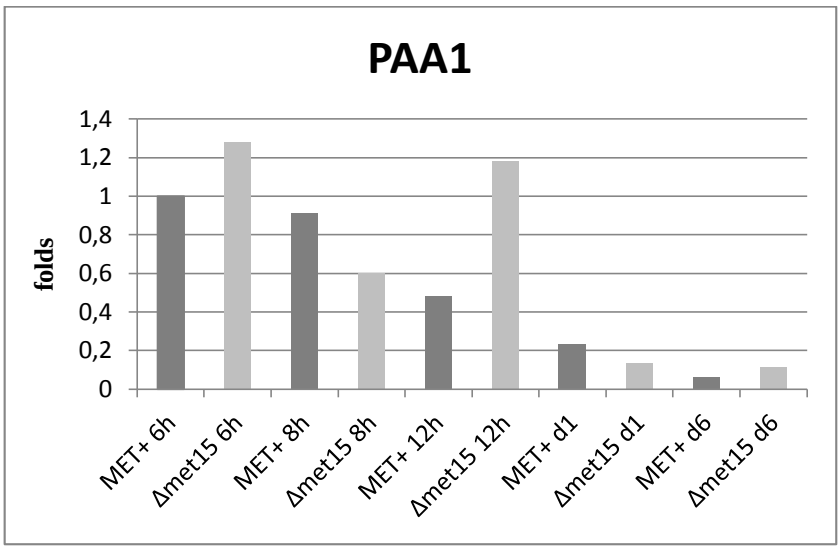

Figure 27. Densitometric analysis of the *PAA1* Northern blot (Figure 24) via Image Lab™

4.3 Real time PCR

To confirm Northern blot results and to get further information, especially about *FMS1* levels, which was not possible via Northern blot, real time PCR was used. Real time PCRs (short: qPCR) were performed with qPCR primers for *FMS1*, *PAA1* and *SPE3*.

These results are limited with their assertion to experiment conditions, which were not optimal, as the quality of RNA was not as good as would have been preferable. Therefore unspecific signals occurred and the reference measurements were quite near to the results themselves. Experiments should be repeated with optimal conditions to get distinct results.

The SPE3 level analysis via qPCR (see Figure 28) partially confirmed the *SPE3* Northern blot results (see Figure 15). A strong increase of *SPE3* in the MetR strain Δ*met15* as compared to *MET+* after eight hours was observable. Spermidine synthesis might be upregulated at this point. The day one levels remained approximately the same.

PAA1 (see Figure 29) and *FMS1* (see Figure 30) showed a similar regulation “trend”, on day one there was a strong increase of both in *MET+* strain. In contrast to Δ*met15* where the levels stayed relatively low, it seems that there was a lot of degradation and rebuild going on in *MET+* strain whereas the Δ*met15* seemed to be anxious to retain constant polyamine levels. In addition it seems that *FMS1* is stronger upregulated under MetR (in a Δ*met15* strain) compared to *PAA1* hinting to increased rebuilding of acetylated spermidine (and spermine).

Unfortunately, as mentioned above, these results must be treated with caution and should be confirmed again.

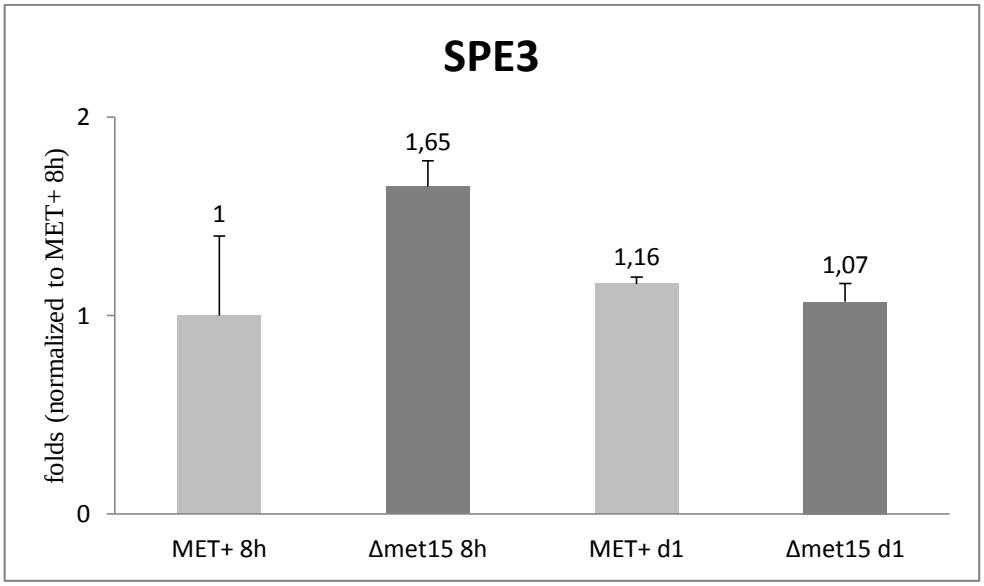

Figure 28. Upregulation of *SPE3* levels in the MetR strain Δ*met15*. qPCR measurement of *SPE3* levels in *MET*+ and semi-auxotroph Δ*met15* strain. Samples aged in SMD+AA, prepared via RNeasy Mini Kit and analysed via qPCR. Folds calculation based on internal benchmark *ACT1* and normalized to *MET*+ 8h.

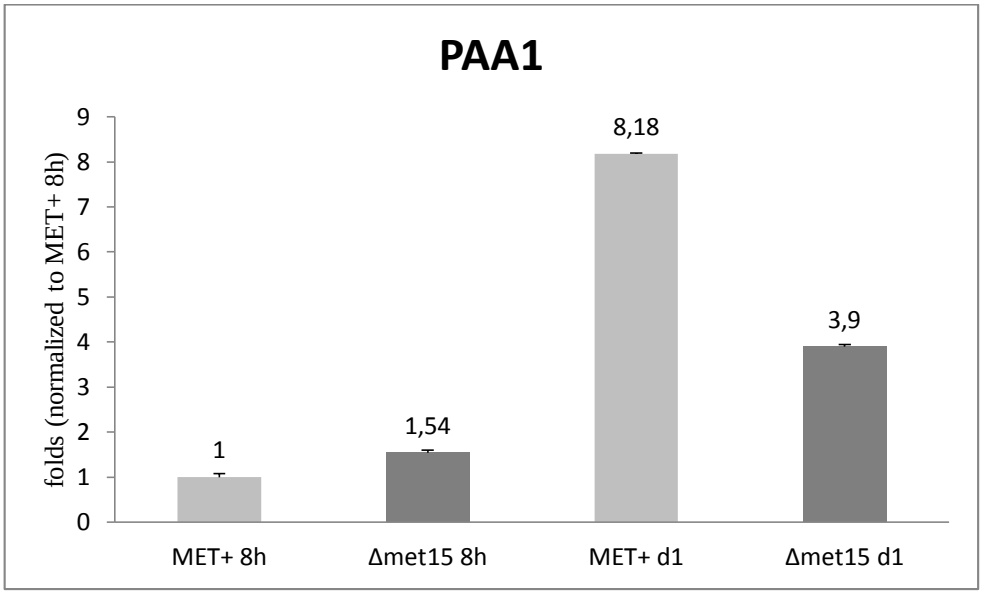

Figure 29. Massive increase of *PAA1* levels in *MET*+ strain on day one detected. qPCR measurement of *PAA1* levels in *MET*+ and semi-auxotroph Δ*met15* strain. Samples aged in SMD+AA, prepared via RNeasy Mini Kit and analysed via qPCR. Folds calculation based on internal benchmark *ACT1* and normalized to *MET*+ 8h.

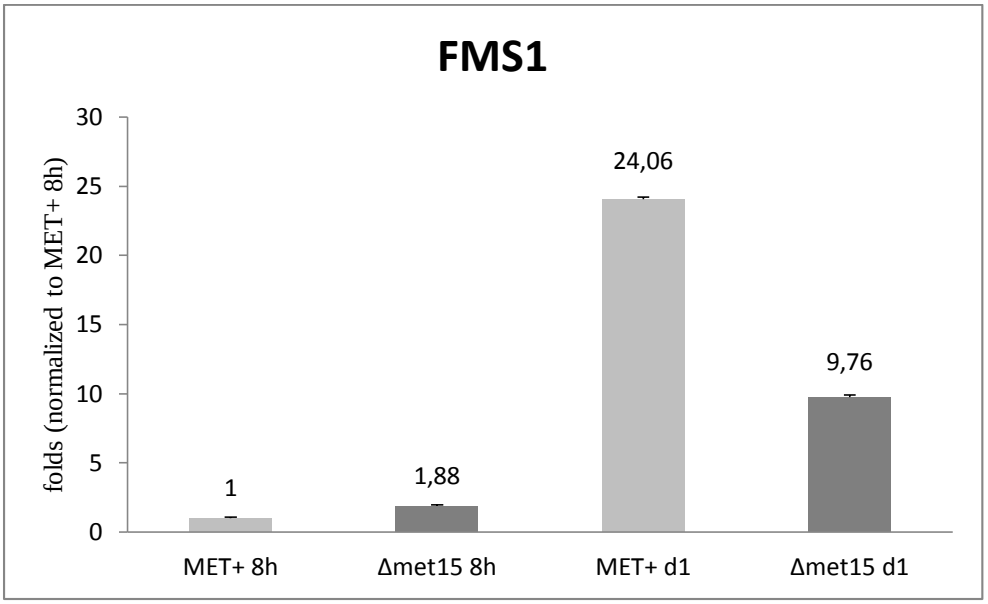

Figure 30. qPCR analysis showed a massive increase of *FMS1* levels in wildtype *MET+* on day one. qPCR measurement of *FMS1* levels in *MET+* and semi-auxotroph Δ*met15* strain. Samples aged in SMD+AA, harvested at defined points in time, prepared via RNeasy Mini Kit and analysed via qPCR. Folds calculation based on internal benchmark *ACT1* and normalized to MET+ 8h.

The next step was to take a look at transcription factors involved in the regulation of important genes in spermidine biosynthesis (see Table 13). At first, a transcription factor called *GCR1* was selected, because of its function as regulator of *SPE3* and *FMS1*, both genes of interest in this research.

Table 13 Transcriptional regulation factors of interest in spermidine biosynthesis (Data: SGD, Christoph Ruckenstuhl)

Regulator for						Gene Systematic Name	Gene Name
PAA1	FMS1	SPE1	SPE2	SPE3	SPE4		
				x		YIL036W	CST6
	x			x		YPL075W	GCR1
				x		YGL073W	HSF1
				x		YNL216W	RAP1
				x		YLR176C	RFX1
			x	x	x	YLR403W	SFP1
				x		YOL004W	SIN3
		x		x		YJL127C	SPT10
		x		x		YIL101C	XBP1
		x			x	YDR463W	STP1

To get information about the influence of *GCR1* on *SPE3* levels in our strains, ∆*gcr1* knockout mutants were produced and a Northern blot with *SPE3* probe was performed. As it is seen in this blot (Figure 31) a slight decrease in *SPE3* levels in the ∆gcr1 knockout clone one (K1) and clone three (K3) was determined. The second clone (K2), might be an aberration, because of bad knockout strain quality. It should be noted that we had to change to Gal based media due to the, in ∆gcr1 mutants current, glucose repression. As no obvious differences were detectable, *GCR1* might not play an important role in this specific case.

What must be kept in mind is that the properties of all used strains under these conditions (YPGal medium) are unknown. Control experiments with wildtype strains, respectively the used MetR strains, under this media conditions should be done to allow a definite assessment. Several other transcription factors come into question as important regulators (as seen in Table 13), but have not been studied yet. Due to the obtained results, namely the massive increase of SPE3 levels under MetR conditions,

regulators of SPE3 would be the obvious next of great interest, for instance *CST6, RAP1, SIN3, HSF1* and *RFX1*.

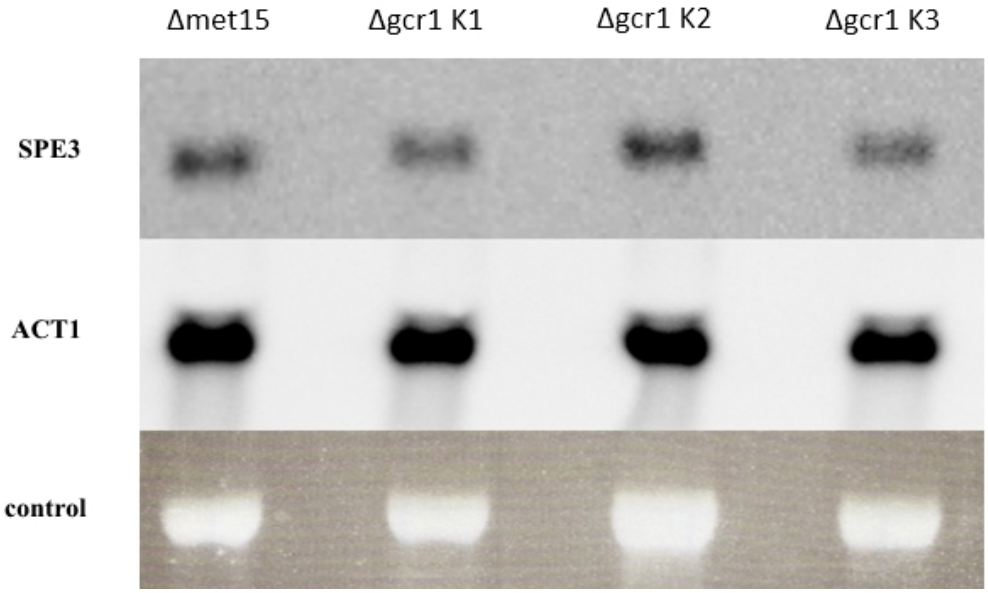

Figure 31. Northern blot analysis of Δgcr1 mutants showed a slight decrease of SPE3 transcription levels. Northern blot with RNA preparations of 8 hour samples of the Δmet15 wildtype strain and three Δgcr1 knock out strains (K1, K2, K3). Samples were prepared via RNeasy Mini Kit, separated on 1,2% agarose gel and probed with SPE3. Medium: YPGal. 2,5 µg RNA applied. 25 S RNA loading control. ACT1 probe as internal control for media conditions in YPGal.

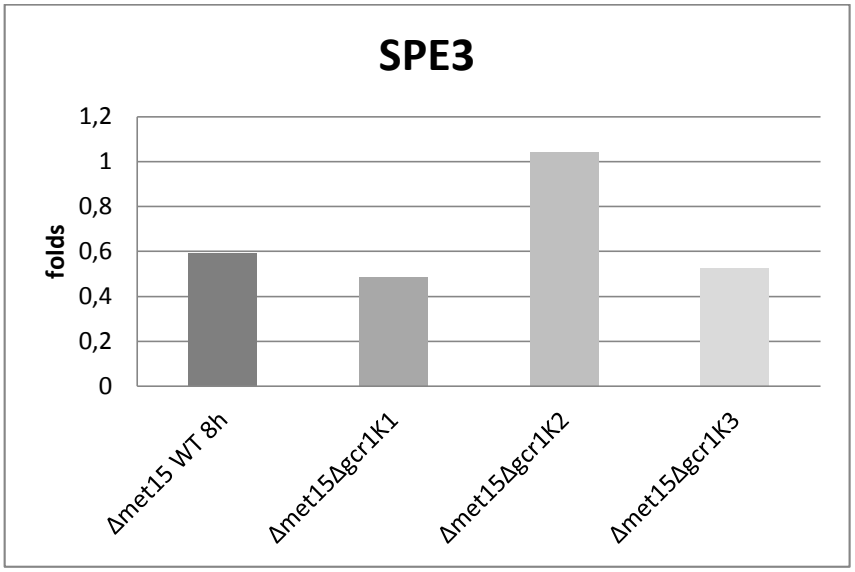

Figure 32. Densitometric analysis of *SPE3* levels in Δ*gcr* knock out mutants (Figure 31) via Image Image Lab™

5 Discussion

5.1 Methionine restriction did not affect H3 acetylation (Lys 9, 14, 18) and dimethylation (Lys 36)

Recent studies have linked the beneficial effects of spermidine on aging and lifespan to, among others, histone acetylation. Spermidine treatment reduced acetylation on all researched lysine residues (Lys 9, 14, 18) on histone H3 in yeast and the generation of a Δ*spe1* mutant, unable to synthesize polyamines, led to premature death associated with hyperacetylation. Hypoacetylation via spermidine is mainly attributed to an inhibition of histone acetylases, rather than to activation of histone deacetylases. By modulating acetylation levels and protein synthesis, polyamines seem to be able to cause several cellular responses, which might help to explain how it is possible that they can affect and promote cell death and cell growth and how they are involved in the complex mechanisms of aging, stress and diseases. These results further suggest a possible link between histone acetylation and longevity in general, but this is not proved yet (Minois, Carmona-Gutierrez, Madeo, 2011).

The decreased lifespan of the Δ*spe1* mutant could be prolonged through spermidine and in addition putrescine supplementation.lit increased chronological lifespan in *S. cerevisiae* wild-type as well as replicative lifespan in old yeast cells. An increase in lifespan could also been shown in *C. elegans* and *Drosophila melanogaster.* In human blood, spermidine treatment led to a prevention of death from necrosis from 15% in wildtype control to 50% in treated mononuclear cells. This provides strong evidence that spermidine might be an efficient “weapon” in the fight against ageing (Minois, Carmona-Gutierrez, Madeo, 2011).

Another important link between polyamines and epigenetic regulation is S-adenosylmethionine as an aminopropyl-group donor, because DNA methylases and AdoMetDC share AdoMet as a common substrate. As a result of this a negative relationship between AdoMetDC levels and DNA methylation and a positive relation with cell differentiation, were found. (Valdés-Santiago et al., 2012)

The acetylation blots (see Figure 12) showed no differences between *MET*+ wildtype and methionine restriction strain Δ*met2* in H3 acetylation on none of the specific lysine residues we determined (H3K9, H3K14, H3K18). The same pattern was seen in the dimethylation blot (Figure 11), which showed no differences between the MetR strain and the prototroph *MET*+ wildtype strain in dimethyl levels at lysine H3K36. We had supposed a decrease in methylation levels in methionine restriction strains in conjunction with the visible decline in S-adeonsyl-methionine levels, as AdoMet is the main substrate for DNA methylases. This hypothesis could not be confirmed by the conducted experiments. As this hypothesis was not extensively examined, future work should involve more detailed research on this topic.

Regarding the acetylation blots, the fact that MetR lowers acetate levels (at early time points) and therefore probably AcetylCoA levels, led to the hypothesis that autophagy might be increased because of a decline in protein acetylation and therefore lead to a better survival of the cell (Marino, Kroemer, 2014; Ruckenstuhl et. al., 2014). There is an inverse relationship of histone H3 acetylation with the transcriptional control of autophagy-essential genes, exemplified by *ATG7* that is influenced by the AcCoA production pathway. Screens with knockdown mutants of genes encoding enzymes involved in AcCoA formation showed that they partly led to a strong induction of autophagy upon chronological aging (Eisenberg, Kroemer, 2014). However, as there was no difference in acetylation levels between the determined strains in our case, we could not confirm that, probably due to the small time frame where differences in acetate levels occur. On the other hand, the slightly reduced spermidine levels, under MetR had no influence on protein acetylation either, which could hind to a balanced state of these two adverse effects under MetR.

It should be noted that these results are limited to the specifity of our antibodies, as there are distinctions on other residues possible. For a definite conclusion an experimental setting, including all possible lysine residues and methylation antibodies, should be set up. Another limitation is the sensitivity of the method. There could still be a difference in acetylation and methylation levels in vivo of up to 10 to 20%, which is too small to detect via western blot analysis, but still may have an influence on the epigenetic regulation in vivo.

5.2 Methionine restriction causes an upregulation in the synthesis of spermidine and a downregulation of following degradation and interconversion in polyamine biosynthesis

In living organisms, an appropriate polyamine homeostasis is essential for replication and cell growth. A malfunction in this homeostasis has massive impacts on lifespan, leading to, for example, cell death or cell cycle arrest (Loikkainen, 2005). As recent data could show, SAM, the aminopropyl group donor in polyamine biosynthesis, is decreased in MetR strains (Ruckenstuhl, unpublished data). Nevertheless, the levels of putrescine and spermidine remain relatively high (see Figure 5 & Figure **6**). This led to the hypothesis that there might be an up- or deregulation of various enzymes in spermidine biosynthesis to retain relatively high spermidine levels.

In the Northern blot analysis (see Figure 18) we detected an increase of *SPE1* mRNA levels in Δ*met2* strain in the 8h and d6 samples. Regarding the role of *SPE1* in spermidine biosynthesis, namely the reaction of ornithine to putrescine, this trend agrees with the MS data we have, showing an increase in putrescine levels in MetR strain Δ*met2*.

The transcription levels of *SPE2*, analysed via Northern blot (see Figure 21), were decreased over time in all strains but there was no detectable distinction between MET+ and the MetR strains Δ*met2* and Δ*met15*. As our hypothesis contains the assumption that the reduced SAM levels under methionine restriction conditions have an influence on the synthesis of polyamines, the fact that there is no deregulation in this first SAM-dependent step of polyamine biosynthesis does not indicate a direct influence here. *SPE1* and *SPE2* are the rate-limiting enzymes in the synthesis of polyamines, both having a fast turnover rate with a half-life period of less than one hour (Loikkanen, 2005). The intracellular levels of polyamines influence the expression of both enzymes in various ways such as transcription, translation and enzymatic activity. *SPE2* is, for example, regulated by both: putrescine, which enhances the levels of enzyme activity and gene expression and spermidine, which decreases it (Valdés-Santiago et al., 2012).

SPE3, the gene encoding spermidine synthase, was found to be upregulated under MetR conditions in microarray experiments by Johnson et al. (2014). In our Northern

blot analysis (see Figure 15) we could reproduce this finding. In early 8h samples we could show a massive increase of *SPE3* levels and also an increase on day 6 in both MetR strains. *SPE4* levels (see Figure 24) in Δ*met15* are decreased. We also do indeed see a decline of *SPE4* in Δ*met2*. As *SPE4* is involved in the synthesis of spermine from spermidine, this is interesting regarding the suggestion that the cell might try to retain high levels of spermidine, maybe because of its beneficial effects in living organisms and maybe also with an influence on the observed lifespan extension in MetR strains. *SPE3* and *SPE4* are stable enzymes, regulated primarily by the amount of the disposability of their substrate, decarboxylated SAM (Loikkainen, 2005). Bearing in mind that SAM levels are decreased in MetR strains, the found upregulation of *SPE3*, using SAM as important aminopropyl donor, is a possible compensation mechanism in response to lower substrate availability while a decline in *SPE4* most likely further enhances spermidine levels. The data of increased *SPE3* levels in MetR strain Δ*met15* in the early 8h sample could be confirmed in the qPCR analysis.

Through an interconversion step, spermine and spermidine can be reconverted to putrescine in vivo. *PAA1* is the first gene involved in this process in yeast, encoding the polyamine acetyltransferase, an enzyme using acetyl-CoA for the production of N-acetylspermidine or N-acetylspermine. These acetylated intermediates are exported out of the cell or they participate in the interconversion to putrescine via polyamine oxidase (*FMS1*). We determined *PAA1* (see Figure 29) and *FMS1* levels (see Figure 30) in our strains with qPCR analysis, showing a similar regulation “trend” of both: on day one there was a strong increase of *FMS1* and *PAA1* levels in *MET*+ strain. In contrast to Δ*met15* where the levels stayed relatively low, it seems that there was a lot of degradation and rebuild going on in *MET*+ strain, whereas the Δ*met15* seemed to be eager to retain constant polyamine levels.

These results have to be treated with caution as the quality of RNA was not ideal and we had problems with high background signals, but we can see a trend with these results. The partway strong induction in our qPCR analysis was probably due to problems with our endogenous control, as we had strong decreasing *ACT1* mRNA levels, which led to massive effects in calculation.

Furthermore, we started to investigate the transcriptional regulation of spermidine biosynthesis. At first we produced mutants with a knockout of the transcription factor *GCR1*, which is a proposed regulator of the genes *SPE3* and *FMS1*. We conducted Northern blot analysis of *SPE3* levels in these knockout strains (see Figure 31) and found out that the deactivation of *GCR1* did not lead to any verifiable effects. *FMS1* levels were beyond our Northern blot detection limit throughout our research. Tests of the used Northern blot probe showed that we were only able to detect very high concentrations, even of pure *FMS1* PCR product (undiluted and 1:10 dilution detectable) with it. As many transcription factors are involved in the regulation of important genes in polyamine biosynthesis, a closer look at the underlying transcriptional regulation is planned for future studies.

As mentioned above, an appropriate polyamine homeostasis is essential for replication and cell growth (Loikkainen, 2005). The downside of these important polyamines is, for example, a particular disturbance in polyamine homeostasis, found in cancer cells. In cancer cells the polyamine levels are increased (Minois, Carmona-Gutierrez, Madeo, 2011) and *SPE1* and *SPE2* levels are elevated. Polyamine concentration as well as gene expression and activity of enzymes involved in polyamine biosynthesis, especially *SPE1*, are higher in cancer tissues than in normal surrounding tissues, which makes polyamine biosynthesis to a potential target for cancer treatment (Soda et al., 2011). Previous research has demonstrated, that an inhibition of polyamine biosynthesis by DL-α-difluoromethylornithine, an inhibitor of ornithine decarboxylase, with or without methylglyoxal-bis-guanylhydrazone (an inhibitor of SAM) successfully suppressed tumor growth and prolonged survival of tumor-bearing animals (Soda et al., 2011).

Eisenberg et al. (2009) showed the beneficial effects of spermidine administration in aging yeast cells. The treated cells had a prolonged lifespan of up to four times longer than that of untreated cells, related to an induction of autophagy, suppression of necrosis and epigenetic modifications (Eisenberg et al., 2009). Regarding this insight, it is interesting that the cell seems to be anxious to retain high spermidine levels during chronological aging in our research. The upregulation of SPE3 levels and also the decrease in interconversion in the MetR strains, both indicating the attempt to keep a certain spermidine level, may be a part of a survival mechanism including a certain

level of spermidine as a requirement for other lifespan-prolonging factors. Further experiments regarding this mechanism need to be conducted for a clarification of the role of spermidine in the observed lifespan extension under MetR conditions.

6 Summary

In summary, our results suggest:

- no differences in H3 acetylation and di-methylation on investigated lysine residues in MET+ strain vs. Δmet2
- an upregulation in spermidine biosynthesis in MetR strains Δmet2 and Δmet15
- a downregulation of SPE4 levels in both MetR strains, where the cell might downregulate spermine synthesis to keep higher spermidine levels
- an increase in FMS1 and PAA1 levels in MET+ on day one, which leads to the expectation that there is less degradation in MetR strains to retain constant spermidine or spermine levels

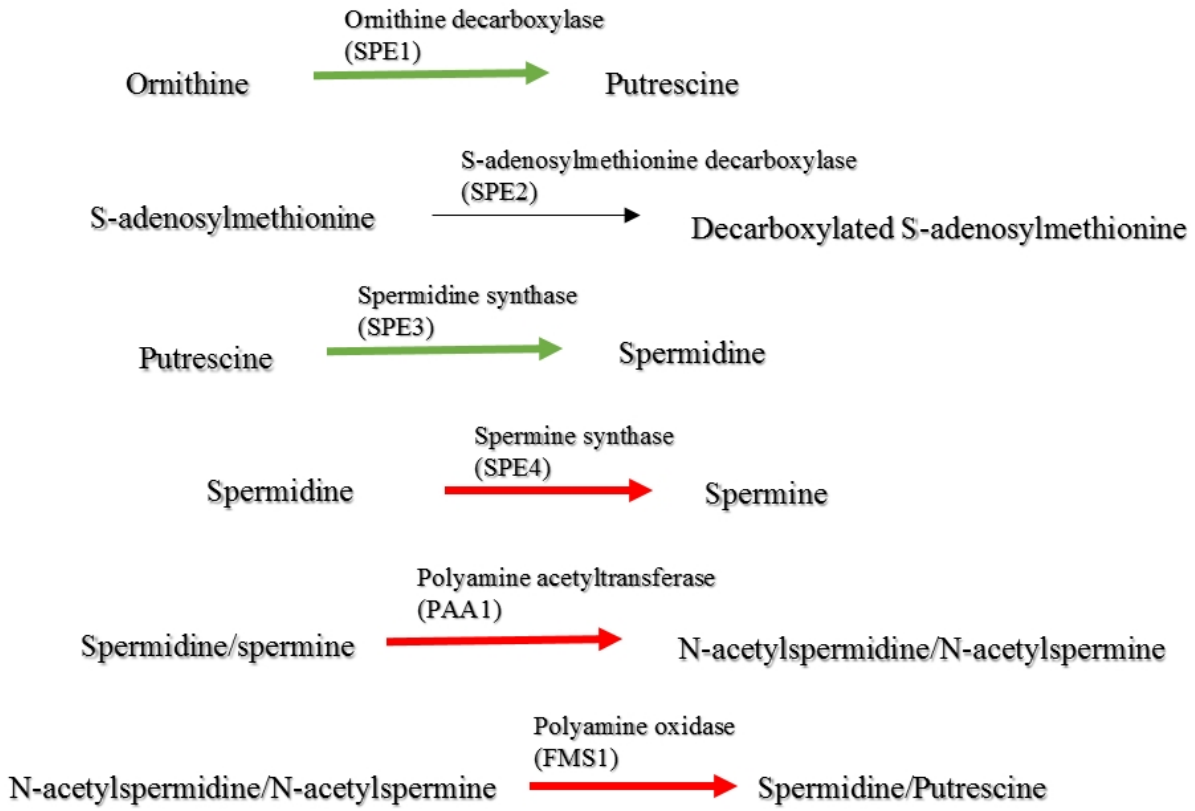

Figure 33 Regulation in MetR strains compared to MET+, green: upregulation, red: downregulation

7 Conclusion

This research indicates distinct changes in spermidine biosynthesis during chronological aging under MetR conditions. The results obtained suggest that the cell seems to be eager to keep high levels of spermidine, which might have beneficial effects for the cell and also an impact on the observed lifespan extension in MetR strains. In the next planned experiments spermidine biosynthesis will be investigated on protein level, to get more evidence for the changes in polyamine biosynthesis. Another interesting point will be the examination of transcriptional regulation factors (see Table 13), to get a closer look at the present mechanism for SPE3 upregulation. Many important questions remain to be answered but the planned experiments will provide a more detailed insight into this specific mechanism and its role in aging.

8 Abbreviations

Table 14 Abbreviations

AcCoA	Acetyl-CoA
AdoMetDC	Decarboxylated S-adenosylmethionine
C. elegans	*Caenorhabditis elegans*
DNA	Deoxyribonucleic acid
MetR	Methionine restriction
MS	Mass spectometry
OD	Optical density
ONC	Overnight culture
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAM or AdoMet	S-Adenosyl-methionine
S. Cerevisiae	*Saccharomyces cerevisiae*
qPCR	Real-time polymerase chain reaction

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