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The Interaction of Aging, Caloric Restriction, and the Sulfur Assimilation pathway explored via Quantitative Time-lapse Flow Cytometry

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The Interaction of Aging, Caloric Restriction, and the Sulfur Assimilation Pathway explored via Quantitative Time-lapse Flow Cytometry

by

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Dedication and Acknowledgements

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The Interaction of Aging, Caloric Restriction, and the Sulfur Assimilation pathway explored via Quantitative Time-lapse Flow Cytometry

Peter Fellowes

Both Caloric Restriction (CR) and Methionine Restriction (MR) extend lifespan in many organisms. Recent results have shown that excess methionine can prevent lifespan extension by CR, suggesting that both MR and CR act by related mechanisms. Reporter strains' expression for genes involved in the sulfur assimilation pathway, as well as a handful of genes suspected to be involved in aging, were measured in a variety of metabolic conditions including CR and MR. By measuring expression levels over time, it was possible to determine the strength and speed of each gene's response to each stimulus. Notably, while large parts of the sulfur assimilation pathway responded to MR, parts only responded to GCN-4 activation via 3-AT, and oxidative stress response genes did not respond significantly to either stress. Met4p, the central sulfur assimilation pathway transcription factor, showed a unique response, by increasing transcriptionally in response to MR, but when glucose was absent. Future experiments can be built off of the tools built here, in order to gain an even deeper understanding of both the aging process as well as the sulfur assimilation pathway.

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Introduction

Lifespan

The aging, senescence, and eventual death of an organism are an aspect of life that is virtually universal. As the medical field in humans advances to the point where aging-related complications (including cancer, Alzheimer's, and other end-of-life illnesses), interest naturally grows in this field, which is not completely understood. Important, unanswered questions include such seemingly basic inquiries such as: what the exact negative effects of aging are and how they can be measured, why organisms do not expend energy to reverse the aging process or how much would be required to do so, and even how even old organisms are able to create young offspring which do not suffer from the effects of aging afflicting their parent.

Theories of Aging

Aging, while a widely recognized and studied phenomenon, is not fully understood. Most organisms begin their life in a state of growth or development, proceed to reproduction, but then after they have stopped reproducing, linger on for some time before dying.

While some biochemical signs of aging have been identified in organisms such as yeast [1], and worms [2], it is ultimately unclear what all the signs and symptoms of aging are. There does not exist a perfect assay for determining how "old" an organism is – the only two things that can be measured with accuracy are when an organism stops reproducing, and when it dies.

One of the first modern theories of aging is that oxidative damage to important aspects of the cellular machinery, from DNA to enzymes, could result in gradual accumulation of damage over a life, and eventual death [3]. Oxidative stress is typically defined as the presence of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, and the hydroxyl radical. These are highly reactive, and will oxidize many organic molecules including proteins, lipids, or even DNA in ways that can change or inhibit their function.

The source of oxidative stress can be extracellular (for example hydrogen peroxide outside the cell or ultraviolet rays), but many of the ROS the cell is exposed to are derived from inside the cell itself, specifically from metabolism. During respiration, pyruvate is broken down into is broken down into carbon dioxide, generating large quantities of ATP in the electron transport chain. However, due to the nature of the chain, it results in ROS being released at a low frequency [4].

Reproductive Lifespan and Chronological Lifespan

There is a tendency for lifespan studies conducted in yeast to examine the chronological lifespan. While superficially, this seems like a very useful metric, it is not necessarily clear that it holds much similarity to chronological aging in humans. In yeast, chronological aging is generally conducted by growing a population of yeast in a rich media until the carbon resources are exhausted by the consumption of the yeast, or removed by the experimenter. The cells are then left in the depleted media for a time, and at timed intervals, samples are placed into fresh media and assayed for their ability to begin growing again. Reproductive lifespan, on the other hand, keeps track of individual virgin (never budded) mother cells, and tracks the number of offspring they are able to generate before senescence and death.

One of the most notable things about chronological aging, then, is that it primarily assays for resistance to a particular type of stress – the ability to suspend metabolism for a period of time, until introduced to a fresh source of carbon. In fact, it has been shown that the primary determinant of survival under such starvations for a variety of "basic, essential" nutrients (e.g. Carbon, Sulfur, Phosphorous) is the ability to dramatically slow down metabolism under such stresses [5]. While it is important for the cell not to die over this time, it is not immediately clear whether such deaths are better attributed to starvation or aging.

In fact, the slowing of metabolism generally seems to avoid aging. This can be demonstrated by the extremely long survival (years) of yeast strains frozen in glycerol, where their metabolic and cell processes are all but suspended. Nobody

suggests that freezing in glycerol is inherently healthy for the yeast, let alone the higher organisms it is meant to model. It does not seem likely that such conditions induce genes which help to counter aging – rather it seems far more likely that like everything else, the factors which contribute to aging simply accumulate far more slowly in such a case, as virtually all activity in the cell comes to a halt.

On the other hand, replicative lifespan, while not a perfect measure, offers a different form of insight into aging. First, the measurement does not depend on the response to a very particular type of stress. A researcher can stress or starve the cells during the process of measuring, but there is no mandatory starvation phase. Using reproductive lifespan also helps to avoid the question of stalled metabolism slowing the accumulation of aging, but also everything else. Simply stopping cellular processes may extend chronological lifespan, but will not prove beneficial to reproductive lifespan, since no offspring will be produced during such a period. Reproductive aging might also help determine how long an organism is truly healthy, as well. "Aging well," or maintaining good health and functionality, is considered important to aging, and an organism must be in good health to produce healthy offspring, not simply hanging on with the barest functionality.

However, it is important to note that until recently, replicative lifespan was extremely difficult to measure, in comparison to chronological aging. It is likely that the ability to assay chronological aging in high-throughput is one of the reasons for its common use. Originally, it was necessary to manually follow several unbudded

mother cells manually, removing their daughters manually, and counting. This manual process was extremely time consuming, and was not practical to do with large sample sizes or multiple conditions. Even with modern microfluidic techniques, the process is far more complicated and time intensive than performing a comparable experiment for chronological lifespan. However, progress is continually being made in allowing these experiments to be performed in higher throughput.

Caloric Restriction

Previous research has shown that restricting food intake, a process known as Dietary Restriction (DR), can prolong the lifespan of many organisms significantly. This appears to hold true across all organisms so far studied.

In yeast, specifically, it is known that restriction of glucose (the primary source of calories) in the growth media to a .05%-.5% range while leaving other nutrients constant (caloric restriction, CR), can extend lifespan in comparison to the 2% glucose "basal" condition. Further restriction (to .005%, or beyond) also leads to a diminishment of lifespan, probably due to starvation. The optimal concentrations seem to be strain-specific, however.

Theories on Caloric Restriction and Aging

Theories abound as to why DR and/or CR are so universally able to extend lifespan. However, while each of these theories is logically sound, and can offer an interesting way to view the relationship between aging and CR, none of them has been proven conclusively.

TOR. The TOR (Target of Rapamycin) complex is conserved from yeast to mammals, though exact functionality is not the same. Both the yeast TOR Complex 1 (TORC1) and the mammalian TOR complex (mTOR) respond strongly to the drug rapamycin. When it is given to them, TOR activity is repressed. Repression of TOR leads to activation of stress pathways as well as decreased ribosome biogenesis. Repression of TOR generally results in lifespan extension, though the exact mechanism for this extension is unknown.

Notably, glucose levels can impact TOR activity. In yeast, Snf1p is at the heart of a complex that is sensitive to AMP levels. AMP typically only accumulates in large quantities if the cell's energy stores, in the form of ATP and ADP, are depleted and broken down into AMP. Since glucose can be quickly utilized by the cell to refresh ATP stores, Snf1p will not be active in the presence of glucose, but will be active when the cell lacks energy. In that way, caloric restriction can lead to increase of in Snf1p activity. Snf1p activity in turn is believed to decrease TOR activity, and thereby increase in the activity of stress proteins such as Msn2/4p. The Msn proteins are very promiscuous, and have a wide variety of targets, including genes

involved in other explanations for lifespan extension such as Pnc1p, an indirect regulator of Sir2p activity.

Oxidative Stress. Oxidative stress, the presence of charged, highly reactive ROS such as hydrogen peroxide, superoxide, and the hydroxyl radical, which can permanently damage organic molecules, can stem from many sources ranging from environmental to cellular respiration. As discussed earlier, it is possible that accumulation of oxidative damage may be one of the key factors leading to aging. One observation that is quite striking is that organisms experiencing DR typically have far fewer oxidatively damaged proteins, lipids, and DNA than similarly aged non-DR organisms. Therefore, it is easy to conclude that DR provides some form of protection against oxidative stress. If oxidative stress is indeed the primary mechanism for aging, then being able to protect against it would certainly extend lifespan.

Hormesis. Hormesis is the idea that low levels of stress are actually healthier for an organism than no stress at all. Caloric restriction is known to activate general stress response pathways governed by Msn2/4. Hormesis posits that low-level exposure to stresses is healthy for the cell, and does not necessarily distinguish between the exact causes of such stress.

Mitorhormesis. Mitohormesis simply takes the quite general idea of hormesis and applies it to the stress of oxidative stress. Fundamentally, the idea is

that low, unavoidable oxidative stresses cause gradual aging of the cell. When the cell is exposed to high levels, this accelerates despite the cells' attempt to mitigate the damage via oxidative stress responses. However, at moderate levels of stress (for example, those caused by respiration brought on by a glucose shortage), the protective machinery is activated and actually results in lower damage to the cell than at very low levels of stress, when the machinery would be off.

Moreover, it has been discovered that CR does not have a beneficial effect on the lifespan of yeast living in anaerobic conditions, actually leading to a decrease in lifespan [6]. Anaerobic conditions prevent respiration, and therefore the increased respiration and accompanying ROS that are normally the consequence of respiration do not materialize. This suggests a role for CR in extending lifespan by increasing the levels of oxidative stress.

It is also possible that the mitohormesis theory could be partially accurate. Evidence shows that there is a strong basis for finding that moderate levels of oxidative stress can indeed increase lifespan [7], but the effect does not need to stem directly from the redox response. Transcription factors, especially those in stress responses, are notoriously promiscuous, and hit a wide variety of targets, some of which do not have an obvious connection to the stimulus. It is possible that the activation of stress transcription factors such as Msn2/4 leads to off-target regulation of genes, which is responsible for the lifespan extension phenotype. That is, at low basal levels of oxidative stress, damage to the cell is negligible. At

moderate levels, due to protective machinery, damage is still quite minor, but a lifespan-extending target is triggered. At high levels, damage overwhelms the redox response, and the cell becomes damaged and dies earlier. Alternatively, the activation of respiration, instead of the stress response to the ROS respiration puts out, could be responsible for activating genes important for lifespan extension [6].

An interesting question when thinking about mitohormesis, and hormesis in general, is if the slightly activated state is favorable, why evolutionary pressure does not favor organisms that constitutively express low levels of these proteins, instead of requiring moderate levels of stress. Or in the case of the off-target version, why is the off-target reliant on oxidative stress instead of being constitutively activated? Unfortunately, there is no clear answer.

"Disposable Soma" theory. This theory posits that when resources are scarce, the organism is likely to only be able to reproduce infrequently (since creating a new organism is very resource intensive), and that it therefore enters a state of preserving its somatic cells rather than growth and reproduction (or in the case of single cellular organisms, preserving their current cell rather than dividing its resources in two). When resources are abundant enough to reproduce, fewer resources are devoted to preservation: the soma are disposable, and need not be maintained as long as the germ can reproduce themselves. The reasoning makes sense when thinking about an organism under serious caloric restriction: preserving your future ability to reproduce later on in better conditions (and when

your offspring will enjoy better conditions as well) is extremely important compared to trying, and probably failing, to reproduce now. However, the important question becomes when calories are not scarce, why the organism does not still devote some of those resources, of which there is no shortage, to maintaining somatic cells, so that it can continue to reproduce for longer.

It is also possible that time is the resource in question, and that the tradeoff is not between metabolic resources devoted to maintenance of the soma and the reproduction germ, but time required between divisions in order for repair machinery to do its job thoroughly [8]. Presumably, an organism whose ecological niche requires it to have a longer lifespan has few alternatives, although it certainly comes at the cost of being able to grow (and therefore reproduce) less quickly. In fact, it has been shown that longer-lived species arrest cell cycle more fully in order to ensure high fidelity repair [9]. Although this version of the disposable soma theory does not explicitly explain the way in which caloric restriction extends lifespan, it is possible that since when an organism is calorically restricted, it by definition is unable to grow as quickly as it would like, it grows more slowly, allowing repair machinery more time to work.

Sirtuins. In yeast, one established cause for aging is the accumulation of Extrachromosomal rDNA circles (ERCs), which result from a recombination event excising them from the genome, replicating themselves, and eventually reach toxic levels [1, 10]. Sir2p, a deacetylase involved in chromatin silencing, is the gene

primarily responsible for inhibiting accumulation of such ERCs. Sir2p's activity, however, is inhibited by the presence of nicotinamide. In turn, Pnc1p is responsible for catalyzing a step in the NAD+ salvage pathway, converting nicotinamide into nicotinic acid. Pnc1p, in turn, is up-regulated by the general stress factors Msn2p and Msn4p, which are triggered under caloric restriction conditions.

However, Sir2p's ability to suppress ERCs is only relevant in yeast, as other organisms do not share this problem with yeast. However, Sir2 has homologs in more complex organisms, called sirtuins, expand on the genome regulating function of Sir2p [11] and are involved in regulating DNA repair [12], chromatin structure [13], and telomere maintenance [14]. Since repairing damage to DNA is likely to help mitigate at least some sources of aging, this suggests that Sir2p and the sirtuins may be crucial players in the lifespan extension by CR.

Methionine

Like glucose, methionine concentrations can impact the lifespan of yeast. Restricting methionine while keeping glucose constant at 2% (methionine restriction, MR) is able to extend lifespan in a similar magnitude as CR [6], even in non-auxotrophic strains (Ke Zou, unpublished results). Similarly, MR has also been found to extend lifespan in rats [15]. Drosophila receiving fewer proteins (including methionine), in the form of yeast in their food, also showed a lifespan increase compared to those eating a similar number of calories, but fewer proteins [16].

It is also interesting to note that restriction of methionine behaves differently from restriction of other amino acids. If an auxotrophic strain of yeast is starved for most amino acids, it will continue to consume glucose and will not arrest its growth – eventually leading to death. However, if a methionine auxotroph is starved for methionine, it will arrest in an unbudded G1 phase and cut back glucose consumption significantly, entering a resting state where it is able to survive far longer [5, 17]. This response to starvation is much more similar to the response for "natural nutrient" starvation – e.g. Nitrogen or Phosphate – than it is to other amino acid auxotrophies. What is more, this different response appears to be driven by cell cycle arrest accompanied by a sharp reduction in glucose consumption.

Most interesting, however, is the impact that methionine levels have in conjunction with glucose. During CR, if excess methionine (10x) is added to the media, then the lifespan extension phenotype typically generated by CR is negated, whereas addition of excess methionine to cells with normal glucose levels does not impact lifespan. This implies that the lifespan extension by CR likely shares a pathway with the lifespan extension by MR. It is hoped that since the two appear to have at least partially overlapping mechanisms that examining a second source of input, methionine, will provide additional information compared to just examining CR independently. It is important to note that this phenotype was observed not only in methionine auxotrophs, but in prototrophs as well, indicating that the important

factor may not the absence of methionine itself, but the need to activate the sulfur assimilation pathway.

Sulfur Assimilation Pathway

To better understand methionine's impact on the aging process, it can be very informative to understand the Sulfur Assimilation Pathway, a process for taking extracellular inorganic sulfur and eventually incorporating it into sulfur-containing metabolites such as methionine, S-adenosyl-methionine (SAM), cysteine, and glutathione.

An essential ingredient in cell growth and metabolism, sulfur must be imported into the cell. Such import often takes place in the form of inorganic sulfate uptake, but can also be achieved by import of extracellular sulfur-containing metabolites including cysteine, methionine, and SAM via import proteins specific to these metabolites. Without sufficient sulfur available to be incorporated into necessary molecules, yeast will tend to arrest in the G1 phase, without budding [5, 17].

Methionine and cysteine are the two proteinogenic amino acids that contain sulfur. Essential for the translation of proteins, they are among the sulfur-containing molecules necessary for continued cell growth and division. It has been previously discovered that starvation either for methionine or cysteine can lead to

increased transcription of the genes involved in the sulfur assimilation pathway, and thereby lead to production of those amino acids. However, a consensus has not yet emerged as to the exact manner of regulation of the sulfur assimilation pathway. Some research implicates cysteine as a sole regulator [18, 19], while other research implies that this is not the case – methionine or SAM are more likely targets [5]. Methionyl-tRNA (as an indirect measure of methionine) has also been implicated [20].

In addition to triggering the sulfur assimilation pathway for methionine and cysteine biosynthesis, sulfur starvation also causes arrest of the cell cycle in an unbudded G1 phase. Accompanying this arrest is a marked decrease in glucose import and consumption, suggesting an overall reduced metabolic flux [5]. Whether this is an inevitable side effect of the activation of the sulfur assimilation pathway or these are two independent but correlated processes is unknown.

The Sulfur Assimilation Pathway Enzymes

The sulfur assimilation pathway consists of several branches and cycles (Figure 1). First, after Sul1p and Sul2p have imported sulfate into the cell, it is converted into sulfur by the action of several enzymes (Met3p, Met14p, Met16p, Met5p, Met10p). Meanwhile, L-Aspartate is converted into O-Acetyl-L-homocysteine (Hom3p, Hom2p, Hom6p, Met2p). Met17p (also known as Met15 or

Met25) combines these into homocysteine. From homocysteine, metabolism can continue on one of two different paths.

One option for homocysteine is to be converted (reversibly) to L-cysteine. Cysteine, in turn, can be converted into a potent antioxidant, glutathione, which can be converted back into cysteine. The other path requires an input from the folate pathway (via Met12p, Met13p, and Met7p), 5-methyltetrahydropteroltri-L-glutamate, to donate a methyl group to homocysteine, converting it to L-methionine via Met6p, in an irreversible reaction. Methionine can convert into SAM reversibly (Sam1p and Sam2p can each catalyze the forward reaction, Sam4p and Mht1p can each catalyze the reverse). SAM is itself a very potent methyl-donator in many reactions in the cell, and after performing such a donation becomes SAH. SAH can in turn be converted back to homocysteine via Sah1p.

Evidence for Cysteine as a sole Regulator

Some papers purport to have implicated SAM over methionine, in *Δsam1* or *Δsam2* strains, to prevent conversion of methionine into SAM, and showed that SAM was able to restore growth and/or repress methionine pathway genes, but methionine could not [21]. However, through the activity of Sah1p, S-Adenosyl-homocysteine (SAH), the product of SAM demethylation, can be converted back into homocysteine, and thence to cysteine or methionine. This means that in such strains, providing methionine as the sole sulfur source will cause starvation for

cysteine (because the Met6 catalyzed reaction is not reversible), while providing SAM will allow the cell to convert it into SAH, homocysteine, methionine, and cysteine.

The evidence for cysteine is most clearly demonstrated by a pair of experiments in mutant strains. First, a Δsah1 strain, unable to convert SAH back into homocysteine, was fed excess methionine, but the methionine pathway genes were still highly expressed. However, upon being fed cysteine (and therefore able to make all sulfur compounds), expression of the methionine pathway was repressed. Similarly, in a Δcys4 mutant unable to convert homocysteine into cysteine, only addition of cysteine was able to again repress the pathway [19].

Moreover, this activity can be attributed to cysteine (or at least cystathione, the intermediate metabolite in cysteine biosynthesis). In a Δgsh1 strain unable to convert cysteine into glutathione, repression could be restored by any metabolite, meaning that production of glutathione was unimportant to the regulation of sulfur metabolism [19].

Evidence against Cysteine as a sole Regulator

However, it is not clear that this is the whole story. One major indication is the behavior of Δmet6 mutants, which are able to synthesize homocysteine but are unable to convert it into methionine or SAM. The cysteine biosynthesis pathway

remains fully intact in these organisms. Nonetheless, growth in minimal media activates the methionine pathway, although ample cysteine can be, and is, synthesized [5, 17]. Therefore, a molecule downstream of Met6p, such as methionine or SAM, must somehow be able to contribute to the regulation of the methionine pathway.

Methionyl-tRNA is also implicated, at least in the arrest process. While characterizing an unusual mutant that showed both temperature sensitivity and methionine auxotrophy, it was discovered that this particular mutant had methionyl-tRNA with a pair of unique properties [20]. Not only was it temperature sensitive, but it also had a very weak affinity to binding methionine, which could only be overcome by addition of excessive levels of methionine into its growth media. This strain would arrest whenever the tRNA was unable to bind much methionine, whether due to concentration or temperature. This was taken to mean that the arrest necessary for extended chronological lifespan was caused by the tRNA or something downstream – not upstream. However, it is known that GCN4, which operates a separate, general, amino acid starvation response and can cause cell cycle arrest, responds in a TOR-dependent manner to the levels of unbound tRNA. This response, rather than methionine restriction itself, may explain the observed phenotype of cell cycle arrest, associated with increased chronological lifespan.

Transcription Factor Regulation: Degradation Control

Regardless of the ultimate intracellular driver of the sulfur assimilation pathway, much is known about the way that it comes to be activated. Met4p, the primary methionine transcription factor, forms three complexes: Met4p-Met28p-Cbf1p, Met4p-Met28p-Met31p, and Met4p-Met28p-Met32p. Met4p is recruited to the binding cofactor (Cbf1p, Met31p, or Met32p) by Met28p, and then is able to facilitate transcription. The Cbf1 complex will bind the CACGTG motif [22], while the Met31 and Met32 complexes bind AAACTGTGG [23]. Most genes in the methionine pathway contain either or both of these motifs, and respond to elevated levels of these transcription factors.

Under conditions of methionine starvation, Met30p is de-activated. Normally part of a protein degradation complex (SCF^{MET30}) which ubiquitinates many targets, its activity is positively correlated with abundant sulfur metabolites. SCF^{MET30} binds Met4p, which is constitutively expressed at a constant level, and ubiquitinates it [24]. However, this ubiquitination does not actually result in the degradation of pMET4, instead only inactivating it, and preventing it from initiating transcription [25].

However, SCF^{MET30} is able to ubiquitinate and degrade the co-factors Cbf1p, Met31p, and Met32p. Interestingly, enough, however, this process is Met4p dependent: Met4p must be bound to SCF^{MET30} in order to facilitate this

ubiquitination and degradation. In this way, in the presence of active Met30p, Met4p actually leads to the degradation of its cofactors.

SCF^{MET30} is, however, not the only cause of Cbf1p, Met31p, and Met32p degradation. All of these proteins are actually actively degraded in the absence of Met4p, even when SCF^{MET30} is not present. The factor responsible for this degradation remains unknown. However, Met4p is able to prevent this degradation by binding to its cofactors, as they are only degraded when unbound [24].

This means that in an interesting set of interactions, Met4p can be responsible for either the degradation of its cofactors (in the presence of SCFMET30) or responsible for preventing such degradation (in the absence of SCFMET30).

This pattern of regulation would suggest that when the cell has abundant sulfur metabolites, SCFMET30 will be active, resulting in inactive Met4p and degradation of the Cbf1p, Met30p, and Met32p cofactors. When the cell lacks sulfur metabolites, SCFMET30 will be inactive, allowing active Met4p and its cofactors to accumulate, and initiate transcription of the proteins required for sulfur assimilation.

Transcription Factor Regulation: Transcriptional Control

In addition to a complex relationship of degradation control, the transcription factors of the methionine pathway also regulate each other transcriptionally. Overexpression of some transcription factors, leading to their increased activity, results in changes in the expression levels of other transcription factors. Notably, Met4 overexpression increased levels of Met28, Met30, and Met32 transcripts, CBF1 overexpression decreased Met28, Met 30 and Met31 transcripts, and Met28 overexpression decreased Met31 transcripts [26].

Regulation of Enzymes

The regulation of the sulfur assimilation pathway enzymes by their transcription factors is fairly well understood, although a few unanswered questions remain. The complexes Met4p-Met28p-Cbf1p, Met4p-Met28p-Met31p, and Met4p-Met28p-Met32p are responsible for promoting transcription of these enzymes. Met31 and Met32 are paralogs, and therefore behave very similarly, binding the same motif, although there is some evidence that there are some important minor differences between the two proteins [27]. The responses of each gene to overexpression of various methionine transcription factors have even been characterized [28].

It is worth noting that a thorough analysis of metabolic flux through the system has shown that there is no particular rate-limiting step in this process – no step of the pathway is saturated under most conditions [29]. As a result, both

increased enzyme levels as well as increased metabolite and/or sulfur inputs will increase flux through the pathway.

It is also possible to trigger the sulfur assimilation pathway by exposure to cadmium. Glutathione, an important antioxidant, is crucial in neutralizing cadmium, and is derived from cysteine. Notably, in this case, cysteine synthesis genes are selectively up-regulated, and flux through the pathway is directed disproportionately at cysteine and glutathione, and not at methionine and SAM [29]. This is to be expected since under such stress, the cell has great need of glutathione, and therefor cysteine, but not methionine. However, these results are interesting, since they suggest that the sulfur assimilation pathway can be regulated in different ways by different compounds – the pathway has more than just an "on" and "off" state. This may suggest a role for Cbf1, known for its role as the primary transcription factor in the Cadmium response, in regulating part of the pathway separately from the rest.

Summary

The Sulfur Assimilation Pathway, responsible for the biosynthesis of methionine and cysteine, is quite complex. Between an extremely long non-linear chain of enzymes with multiple substrate inputs and outputs and transcription factors that regulate each other translationally and post-translationally, fully understanding the system is quite a large undertaking. As a result, many questions

remain about the pathway's regulation. Most notably, the substrate(s) that activate the pathway have not yet been positively identified.

Materials and Methods:

Creation of Reporter Strains:

The creation of the reporter strains in wild-type S288C was facilitated by building a number of related plasmids, replicated in E Coli. These plasmids were built off a plasmid backbone (pvc002), containing an intact version of Ura3, as well GFP and resistance to ampicillin. The promoter region of the gene to be reported was replicated via PCR of genomic DNA. This DNA segment was then inserted into the plasmid immediately in front of the GFP via digestion and ligation. The resulting plasmid was then transformed into E Coli, which was then plated onto media containing ampicillin, to select for plasmid incorporation. The colonies were sequenced to verify that the plasmid was indeed correct. The plasmids were then harvested and transformed into S288C cells with a non-functional Ura3 locus. Transformation success was assayed by both confirming growth on media lacking uracil and expression of GFP.

The exception is the Gcn4p activity reporter, which had been already built, designed and tested by Victor Chubukov.

Flow Cytometry:

Flow cytometry was conducted using an automated flow cytometry robot, as described in Zuleta et al. [30]. In each case, strains were kept continuously growing, and then diluted to approximately the same level. They were then monitored and measured in Synthetic Complete (SC) media for at least an hour. Between the final growth measurement and the initial condition measurement, the 96-well plate containing the various reporter strains was spun down and the cells washed with the condition media twice. The reservoir media was also replaced with the condition media, as well. Automatic dilutions and measurements continued overnight at 20-minute intervals.

Media used was SC media, with a few modifications. One condition involved leaving methionine out of the media altogether, forcing the cell to activate the sulfur assimilation pathway to continue growth. Another condition involved Caloric Restriction in the form of leaving out histidine and replacing it with 10mM 3-Amino-1,2,4-Triazole (3-AT), which causes the cell to starve for histidine by inhibiting further synthesis. The final condition was Caloric Restriction – reducing the glucose concentration from 2% to .05%. These conditions (glucose level, methionine level, and presence of 3-AT/histidine) were also mixed and matched.

Data Analysis:

The flow cytometry data obtained as described above was then processed in order to determine the rate of translation at every time point. Based on the measured rates of cell division and dilution of the media, it is possible to calculate the rate of protein synthesis over time. The instrument itself only directly measures the fluorescence level of each cell. However, the change in fluorescence in each cell can be defined as the rate of protein synthesis minus the total degradation flux of the protein. GFP is very stable, so it effectively does not decay over the timespan of the experiment. However, the cells do divide, thereby splitting some accumulated fluorescence away. This allows us to use Equation 1 to determine the rate of protein synthesis at a given time, where A_{cell} is the synthesis rate per cell, F_{cell} is the fluorescence of each cell, and D is the cell density measured by the flow cytometer.

$$A_{cell}(t) = dF_{cell}(t) - F_{cell}(t)*dLog(dD(t)) \quad [1]$$

Within an experiment, the growth rates of different reporter strains under the same conditions were grouped together. This helped reduce the noise in growth rate measurement, since the growth rates of the different reporter strains in the same media should be essentially the same. Without such a correction, the noise of the growth rate calculations caused severe fluctuations in the calculated protein translation level.

Additionally, the spinning down of the cells to switch media also had a notable effect on the measured cell growth rate. As a result of the spinning and

washing, several cells were lost, and the effect was not entirely uniform across wells. This would result in a minor miscalculation of the growth rate for that pair of timepoints.

Results:

Several reporter strains were successfully constructed from the S288C strain as described in Materials and Methods. Table 1 shows genes that were considered for reporter status, as well as the progress made in this library. Unfortunately, difficulties were encountered in the cloning process, so not all constructs were successfully made on the first several attempts. Moreover, of those made, not all constructs could be measured in the initial set of experiments (there was only space for 24 conditions per plate including controls). Overall 30 reporter strains were generated in addition to the Gcn4 activity reporter.

Role:	Gene:	Status:
Sulfate to Sulfur	Met3	Not constructed
	Met14	Constructed
	Met16	Measured
	Met5	Constructed
	Met10	Constructed
Aspartate to O-acetyl homoserine:	Hom3	Constructed
	Hom2	Measured
	Hom6	Constructed
	Met2	Measured
Folic Acid Cycle Inputs:	Met12	Not constructed
	Met13	Not constructed
	Met7	Not constructed

Homocysteine Synthesis:	Met17	Not constructed
Methionine Synthesis:	Met6	Measured
Homocysteine/Cysteine cycle:	Cys4	Not constructed
	Cys3	Not constructed
	Str2	Measured
	Str3	Not constructed
Cysteine/Glutathione cycle:	Gsh1	Measured
	Gsh2	Not constructed
	Ecm38	Not constructed
	Dug1	Not constructed
S-Adenosyl-Methionine Cycle:	Sam1	Measured
	Sam2	Measured
	Sah1	Not constructed
	Mht1	Measured
	Sam4	Measured
Methionine Import:	Mup1	Measured
	Mup3	Measured
Sulfate Import:	Sul1	Measured
	Sul2	Measured
General Amino Acid Starvation:	Gcn4 activity	Measured
Methionine Transcription Factors:	Met28	Not constructed
	Met30	Constructed
	Cbf1	Measured
	Met31	Not constructed
	Met32	Measured
	Met4	Measured
Siroheme biosynthesis	Met1	Measured
	Met8	Constructed
Constitutive gene expression (control):	Act1	Measured
	Hht1	Not constructed
Oxidative Stress:	Trx2	Constructed
	Sod1	Measured
	Pnc1	Not constructed
General Stress (including oxidative):	Hsp12	Not constructed
	Hsp104	Measured
General Stress (Msn2/4p activity):	Hsp82	Measured

Table 1: Reporter Strain Construction Status

Twenty-three of the reporters constructed above were measured in eight different conditions corresponding to presence or absence of methionine, 2% or .05% glucose, and normal hisitidine or 3-AT. The fluorescence of each of these reporters in each of these conditions was then measured for several hours at twenty-minute intervals.

One advantage of performing a time course measurement rather than a single time point before and after is that it allows both the division rate of the cells and the level of translation of the GFP reporter to be assayed. In turn, as explained in the Methods section, this not only gives a general sense of the response over time, but also allows the synthesis rate of the protein over time to be directly calculated. Moreover, before conducting the time course measurements, it cannot be known on what timescale the transcriptional response of a particular gene will occur on, and whether they are comparable across genes. Picking a wrong single time point could severely impact the results. Conducting a time course ensures that the full response, and its magnitude, is captured.

Analysis of the expression patterns measured in this way led to three distinct clusters of behavior among gene types (Figure 2). First, many genes, chiefly those in the sulfur assimilation pathway, responded to methionine restriction but not to glucose or 3-AT by itself. Second, a handful of genes, including Gcn4p, responded strongly to the 3-AT signal, but not to glucose or methionine. Third, two heat shock

proteins responded to increasing cell density. Finally, a handful of proteins did not have significant differential responses at all.

As expected, nearly all sulfur assimilation pathway genes tested were generally up-regulated when methionine was not present in the media. Moreover, the response of these genes was not changed significantly by the presence or absence of glucose in the media (Figure 4). They did not respond to 3-AT (stimulation of the general amino acid starvation stress response) noticeably, though in the cases where methionine was absent and 3-AT was present, exposure to 3-AT resulted in a smaller initial burst of translation, but a higher level of expression over time (Figure 4).

GCN4 activity was not triggered by the absence of methionine in the media. Since the strain was not auxotrophic, activation of the sulfur assimilation pathway was sufficient to ensure that the cell experienced no true starvation – only MR (Figure 5). A few genes in the sulfur assimilation pathway, Hom2, Sam4, and Str2, were regulated in the same manner as GCN4 activity, but in a slightly delayed fashion compared to GCN4 – possibly being activated by it. Also, these genes are not typical genes in the sulfur assimilation pathway. Sam4 and Str2 are responsible for the "reverse" steps of changing non-amino acids SAM and Glutathione into methionine and cysteine, respectively – a useful function if the cell is starving for amino acids. Hom2's regulation by Gcn4p has previously been characterized [31]

and it catalyzes an early step in production of threonine as well – having it regulated by a general mechanism instead of one pathway seems sensible.

Act1p, known to be expressed constitutively, accompanied the control of a completely non-fluorescent strain. Act1p was expressed at constant levels across all conditions, though at slightly lower levels when under 3-AT stress, possibly due to difficulty making large quantities of protein in that case. The oxidative stress response protein Sod1p had insignificantly higher levels in response to lack of glucose, and slightly lower levels in response to 3-AT. The Cbf1p transcription factor showed fairly constant expression profile across all conditions.

Two heatshock proteins implicated in oxidative stress response, Hsp82p and Hsp104p, were also part of the "non-response" cluster, but formed an interesting sub-cluster within it by responding in a fairly unique manner. They tended to rise over time in all conditions, but grew far slower in the presence of 3-AT or CR (Figure 5). Interestingly, this seems to correlate quite well with the increase in cellular density.

Two genes did not cluster well, being loosely affiliated with the methionine response cluster. First, Mup1 was very noisy and only responded mildly to the absence of methionine. Far more interestingly, Met4 behaves very much like a normal member of the methionine response cluster in 2% glucose, clustering very will with them. However, under conditions of glucose restriction, it behaves far

more similarly to the members of the non-response cluster, behaving similarly to genes such as Sod1 (Figure 6). This means that it responds to methionine's absence only when there is abundant glucose.

Discussion

Although it is not possible to conclusively answer all the questions posed for this experiment, the results generated still shed some important light on the way in which glucose and methionine may interact to influence the aging process. For instance, although the response of sulfur assimilation pathway genes to methionine's absence is predictable, the fact that most do not respond noticeably to 3-AT's triggering of Gcn4p nor to glucose restriction is quite informative. Similarly, the fact that a handful of sulfur assimilation pathway genes respond to 3-AT rather than to methionine restriction is also quite interesting. Looking at their position in the sulfur assimilation pathway (Figure 3) suggests that there is an interesting pattern to their regulation, with flow "down" the pathway typically being driven by methionine shortage and flow "up" the pathway being driven by general amino acid starvation.

The experiments show a few new results. First, many sulfur assimilation pathway genes respond in a very time-sensitive manner to methionine shortage: a brief burst of transcription followed by a lull. On the other hand, activation of GCN4

makes such responses far more pronounced over time. It is, however, unclear why this effect occurs.

The most interesting findings of expression patterns have to do with the oxidative stress genes and the methionine transcription factors. The oxidative stress genes that were measured, as a whole, did not respond significantly to any of the environmental changes posed here. This is a point of concern if oxidative stress response is a major factor in the lifespan extension of CR/MR.

Two interesting observations arise from observing transcription factor expression. First, Met4 has a somewhat unique phenotype in that it does become activated in response to MR, but it does not do so if also in CR. It was hoped that a gene would be found that responds to both stresses, especially an important transcription factor like Met4p. However, the response that would have been most informative would have been an "or" relationship between CR and MR. Instead, Met4 demonstrates an "and not", which is not consistent with its up regulation leading to the lifespan extension. Also interesting is the differential regulation of Met32 and Cbf1. Both cofactors of Met4p in the regulation of the sulfur assimilation pathway, only Met32 responds transcriptionally to methionine shortage.

Future Experiments:

Future experiments, including using the already designed constructs to finish the goals of the experiments already conducted, as well as examine two other important questions could be prove quite informative. It is also possible that they will be able to answer some of the questions raised but not yet answered. It is hoped that the results in this paper and that the toolboxes created, both biological and bioinformatical, allow for further insight into the process of aging in yeast. Although it is not possible to perform them at this time, it is hoped that describing possible experimental uses of these resources might help further better understanding of both the sulfur assimilation pathway, as well as the relationship between CR, MR, and lifespan extension.

Inferring Inhibitors of Aging by Correlating Gene Expression and Lifespan Phenotype

Ultimately, the results in this document are insufficient to determine either genes directly implicated in aging, or shed new light on the aging process. Measuring the other constructs under these conditions would give greater insight into the response of the genes of the sulfur assimilation pathway as well as the oxidative stress response. Further measurements, would also be needed to cover the full variety of conditions for which the lifespan is known, namely under conditions of:

1. Highly restricted glucose (.005%)
2. 2% glucose, 10x methionine

3. .05% glucose, 10x methionine

Once these measurements are completed, it should be possible to examine the responses of the genes for any which respond differently in cases that extend lifespan and those that do not. Any gene that did would be a good target for future investigation, since its expression pattern is highly correlated with the lifespan phenotype, meaning that it is likely either the cause or that both are driven by the same cause.

	2% glucose	.5-.05% glucose	.005% glucose	.00005% glucose
10x methionine				
1x methionine	Done	Done		
-methionine	Done	Done		

Table 2:

This table represents a variety of conditions in metabolic space. A green square represents a condition that extends lifespan, yellow represents a condition that has no effect on lifespan, and red shortens it. White means the lifespan has not been measured.

Determining the Key Substrate in Sulfur Assimilation Pathway Regulation:

Although both the enzymatic functions in the Sulfur Assimilation Pathway and the complex interaction between the transcription factors are well characterized, what remains uncertain is the manner in which intracellular metabolite levels regulate these transcription factors' activity. Although different experiments have given some indication that methionine and/or cysteine are involved, none have thoroughly analyzed the entire pathway by systematically removing key enzymes and restricting key metabolites to control the cellular response.

If the metabolite levels can be controlled, it would be fairly straightforward to expose to cell to some, while restricting others, and measure the level of pathway activation in each case. Presumably, such data would conclusively point to the regulating metabolite(s), as the activity varied with them, and not the others. Controlling the levels of metabolites is, however, difficult in the sulfur assimilation pathway because of the branches and loops involved. It is very easy for one metabolite to convert into another. Knocking out a series of genes is the only way to control metabolite levels.

Because Met17p controls all flux of inorganic extracellular sulfate into incorporation into sulfur metabolites, it should be possible to control the levels of sulfur metabolites in a *Δmet17* background more easily (for example, in strain BY4741). Without changing the base nutrients, levels of methionine, SAM, or

cysteine can be controlled to examine the response of the sulfur assimilation pathway to the presence or absence of certain nutrients.

In a *Δmet17 Δmet6 Δsam4* background, synthesis of methionine from homocysteine or SAM is prevented, meaning that only the methionine provided in the media can be used. This allows for the starvation of methionine even with abundant SAM and cysteine, if SAM is fed. Alternatively, if cysteine is fed to the cells, it will be abundant while SAM and methionine are both scarce.

In a *Δmet17 Δsam1 Δsam2* background, SAM synthesis from methionine would be prevented. In turn, this allows for low levels of SAM, and high levels of cysteine and methionine, if cysteine is fed, or just high levels of methionine if methionine is fed (since the HCS -> methionine reaction is not reversible).

In a Δmet17 Δcys4 Δcys3 background, synthesis of cysteine from homocysteine is prevented, meaning that only the only cysteine available to the cell is the cysteine provided in the media, allowing cysteine levels to be held low, even in abundant methionine and SAM.

High SAM and low methionine and cysteine can be examined in a *Δmet17 Δsah1 Δsam4* background, since it prevents the generation of fresh methionine or HCS from SAM, while not preventing SAM's methylation function.

	Δcys4 Δcys3	Δsam1 Δsam2	Δsam1 Δsam2	Δmet6 Δsam4	Δmet6 Δsam4	Δsah1 Δsam4
Methionine	Abundant	Abundant	Abundant	Scarce	Scarce	Scarce
Cysteine	Scarce	Abundant	Scarce	Abundant	Abundant	Scarce
SAM	Abundant	Scarce	Scarce	Abundant	Scarce	Abundant

Table 3: This table shows the different conditions that can be achieved using various strains.

The use of reporters throughout the Sulfur assimilation pathway should allow the detection of any differential regulation, as well. For example, if cysteine restriction channels sulfur towards cysteine/glutathione by an increased level of enzymes facilitating that reaction, as cadmium exposure does, it should be able to be detected by increased CYS3/CYS4 levels relative to MET6.

Specifically, this means that conducting this experiment would involve generating the background strains above (e.g. *Δmet17 Δmet6 Δsam4*), transforming some or all of the reporter plasmids built for the lifespan experiment into them, and measuring their growth and fluorescence in the media discussed above, in order to systematically judge the response of the system to low levels of various key metabolites.

Ultimately, knowing the substrate(s) responsible for activating the methionine pathway is not only an interesting, currently unsolved problem, but potentially useful to further examination of the aging phenotype. Understanding the mechanisms for activating the transcription factors could give a better insight into

how the cell is truly responding to the dual stimuli of glucose and methionine, particularly if they are responsible (even in an off-target manner) for activating lifespan-extending genes.

Mechanism of Glucose/Methionine Interaction:

It should also be possible to use the tools developed for probing the sulfur assimilation pathway to examine the direct mechanism of action responsible for tying the lifespan response for CR and MR together. There are two truly big questions about the glucose/methionine/aging interaction: First, which downstream effect(s) is responsible for the aging phenotype, and second, in what way is the methionine signal able to interfere/interact with the glucose signal? The experiments conducted in this paper aim to answer the first question. But the same tools used to determine the regulation of the sulfur assimilation pathway may prove the best way to address the second question.

On a basic level, by controlling the levels of the metabolites using these mutant strains in a manner similar to the previous aim, but measuring life spans instead of reporter expression, it is possible to rule out entire classes of hypotheses (e.g. methionine requirement for translation if methionine has no impact on lifespan independent of SAM). It would then be possible for more thorough investigations to be performed, to trace the interaction. For example, if methionine level were implicated, one experiment would be to replace the promoter of some methionine

TFs (which may be responding to methionine, and also hitting other genes) with an inducible reporter, and then see if the effect goes away. The result would then either implicate a direct interaction with methionine or the activity of a limited number of genes activated by the TF.

Soon, measuring lifespan should become more straightforward due to advances in the lab. A strain has been developed which is daughter-lethal (upon exposure to a certain chemical, newly budded cells stop development, allowing them to be counted but not contribute any offspring themselves), thereby allowing the more rapid assessment of offspring number (Changhui Deng, unpublished results). Soon, it should be possible to perform genetic manipulations on this strain, and thus have the strains necessary for this experiment able to have their lifespans assayed in high throughput.

In addition to helping to tie together the work on the sulfur assimilation pathway and the lifespan phenotype, an added bonus will be that it may be possible to use this information to help confirm any findings on the cause of the aging phenotype. Since this will hopefully help tie part of the methionine response to the glucose, it should narrow down the parts of the glucose response that could be responsible for the aging phenotype, especially if the connection is further downstream.

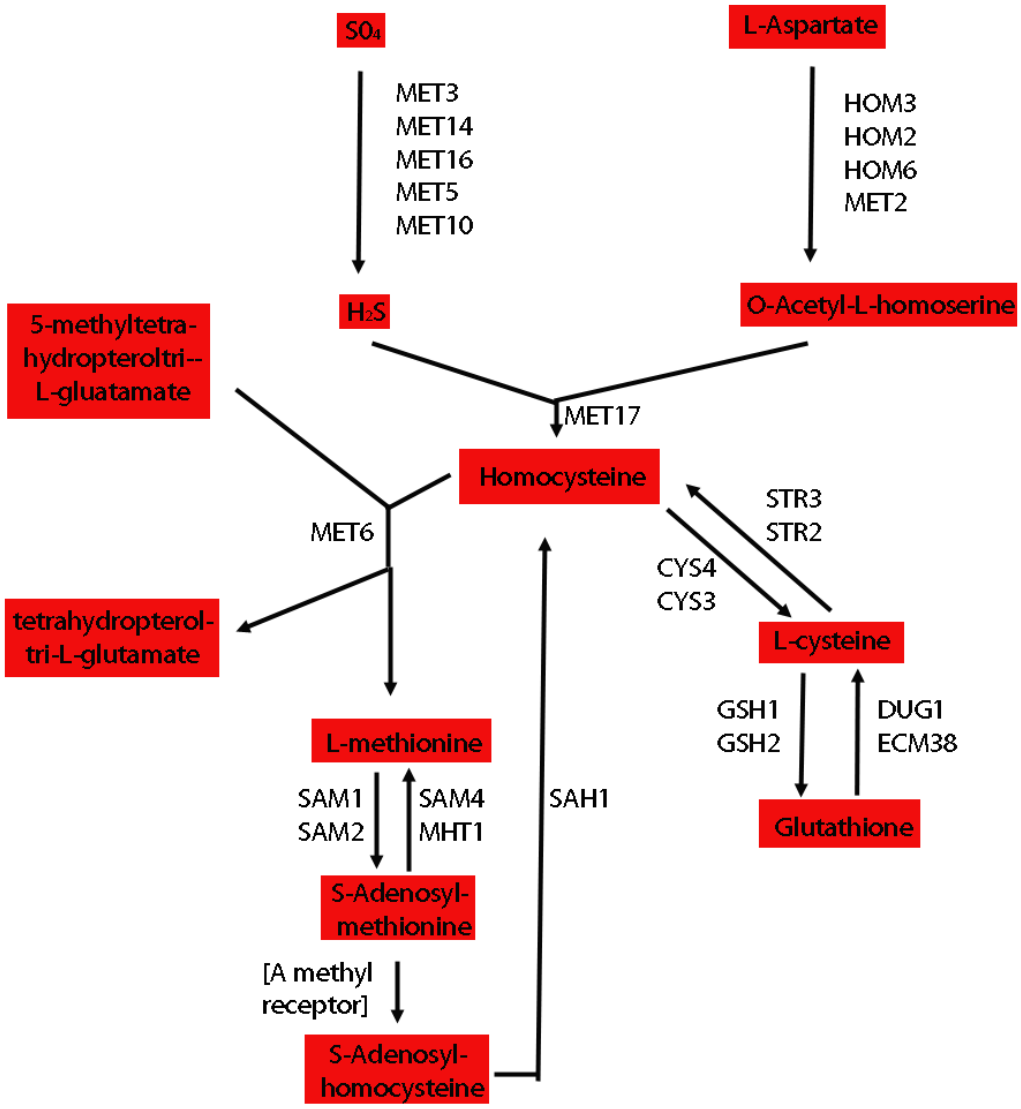

Figure 1: The sulfur assimilation pathway

The key metabolites in the sulfur assimilation pathway are represented by red rectangles, while arrows indicate the enzymes responsible for conversion between them.

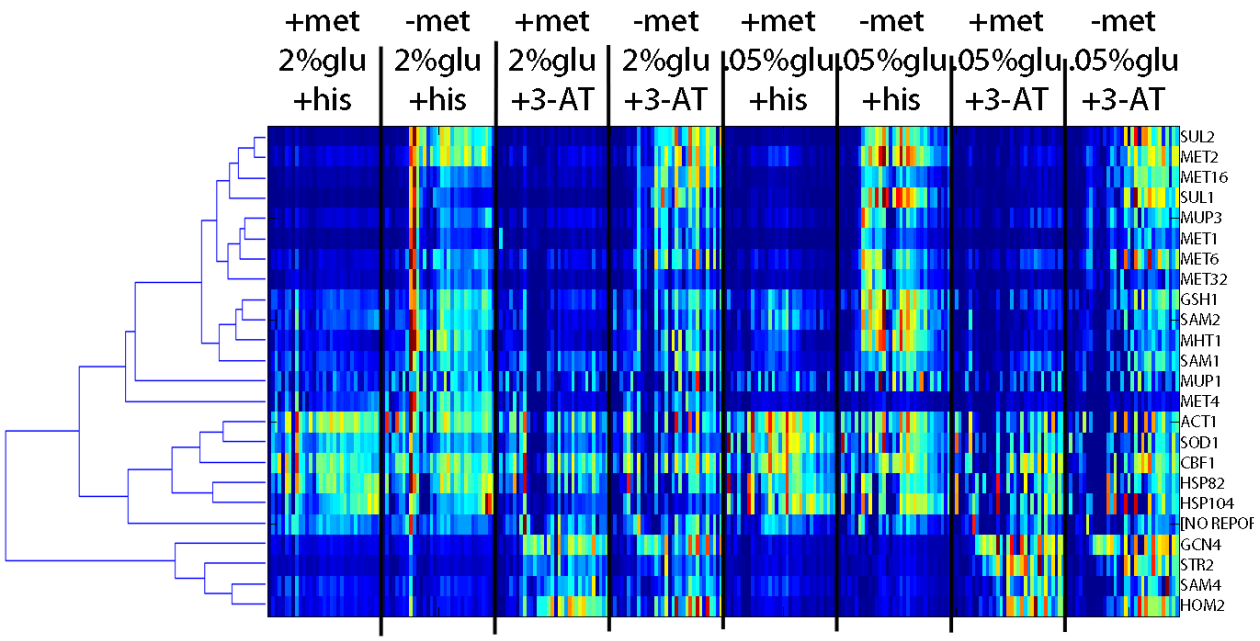

Figure 2: Clustered results of time lapse flow cytometry

In this figure, the normalized expression level of each reporter can be observed under all conditions. Red represents high expression, blue represents low. The genes are also ordered by the results of clustering their expression patterns by correlation.

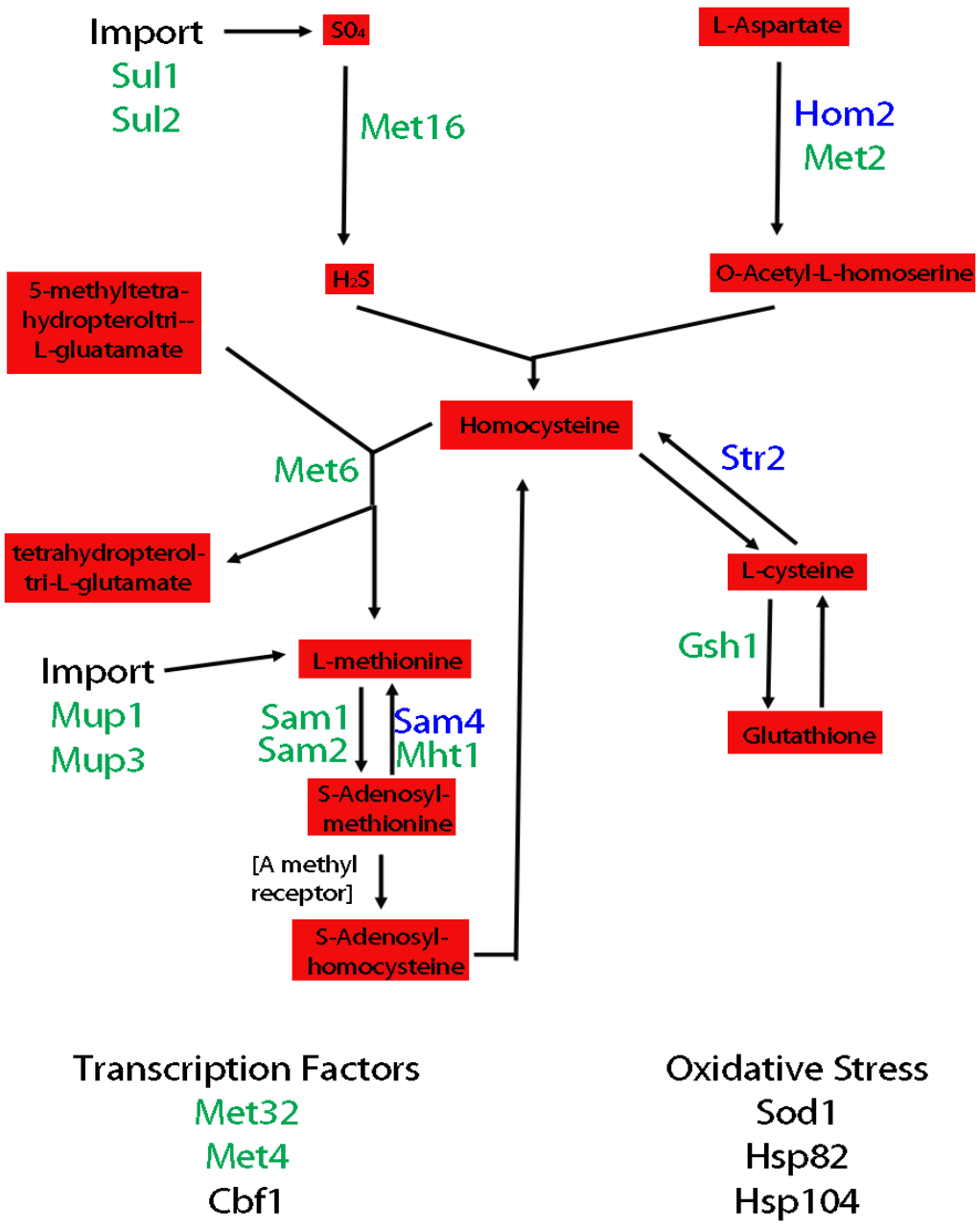

Figure 3: Regulation of sulfur assimilation pathway.

The sulfur assimilation pathway is overlaid with the reporters whose expression was measured. They are color coded according to their cluster: Green represents the methionine-responsive cluster, blue represents the 3-AT responsive cluster, and black represents no concrete response.

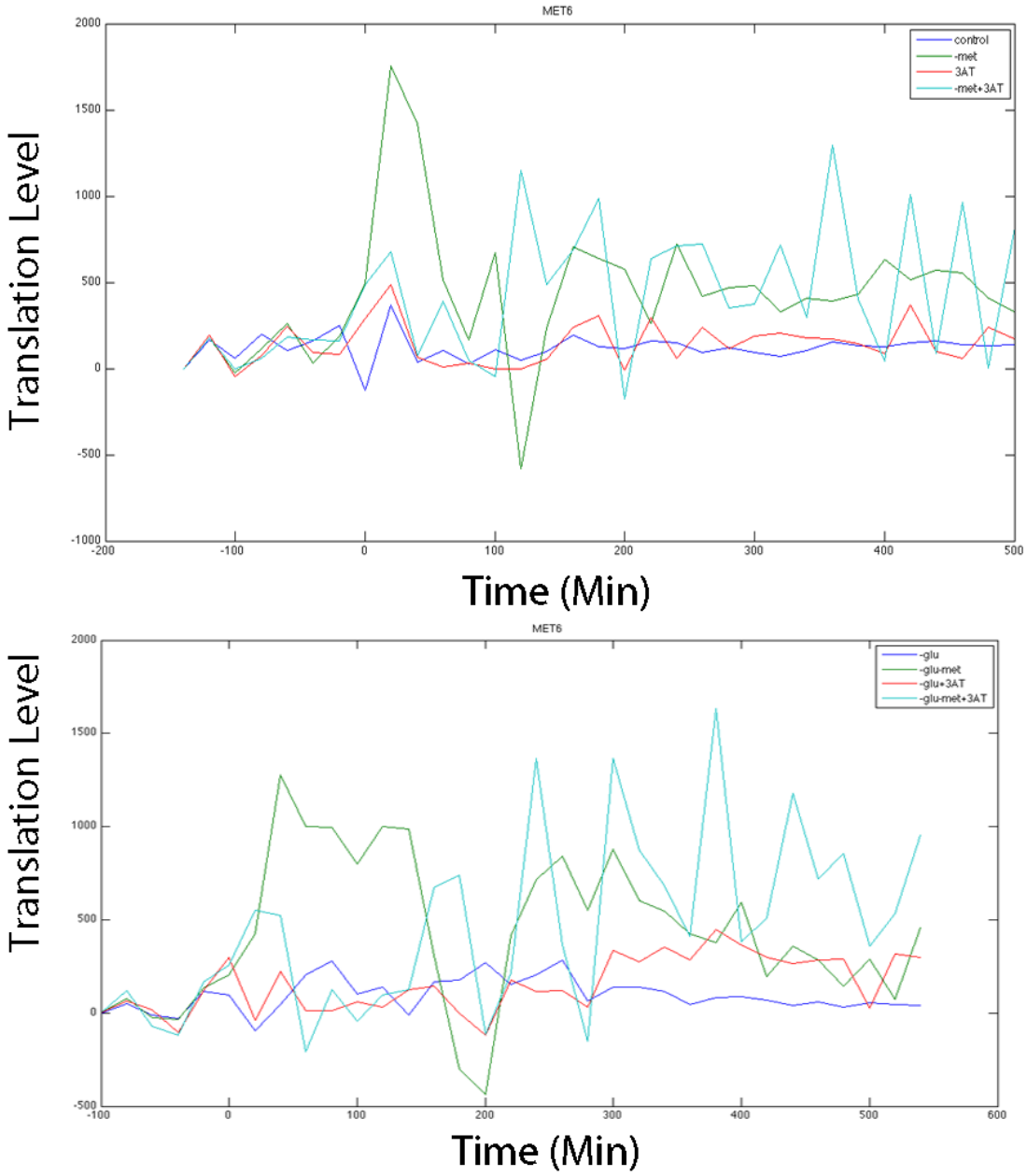

Figure 4: Met6 expression over time

Met6 expression levels are shown under 2% glucose (top) and .05% glucose (bottom) conditions. Met6 is representative of other members of the methionine-sensitive cluster.

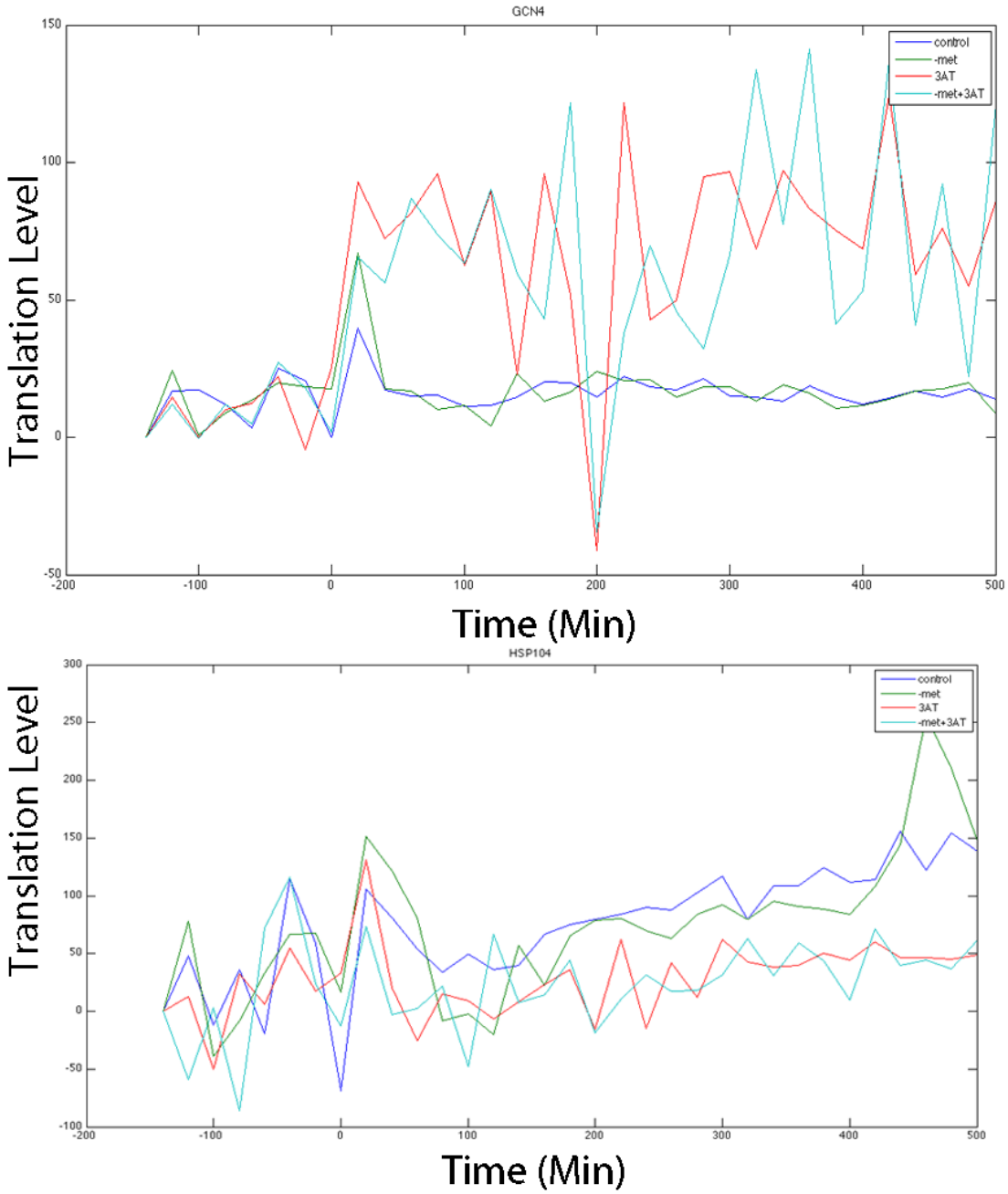

Figure 5: Gcn4 and Hsp104 expression over time

Gcn4 (top) and Hsp104 (bottom) are shown over time. Gcn4 is representative of other members of the 3-AT-sensitive cluster. Hsp104 is characteristic of the climbing over time of both Hsp-family proteins observed.

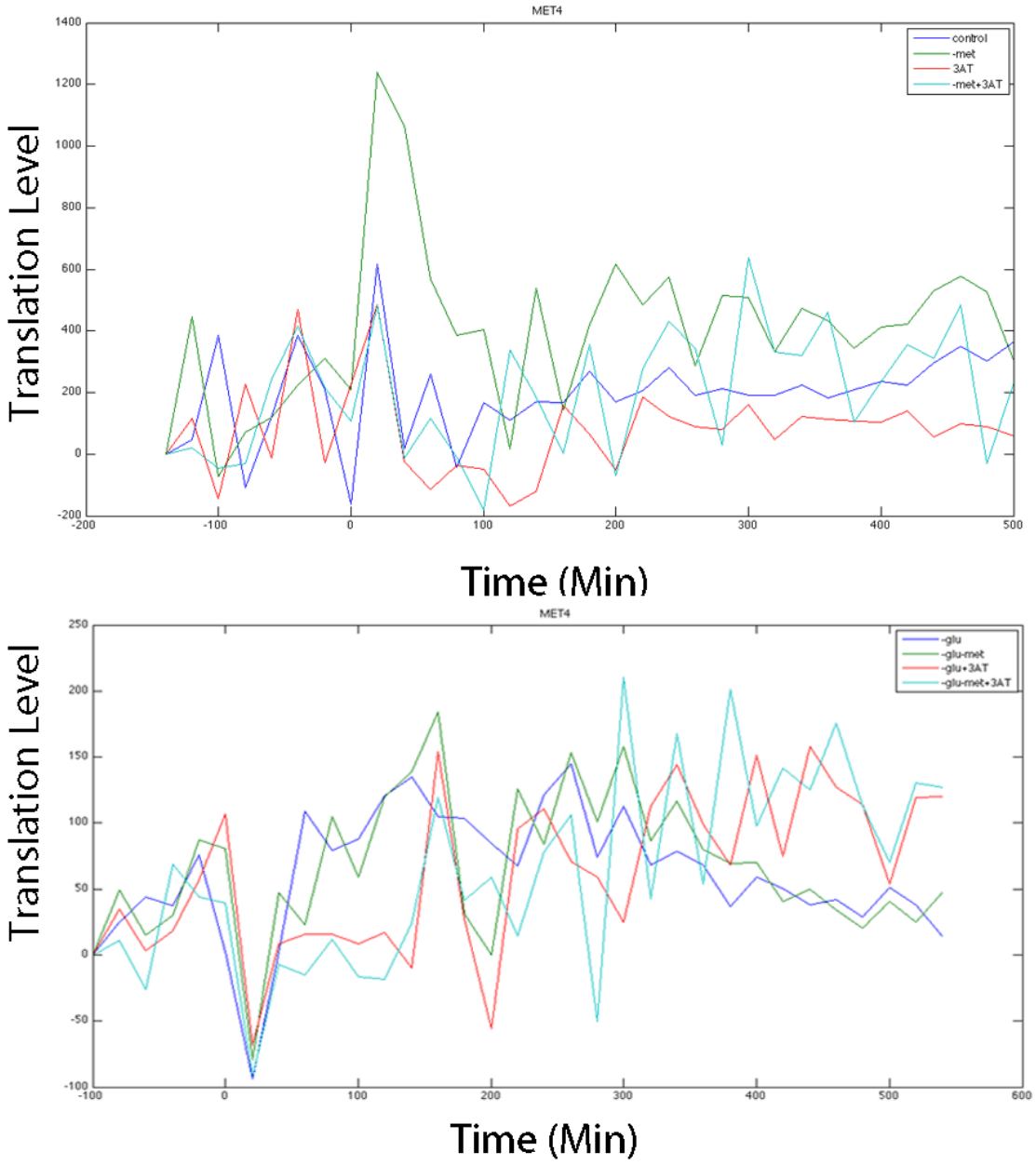

Figure 6: Met4 expression over time

Met4 expression levels are shown under 2% glucose (top) and .05% glucose (bottom) conditions. Met4 has a unique expression pattern in that it responds very strongly to methionine restriction in the 2% glucose case, but not the .05% case.

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