ON SULFUR SENSING IN *SACCHAROMYCES CEREVISIAE*

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DEDICATION

To my mentors Ben, Fay, and Maha, and to the members of my thesis committee, your guidance has been invaluable to my success. I express my greatest gratitude for instilling in me the sense of joy and wonderous excitement that comes with exploring the boundaries of the natural world.

To my family and friends, none of this would be possible without your constant love and support. Especially my mother, Sharah. Any success of my own is yours to share in its entirety.

For my father, David, who qualifies as both. What an amazing gift it is to be able to share the language of science between father and son, I look forward to many more years of it.

ON SULFUR SENSING IN *SACCHAROMYCES CEREVISIAE*

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The unique chemistry available to sulfur compared to oxygen, such as the ability to exist in numerous oxidation states and greater nucleophilicity, makes many of the biochemical reactions requisite for cellular life possible. As a result of this critical importance, organisms have developed several mechanisms for sensing and maintaining levels of sulfur-containing metabolites. In the yeast *Saccharomyces cerevisiae*, regulation of sulfur metabolism can be distilled down to the actions of two proteins; the F-box protein Met30, and the transcriptional

coactivator Met4. Met30 belongs to the family of SCF (Skp1-Cul1-F-box protein) E3 ubiquitin ligases, and negatively regulates the transcriptional activity of the master transcriptional activator of sulfur metabolism genes, Met4, via oligo-ubiquitination when sulfur metabolite levels are high. When yeast are starved of sulfur, Met30 ceases to ubiquitinate Met4, releasing it to be deubiquitinated and transcriptionally active to boost levels of a network of sulfur metabolic genes known as the *MET* regulon to restore sulfur metabolite levels. While the molecular activities of both Met30 and Met4 have been extensively studied over the last two decades, the biochemical basis for sulfur-sensing by the Met30 E3 ligase has remained unknown. Herein, I reveal the biochemical details by which Met30, the master regulator of sulfur metabolism, senses the availability of sulfur metabolites to modulate its E3 ligase activity to regulate sulfur metabolism in yeast. Utilizing a combination of yeast genetics and biochemical assays, I show that Met30 uses redox-active cysteine residues in its C-terminal WD-40 repeat region to modulate binding between itself and its substrate Met4 in accordance with the availability of sulfur metabolites. These insights represent significant advances in the understanding of sulfur metabolic regulation in yeast.

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PRIOR PUBLICATIONS

Johnson, Z., Venters, J., Guarraci, F.A. and Zewail-Foote, M., 2015. Methamphetamine induces DNA damage in specific regions of the female rat brain. *Clinical and Experimental Pharmacology and Physiology*, *42*(6), pp.570-575.

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SCF – Skp1-Cul1-F-box

SAM – *S*-adenosyl methionine

GSH – Glutathione

SAH – *S*-adenosyl homocysteine

GSSG – Oxidized glutathione

PPP – Pentose phosphate pathway

ROS – Reactive oxygen species

UIM – Ubiquitin-interacting motif

bHLH – Basic helix-loop-helix

mPEG2k-mal – methoxy-polyethylene glycol maleimide (average mw of 2000 daltons)

CHAPTER ONE

Literature Review

SULFUR METABOLISM

Introduction

Sulfur-containing amino acids represent only two of the twenty canonical amino acids, yet in addition to methionine and cysteine, the sulfur amino acid metabolic pathway yields two of the most consequential small molecule metabolites in the cell – glutathione (GSH) and *S*-adenosyl methionine (SAM). Glutathione, a cysteine-containing tripeptide which exists in millimolar levels in cells, is the major cellular reductant and buffers cellular macromolecules against oxidative stress as well as maintains the reduced state of protein thiols. *S*-adenosyl methionine (SAM), the biochemical conjugate of methionine and adenosine, produces a sulfonium ion-containing metabolite responsible for the vast majority of cellular methylation reactions. In this chapter I detail the history and current understanding of sulfur metabolism, the sulfur metabolic pathway in yeast, and its regulation by Met30 and Met4.

History

Much of the focus of biochemists in the 19th century and the first half of the 20th century was centered on the discovery and identification of small molecule metabolites (MW < 1500 Da) such as methionine and other amino acids (Mueller, 1923, Vickery and Schmidt,

1931), while the latter half of the 20th century was dedicated to the identification of the gene products and network of gene products responsible for the enzymatic synthesis and interconversion of these small molecules, now referred to as metabolic pathways. Of the amino acids to be first discovered, cysteine (in this case, cystine) was amongst the earliest when in 1811, William Hyde Wollaston isolated "cystic oxide" from urinary calculi (Wollaston, 1811). The hilarious and often unfortunate efforts of scientists and their contentious debates over the nature of this one amino acid would not be resolved until nearly a century later, and the history is entertaining to read (Vickery and Schmidt, 1931). Shortly after the characterization of cysteine, methionine was discovered in an effort to identify the component in casein protein hydrolysates that stimulated growth in hemolytic streptococci (Mueller, 1923). While the chemistry and nature of these sulfur amino acids was known by the mid 1920s, the challenging work of understanding their metabolism and biological context would take nearly another century.

The aforementioned "network of gene products" responsible for the interconversion of sulfur metabolites in yeast follow the simple nomenclature of *Saccharomyces cerevisiae* as the "*MET*" genes, which comprise the methionine biosynthetic pathway. In yeast, much of the work done to identify the genes in this pathway was performed by Huguette de Robichon-Szulmajster, Yolonde Surdin-Kerjan, and later Dominique Thomas at the Centre de Génétique Moléculaire, CNRS, in Gif-sur-Yvette, France. A collaboration between de Robichon-Szulmajster, Surdin-Kerjan, and Robert K. Mortimer characterized the partially overlapping threonine and methionine biosynthetic pathway, ultimately branching at the

common precursor homoserine (de Robichon-Szulmajster et al., 1966). Ten years later, the biochemical pathway was largely completed, and most of the *MET* genes named and their enzymatic activities established (Masselot and de Robichon-Szulmajster, 1975, Masselot and Surdin-Kerjan, 1977). After a lifetime of work on the subject, Thomas and Surdin-Kerjan wrote their swan song in an epically comprehensive review on sulfur metabolism in yeast (Thomas and Surdin-Kerjan, 1997). While much has been learned over the course of the last two hundred years regarding sulfur metabolism and sulfur metabolites, the metabolic heterogeneity of life lends ample room for discovering the peculiarities in sulfur metabolism and sulfur metabolic regulation unique to various species. What follows is a brief summary of the sulfur amino acid biosynthetic pathway of *Saccharomyces cerevisiae*.

Overview of Sulfur Metabolism in Yeast

Sulfur Assimilation

Unlike humans, yeast are able to synthesize their own sulfur-containing metabolites *de novo* via sulfate assimilation. However, reduction of sulfur in the form of sulfate to the usable sulfide is not straightforward, and it should be noted how energetically expensive sulfate assimilation is – to go from one atom of sulfur in the form of inorganic sulfate (SO_4^{2-}) to the biologically useful sulfide (H_2S) requires two ATP and four NADPH. The first step requires the adenylation of intracellular sulfate to adenylyl sulfate (APS) by the action of *MET3* (ATP sulfurylase), followed by the phosphorylation of APS by *MET14* (APS kinase) to produce phosphoadenylyl sulfate (PAPS), thus thermodynamically activating sulfate in

order to make sulfate reduction to sulfite by NADPH oxidation energetically favorable (De Meio, 1975). Sulfite formation from PAPS is carried out by *MET16* (PAPS reductase) at the expense of one reducing equivalent in the form of NADPH. The liberated sulfite is then reduced to sulfide by sulfite reductase, a heterotetrameric enzyme encoded by *MET5* and *MET10* that oxidizes three molecules of NADPH, donating six electrons to produce sulfide (Thomas and Surdin-Kerjan, 1997). The final step of sulfur assimilation is the incorporation of sulfide into the four-carbon chain of *O*-acetylhomoserine by *O*-acetylhomoserine sulfhydrylase (*MET17*, sometimes referred to as *MET15* or *MET25*) to produce homocysteine, the key intermediate at the core of sulfur metabolism. The sulfur metabolic pathway bifurcates at homocysteine, where the newly incorporated sulfur atom will be used either to produce methionine and SAM via the SAM branch of sulfur metabolism, or cysteine and glutathione via the GSH branch.

Methionine, SAM, and the methyl cycle

Synthesis of methionine from homocysteine requires the donation of a methyl moiety from 5-methyltetrahydrofolate to the free thiol of homocysteine catalyzed by methionine synthase (*MET6*) (Masselot and de Robichon-Szulmajster, 1975, Thomas and Surdin-Kerjan, 1997). Once formed, methionine can serve in proteinogenic functions or can be converted into *S*-adenosyl methionine (SAM) by the action of SAM synthetase (*SAM1/2*), which couples the attack of the methionine sulfide on the ribose ring of ATP to the release of pyrophosphate and inorganic phosphate to drive the reaction towards SAM accumulation.

The functional importance of SAM as a metabolite is difficult to overstate – nearly all cellular methylation events use SAM as a substrate, and it is involved in such a large number and variety of reactions that it is thought to be only second to ATP with respect to group transfer reactions in cells (Cantoni, 1977, Walsh et al., 2018). It is also unusual in that its group transfer potential comes from the generation of a high-energy sulfonium cation, with group transfer returning sulfur to a divalent state typically in the form of *S*-adenosylhomocysteine (SAH) after methylation by a SAM-dependent methyltransferase. As cellular levels of SAM are relatively quite low compared to ATP, the high utilization of SAM in biochemical reactions places a burden on cells to regenerate SAM via the recycling of *S*-adenosylhomocysteine (SAH) by the enzyme *S*-adenosylhomocysteine lyase (*SAH1*). This enzyme strongly favors consumption of SAH and production of adenosine and homocysteine, the latter capable of being metabolized to cysteine or back to methionine and SAM (Mato et al., 1997, Ueland, 1982).

This cyclic regeneration of SAM by the thermodynamically-favorable splitting of the transmethylation byproduct SAH drives the methyl cycle, and maintains the methylation potential (the SAM/SAH ratio) of the cell (Mato et al., 1997, Ueland, 1982). Maintenance of this ratio is crucial *in vivo*, as SAH is a potent inhibitor of SAM-dependent methyltransferase enzymes, and high SAH levels result in widespread inhibition of cellular methyltransferases (Richon et al., 2011). Perhaps unsurprisingly, dysregulation of the methyl cycle has implications in human health and disease (Barroso et al., 2017, Mato et al., 1997).

Cysteine, GSH, and transsulfuration

Aside from the methyl cycle intermediates, the alternative fate of homocysteine is the production of cysteine and GSH. The interconversion of cysteine and homocysteine is known as transsulfuration, and while humans are only capable of transsulfuration in the direction of cysteine synthesis, yeast are able to interconvert these metabolites bidirectionally. Synthesis of cysteine from homocysteine requires the intermediate metabolite cystathionine, produced by the enzyme cystathionine beta-synthase (*CYS4*) and subsequently consumed by cystathionine gamma-lyase (*CYS3*) to yield cysteine; the deletion of either gene results in cysteine auxotrophy in yeast (Thomas and Surdin-Kerjan, 1997).

Downstream of cysteine is the critically important small molecule metabolite glutathione (GSH). Synthesis of GSH from cysteine starts with the ligation of glutamate and cysteine by gamma-glutamyl-cysteine ligase (*GSH1*) to form the intermediate gamma-glutamylcysteine, followed by the conjugation of glycine by glutathione synthetase (*GSH2*) to generate the final tripeptide product. Interestingly, deletion of *GSH1*, but not *GSH2*, resulted in a yeast strain with impaired mitochondrial function and increased sensitivity to various oxidative stressors – implying that the gamma-glutamylcysteine dipeptide intermediate is capable of at least partially substituting for GSH (Grant et al., 1997).

The GSH tripeptide exists in millimolar concentrations in cells and is the major cellular reductant, buffering biological macromolecules against oxidative damage and preserving the reduced state of protein thiols (Cuozzo and Kaiser, 1999, Wu et al., 2004). With a redox potential of –240 mV (for thiol/disulfide exchange), GSH is a powerful reductant, and additionally, serves to detoxify heavy metals in yeast (Penninckx, 2000). In a

particularly well-studied example, the heavy metal cadmium is detoxified via the *YCF1*-dependent transport and sequestration of its GSH-chelated form, bis(glutathionato)cadmium, into the vacuole (Li et al., 1997). In addition to its cellular role as a reductant, the abundance and nucleophilic nature of GSH also serves to protect cells by quickly reacting and neutralizing electrophilic or oxidizing chemical species that might otherwise go on to damage biological macromolecules (Pompella et al., 2003).

Conclusions

Considering the importance of sulfur-containing metabolites, it is not surprising that organisms have developed sophisticated and clever mechanisms to regulate their levels over the course of evolutionary history. This evolutionary pressure is well illustrated by *S. cerevisiae*'s sulfur-sparing response, in which depletion of sulfur metabolites results in the expression of genes involved in sulfur metabolite production (the *MET* genes) as well as isozymes of genes involved in central carbon metabolism that themselves encode relatively few cysteine and methionine residues (Fauchon et al., 2002). In fact, *MET4*, the *MET* gene that orchestrates this transcriptional response, contains only the initiating methionine residue in its 672 amino acid primary sequence, and is otherwise devoid of sulfur-containing amino acids.

The example is revealing in its parallels to other organisms, which appear to cope with sulfur starvation or redox stress primarily by transcriptional means to boost levels of critical enzymes in order to alleviate the stress (Imlay, 2013, Manford et al., 2020,

Maruyama-Nakashita et al., 2006, Pendleton et al., 2017, Yamamoto et al., 2018). In yeast, the major regulator of the sulfur metabolic gene network is the aforementioned transcription factor Met4, which is activated in response to sulfur starvation or heavy metal stress and is negatively regulated by the SCF E3 ligase Met30 in the absence of these stimuli (Ljungdahl and Daignan-Fornier, 2012). The following sections review the current understanding of these regulators of sulfur metabolism.

MET4, THE SULFUR METABOLISM TRANSCRIPTIONAL ACTIVATOR

Introduction

It is a common feature of life to organize genes involved in similar functions into discrete groups bar-coded in such a way as to couple their transcriptional regulation. For sulfur metabolism in yeast, this discrete group is known as the *MET* regulon, and its transcriptional regulation is governed by the transcriptional co-activator Met4. It has been known for some time that genes involved in sulfur metabolism in yeast are under tight transcriptional control (Cherest et al., 1969, Gierest et al., 1985). This control is so tightly governed that analysis of cell extracts isolated from a *met4*Δ strain revealed near-zero levels of activity from any sulfur assimilation enzyme (Thomas et al., 1992). However, the molecular mechanisms that permit *MET4* to regulate *MET* gene expression are complex and still being uncovered, and a brief history and review of the current understanding of these mechanisms follows.

Met4 gets by with a little help from its friends

Identification of cis-regulatory elements

An analysis of the promotor region of *MET17* uncovered two regulatory sequences that effected the transcription of homocysteine synthase in yeast, the first being CACGTG palindromes and the other being AAANTGTG, the first and last sequences being defined more specifically as CACGTGA and CTGTGGC in later analysis (Lee et al., 2010, Thomas et al., 1989). The palindromic sequence CACGTG was shown to be bound by *CBF1* (centromere binding-factor 1), is shared by numerous *MET* genes (as well as all yeast centromeres), and is necessary but not sufficient for proper *MET* gene regulation (Mellor et al., 1990, Thomas et al., 1992, Thomas and Surdin-Kerjan, 1997). It was later discovered that the AAANTGTG consensus sequence was bound by two highly similar proteins, Met31 and Met32, and that disruption of these genes results in dysregulated gene expression at some *MET* genes but were less important for transcriptional activation at other *MET* genes (Blaiseau et al., 1997).

These unusual results, combined with results showing that Met4 can assemble into multiple different complexes on different *MET* genes, led to the hypothesis that Met4 acts as the master transcriptional activator, but requires the mixing and matching of specific auxiliary factors to form active transcriptional activation complexes at the promotors of *MET* genes (Blaiseau and Thomas, 1998). This is all the more interesting as Met4 has no intrinsic DNA binding activity itself, and so must be recruited to the promotors of *MET* genes via

these multi-protein complexes (Blaiseau et al., 1997, Blaiseau and Thomas, 1998, Kuras et al., 1996).

Cbf1-Met28-Met4

Cbf1, a member of the basic helix-loop-helix (bHLH) protein family, was initially characterized by an analysis of proteins that specifically bound the yeast centromere DNA element 1 (CDE1), and its potential as a transcriptional regulator was quickly hypothesized by the fact that Cbf1 binding sites were identified at multiple upstream sequences of protein encoding genes (Bram and Kornberg, 1987). This was confirmed with respect to yeast, demonstrating the bifunctional role of *CBF1* in DNA segregation as well as transcriptional regulation – particularly in the case of sulfur metabolic genes (Mellor et al., 1990, Thomas et al., 1992, Thomas and Surdin-Kerjan, 1997). An additional factor, Met28, is a leucine zipper-containing protein that was identified as an additional member of the Cbf1-Met4 heteromeric complex (Kuras et al., 1996). While Met28 has no intrinsic DNA-binding or transcriptional activity, its role in *MET* gene transcriptional activation appears to be based on its ability to form and stabilize a complex with Met4 in the presence of Cbf1 capable of binding DNA (specifically around the CACGTG consensus sequence), and enhances the DNA-binding activity of Cbf1 (Kuras et al., 1997). In addition to the specificity conferred by Cbf1 at CACGTG palindromes, it was discovered that an additional *cis*-regulatory element, the RYAAT motif, when positioned adjacent to the Cbf1 binding site resulted in enhanced binding of the heterotrimeric complex to DNA (Siggers et al., 2011). Additionally, loss of the RYAAT motif reduces *MET* gene transcription in low-sulfur conditions *in vivo* (Siggers

et al., 2011). The implication of these data is that the use of non-DNA-binding transcriptional co-activators in conjunction with a DNA-binding co-activator in complex together can modulate the affinity and specificity of the entire transcriptional activating complex, correctly directing a bifunctional protein like Cbf1 to promote transcription of *MET* genes when sulfur metabolites are low and Met4 (and Met28) are active.

Met31/Met32-Met28-Met4

Further expanding on the concept, Met4 also works together with two paralogous zinc-finger transcription factors, Met31 and Met32, to be recruited to the promotors of *MET* genes to regulate the sulfur metabolic transcriptional program. Initially discovered by two different techniques published in the same paper — *MET31* identified by the one-hybrid method and *MET32* by a screen for mutants defective in methionine uptake — both were demonstrated to activate transcription of a LexA reporter in a *MET4*-dependent manner (Blaiseau et al., 1997). Although highly similar in sequence, the two genes appeared to have opposite phenotypes as single mutants with respect to *MET3* and *MET14* expression, but analysis of the double mutant and the *met4*Δ mutant suggested the two worked as transcriptional activators of at least a subset of the *MET* genes (Blaiseau et al., 1997). Biochemical analysis the following year demonstrated that both proteins form high molecular weight complexes with Met28 and Met4 on the AAACTGTG consensus sequence, and genetic analysis demonstrated that Met4 requires the assembly of this complex to activate transcription of the *MET31/32*-dependent *MET3* gene, but not the *CBF1*-dependent *MET16* gene (Blaiseau and Thomas, 1998).

Met4 meets microarrays

The complex nature of such a variable multi-component transcriptional activation system makes analysis by low-throughput means tedious and difficult to interpret, and more detail would have to wait until the development of more sophisticated technologies that permit genome-wide analysis of transcriptional activity. The metaphorical call would be answered in 2010, when genome-wide expression profiles of *MET4* target genes were studied in the background of various Met4 co-factor deletion mutants to dissect the "combinatorial control of the Met4 transcriptional complex" (Lee et al., 2010). The study revealed a core regulon of 45 genes under the control of Met4 which were divided into three classes, all of which depended on *MET31* and *MET32*, with a subset dependent on *MET28* and *CBF1* to various degrees. Interestingly, Cbf1 bound all genes in the regulon constitutively regardless of the sulfur-status of the cell, while binding by Met4, Met31, and Met32 was dramatically lower in repressive conditions (Lee et al., 2010). Importantly, the authors found that Cbf1 and Met28-dependent genes mapped to portions of the sulfur metabolic network that appear to segment the transcriptional regulation of genes involved in inorganic sulfur assimilation versus organic sulfur metabolite synthesis – with genes that were strictly dependent on the two proteins regulating the transcriptional regulation of sulfur assimilation genes (Lee et al., 2010). These results fit well with previous reporting that *cbf1*Δ and *met28*Δ strains are incapable of growth when sulfate is the sole sulfur source (Thomas et al., 1992, Thomas and Surdin-Kerjan, 1997).

It should be emphasized how variable the system is – the number of binding sites, type of consensus sequence, their orientation (whether the *CBF1* binding site is in front or behind the *MET31/32* site), and the relative distance between binding sites all influence the transcriptional output of any individual *MET* gene (Kuras et al., 1997, Lee et al., 2010, Siggers et al., 2011). In 2012, the Botstein lab developed a method that allowed for the rapid overexpression of Met4 and each of its' co-activators to test the effect of each component on *MET* gene target expression, abusing mass action to reveal specific roles even between Met31 and Met32 which are nearly 50% identical (McIsaac et al., 2012). Their microarray data and computational analysis further separated *MET4*-dependent genes into nine clusters based on expression profiles, finding that subtle differences in nucleotides in and adjacent to Cbf1/Met28 or Met31/32 consensus sequences can result in activation or repression of gene transcription depending on context (McIsaac et al., 2012). Also evident is the presence of feedback loops in the *MET* gene system, such as Met4 induction of co-activators like *MET28* and *MET32* as well as its repressor *MET30*. The authors point out that historically *MET31* and *MET32* have been characterized as largely redundant, even though genetic analysis has found that *MET32* has a dominant role over *MET31*, and that a likely basis for this is the existence of feedback loops for Met32 and Met4 that do not exist for Met31 (Patton et al., 2000, McIsaac et al., 2012).

Conclusions

The heterogeneity of Met4 transcriptional complexes and their diverse range of specificities and outputs are truly impressive, but how does Met4 actually activate transcription? Adding to Met4's repertoire of protein-protein interactions, the master sulfur transcription factor recruits both Mediator (a RNA Pol II cofactor) and the SAGA complex (Spt-Ada-Gcn5 acetyltransferase complex) to *MET* genes, and can do so independently (Leroy et al., 2006). The biology of the two complexes is outside the scope of this dissertation, but the ability of Met4 to use a half dozen protein-protein interactions to activate *MET* gene transcription in such a specific and well-regulated manner has made the *MET* regulon an excellent and illuminating model for eukaryotic transcriptional activation. However, this is not the only model system Met4 belongs to. The following section will review the equally complex molecular mechanisms governing *MET* gene repression by the SCF^{Met30} E3 ligase.

MET30, THE NEGATIVE REGULATOR OF SULFUR METABOLISM

Introduction

The assimilation of sulfate and biosynthesis of sulfur metabolites comes at a significant energetic and metabolic cost to cells. Sulfate assimilation requires two ATP and four NADPH to produce homocysteine from inorganic sulfate, and biosynthesis of methionine from homocysteine requires donation of a methyl group from 5-methyltetrahydrofolate, which itself requires the investment of NADPH to produce – making methionine biosynthesis one of the largest sinks of NADPH in the cell (Walvekar and

Laxman, 2019). As previously noted, the sulfur metabolic transcriptional program is tightly repressed under normal growth conditions so as to avoid wasteful spending of finite metabolic capital when sulfur metabolite levels are already high, information that has been known for more than fifty years (Cherest et al., 1969, Thomas and Surdin-Kerjan, 1997). The molecular basis of this transcriptional repression would not be uncovered until the turn of the millennia, at the discovery that Met30 was an E3 ligase that utilized the posttranslational modification of Met4 by ubiquitin to negatively regulate transcription of the *MET* regulon. This last section of the chapter will focus on the history and current understanding of Met30, and briefly review the biology of SCF E3 ligases.

Discovery and characterization of Met30

A color-based screen utilizing a fusion between the *MET17* promotor and catechol oxidase from *Psuedomonas putida* was utilized to identify genes that failed to repress *MET* gene transcription when cells were grown under repressive conditions, leading to the cloning and discovery of *MET30* (Thomas et al., 1995). Sequencing revealed that *MET30* was 640 amino acids in length, with five WD-40 repeats spanning the C-terminal portion of the protein (the real number is likely seven or eight), and genetic analysis revealed that *MET30* was an essential protein that interacted directly with Met4 – and that this interaction is required to repress *MET* gene transcription under repressive growth conditions (Thomas et al., 1995). Shortly thereafter, a new motif, the F-box domain, was discovered in a screen for suppressors of the *cdc4* mutant cell cycle defect that identified *SKP1* as a gene whose

overexpression rescues the ability of mutant *cdc4* to degrade the CDK inhibitor Sic1, and that *MET30* shared this motif (Bai et al., 1996). Among other prescient observations, it was speculated that Met30 might use its hypothetical interaction with Skp1 via the F-box motif to negatively regulate Met4 by proteolysis, and that "it is possible Skp1 functions in non-cell cycle pathways, e.g., through the *MET30* gene, and perhaps in non-proteolytic capacities" (Bai et al., 1996).

The observation is striking in retrospect, as the authors could not have known the contentious debate that would take place just a few years later surrounding SCF^{Met30} and Met4 degradation. Later, two papers tied together years of genetic experiments that ultimately demonstrated that the multi-protein complex of Cdc53-Skp1-Hrt1 and the variable F-box protein subunit compose an E3 ligase, with the F-box conferring substrate specificity while the entire complex (Hrt1 in particular) recruited the E2 conjugating enzyme Cdc34 to promote ubiquitination of a diverse set of substrates that regulated cell cycle progression and sulfur metabolism in yeast (Patton et al., 1998, Seol et al., 1999). Before continuing, a brief digression on SCF E3 ligases is necessary to appreciate the research on Met30 and Met4 that would take place at the beginning of the 21st century.

A brief digression on SCF E3 ligases

The general principle behind SCF E3 ligases is the use of a common cullin scaffold (Cdc53 in yeast) flanked by two adaptor proteins on either side of the scaffold to facilitate substrate ubiquitination. Skp1connects the F-box protein substrate recognition factor while

Hrt1 recruits an E2 enzyme, and the complex holds the substrate and E2 together so that the E2 conjugating enzyme can directly pass ubiquitin onto the substrate (Zheng and Shabek, 2017). While the general architecture of SCF E3s is simple enough, their regulation is highly complex. The earliest studies of SCF E3 ligases were done in the context of cell cycle progression, and key regulatory aspects quickly became apparent (Krek, 1998). The major regulatory aspect in this particular context is phosphorylation-dependent ubiquitination, the idea being that phosphorylation of a cyclin or CDK inhibitor on a particular residue permits the recognition of the phosphorylated substrate by the F-box protein, leading to its ubiquitination and degradation to permit cell cycle progression (Skowyra et al., 1997, Willems et al., 1999).

The focus on substrate recognition by the F-box protein would be key, as it would be discovered later that binding between the F-box protein and its substrate regulates incorporation of F-box/Skp1 heterodimers, which exist in stoichiometric excess relative to the Cdc53/cullin scaffold protein, into active SCF complexes which can then enable ubiquitination of the substrate. The best studies of this phenomenon have come out of the Deshaies lab (Liu et al., 2018, Reitsma et al., 2017). They find that the SCF E3 ligase cullin scaffold undergoes constant and rapid scanning of the cellular population of F-box/Skp1 heterodimers. By using cyclic changes in the affinity of these heterodimers for the scaffold protein, and using the addition of the Nedd8 (a small ubiquitin-like protein) modification on the cullin scaffold to stabilize the complex, the fully incorporated and modified complex can facilitate ubiquitination when the F-box has substrate bound. Conversely, destabilization of

the complex by the COP9 signalsome (CSN complex) occurs when substrate is no longer bound to the F-box protein via the removal of the Nedd8 modification. The result is constant turnover of these complexes in such a way as to ensure timely ubiquitination and degradation of substrate proteins as they become available (Liu et al., 2018, Reitsma et al., 2017).

The benefit of such a system is that it avoids turning SCF composition into a mass action problem, with the most abundant F-boxes hoarding the cullin scaffold while less abundant F-boxes are unable to ubiquitinate their substrates. Why does the cell then not simply increase the levels of the cullin scaffold to match F-box levels? One reason might be that it is actually beneficial to have a delay in the system so as to increase its specificity by "setting a ceiling on the maximal k_{off} of a substrate" (Liu et al., 2018). Another reason might be evolutionary – if the organism does not need to carefully and constantly regulate the stoichiometry between scaffold and substrate recognition factor, that frees genes encoding substrate recognition modules to be duplicated and diverge to acquire new functions without affecting cullin dynamics with already existing substrate recognition factors. The details of the regulatory system are incredibly complicated, but the simplified takeaway is that F-box proteins exist as free heterodimers with Skp1 in cells, and it is the recognition and binding of the substrate to the F-box protein which incorporates the F-box/Skp1/substrate trimer into the fully active E3 complex, and that this is the key step for substrate ubiquitination. While this research is relatively new and there are still many outstanding questions, the best reviews on E3 ligase biology and the ubiquitin-proteasome system in yeast to date can be found here (Finley et al., 2012, Zheng and Shabek, 2017).

Met30 and Met4 in the 21st century

Met30 and Met4 degradation: what's in the media?

The year 2000 would kick off an interesting debate between the groups of Dominique Thomas and Peter Kaiser on the mechanism by which Met30 represses Met4 activity, namely whether or not Met30 ubiquitination of Met4 leads to degradation of the transcription factor at all. Rouillon et al. reported in January of that year that the addition of methionine to yeast grown in minimal "B media", which contains no sulfur source, resulted in proteolysis of Met4 by the 26S proteasome through ubiquitination by SCF^{Met30} (Rouillon et al., 2000). A few months later, it was reported that Met4 ubiquitination by Met30 in cells cultured in "rich media" did not result in changes to its proteolytic stability, but instead modification by ubiquitination altered the ability of Met4 to form active transcriptional complexes at *MET* gene promotors (Kaiser et al., 2000). The authors suggest that differences in protein extraction techniques might be responsible for the apparent "degradation" of Met4 in Rouillion et al.'s study (Kaiser et al., 2000). Two years later, a clever effort by the Thomas group to reconcile these flatly contradictory results using GFP fluorescence demonstrated that it was the difference in media composition that explained the apparent discrepancy, with "rich media" resulting in stability and "B media" resulting in instability upon methionine addition (Kuras et al., 2002).

Recognition and degradation of ubiquitinated E3 ligase substrates by the 26S proteasome requires a chain of at least four K48-linked ubiquitin monomers (Deveraux et al.,

1994, Thrower et al., 2000). While it was clear that Met4 was oligo-ubiquitinated, a reasonable explanation for its stability proposed by Kuras et al. is the modification of Met4 by mono-ubiquitination on multiple lysine residues (Kuras et al., 2002). This hypothesis was nullified by a report from the Kaiser lab which demonstrated that the nature of the ubiquitinated species of Met4 was of a single K48-linked ubiquitin chain on a single Met4 lysine residue (Flick et al., 2004). The group went on to show later that protection of ubiquitinated Met4 from proteasomal degradation was the result of an internal ubiquitin-interacting motif (UIM) in Met4, which folds over and caps the growing ubiquitin chain, simultaneously preventing further chain elongation and recognition of Met4 by the 26S proteasome – and point out that this is independent of media condition (Flick et al., 2006). A few months earlier, genetic manipulation of the sulfur metabolic pathway demonstrated that it was the accumulation of cysteine, not methionine or SAM, that signaled for the degradation of Met4 in minimal media as had been previously thought (Menant et al., 2006). These results conform well with an earlier report demonstrating that it was the cysteine branch of sulfur metabolism that was sufficient to repress *MET* gene transcription (Hansen and Johannesen, 2000). Nonetheless, this squabble seemed to fizzle out by the late 2000s, but there was no dearth of interesting biology yet to uncover between these two proteins.

Met30 and cadmium

Regardless of the exact fate of ubiquitinated Met4, the regulation of Met30's E3 ligase activity was found to be of two types; that of sulfur starvation and that of heavy metal toxicity – particularly that of cadmium (Patton et al., 1998, Yen et al., 2005). Purging of

toxic cadmium ions from the cell requires the use of glutathione to chelate and transport cadmium into the vacuole (Li et al., 1997). Unlike starvation of sulfur metabolites, the addition of cadmium to yeast quickly results in the abrupt dissociation of Met30 from the SCF core complex, permitting the deubiquitination and activation of Met4 and the *MET* gene program (Barbey et al., 2005). This mechanism was found to proceed through the Cdc48/p97 AAA+ ATPase complex, which binds and strips the cadmium-specific, auto-ubiquitinated form of Met30 from Skp1 (Yen et al., 2012). It was recently shown that this stripping mechanism was dependent on the Cdc48 cofactor Shp1 (Lauinger et al., 2020). To my knowledge, this was the first example of negative regulation of SCF activity by the active and deliberate separation of a substrate recognition factor from the cullin scaffold of a cullin-based E3 ligase (Barbey et al., 2005).

Met30 links sulfur metabolism to cell cycle progression

MET30 was found to be an essential gene upon its discovery, and interestingly it was discovered that the lethality of *met30*Δ mutants could be suppressed by the deletion of *MET4* as well as *MET32* (Patton et al., 2000, Su et al., 2008, Thomas et al., 1995). Met30 is also reportedly responsible for the degradation of the inhibitory cell cycle kinase Swe1, and is in fact required for multiple steps in cell cycle progression (Kaiser et al., 1998, Su et al., 2005). While it is clear that the essential nature of *MET30* is due to its ability to negatively regulate the transcriptional activity of *MET4,* specifically through the *MET32* co-activator, the exact mechanism for the lethality caused by hyperactivation of Met4 is still not understood – although genetic experiments have shown that deletion of Met30 affects DNA replication (Su

et al., 2005). Recent work has also implicated SCF^{Met30} in a more direct role in the maintenance of genomic stability by showing that the SCF^{Met30} and SCF^{Cdc4} E3 ligases work together to degrade the H3 variant CENP-A (Cse4 in yeast), and that this prevents the chromosomal instability phenotype resulting from Cse4 mislocalization during DNA replication and segregation (Au et al., 2020).

Conclusions

The study of Met30, Met4, and sulfur metabolism in yeast has revealed much about the sophisticated biology of eukaryotic life, including principles of transcriptional regulation, controlled protein degradation, and the interconnectedness and importance of sulfur metabolites and sulfur metabolism for life (Bai et al., 1996, Krek, 1998, Siggers et al., 2011, Thomas and Surdin-Kerjan, 1997, Walvekar and Laxman, 2019). A surprising relationship between sulfur and lipid metabolism was discovered in 2014 when a screen for mutants that failed to repress transcription of the *MET* regulon uncovered *CHO2*, which encodes the methyltransferase that synthesizes phosphatidylcholine (PC) from phosphatidylethanolamine (PE), as a gene whose deletion causes constitutive induction of the *MET* gene transcriptional program (Sadhu et al., 2014). As our lab would also find, loss of *CHO2* results in the intracellular accumulation in SAM and reduced capacity for the synthesis of cysteine, findings that fit nicely with previous reports that demonstrate that synthesis of cysteine is more important that methionine in the regulation of *MET* gene transcription (Hansen and Johannesen, 2000, Menant et al., 2006, Sadhu et al., 2014, Ye et al., 2017). A key question

remains; specifically how does Met30 mechanistically sense the presence or absence of sulfur metabolites and how does that influence its E3 ligase activity? Sadhu et al. in their report find that starvation of sulfur results in the synthesis of a faster-migrating proteoform of Met30 detectable by Western blot, and hypothesize that this proteoform might be important for the regulation of the sulfur metabolic transcriptional program. They also speculate that cysteine oxidation could be involved in the mechanism (Sadhu et al., 2014). The beginning of my doctoral studies start at the investigation of this proteoform, and while my findings demonstrate its limited role in the regulation of Met4 during sulfur starvation, eliminating that hypothesis allowed me to uncover Met30's mechanistic role as a redox sensor.

CHAPTER TWO

Research Approach and Methodology

MATERIALS AND METHODS

Yeast strains and media

All strains used in this study are of the prototrophic CEN.PK background, and are listed in Appendix A. Gene deletions and epitope tagging were carried out using standard PCR-based strategies to amplify a resistance cassette flanked on both ends by homology to the gene of interest to delete or tag a given gene by homologous recombination (Longtine et al., 1998). Met30 cysteine point mutant strains were generated by cloning the Met30 ORF into the pFA6 Flag-tagging plasmid, using PCR to make the cysteine to serine mutations, then amplifying the entire ORF and resistance cassette and transforming into yeast using the lithium acetate method. Clones positive for drug resistance had the relevant section of the *MET30* ORF amplified by PCR and sequenced to verify each mutation. A similar strategy was used to generate strains expressing the long and short isoforms of Met30 from the HO locus in the background of an endogenous *met30*Δ diploid strain before sporulation and tetrad dissection.

The following media are used in this study: YPL (1% yeast extract, 2% peptone and 2% lactate); sulfur-free glucose and lactate media (SFD/L) media composition is detailed in

Appendix A, with glucose or lactate diluted to 2% each; YPD (1% yeast extract, 2% peptone and 2% glucose).

Western Blots

Whole cell lysate protein sample preparation

Five OD_{600} units of yeast culture were quenched in 15% TCA for 15 min, pelleted, washed with 100% EtOH, and stored at −20°C. Cell pellets were resuspended in 325 µL EtOH containing 1 mM PMSF and lysed by bead beating. The lysate was separated from beads by inverting the screwcap tubes, puncturing the bottom with a 23G needle, and spinning the lysate at 2,500xg into an Eppendorf for 1 min. Beads were washed with 200 µL of EtOH and spun again before discarding the bead-containing screwcap tube and pelleting protein extract at 21,000xg for 10 min in the new Eppendorf tube. The EtOH was aspirated and EtOH precipitated protein pellets were resuspended in 150 µL of sample buffer (200 mM Tris pH 6.8, 4% SDS, 20% glycerol, 0.2 mg/ml bromophenol blue), heated at 42°C for 45 min, and debris was pelleted at 16,000xg for 3 min. DTT was added to a final concentration of 25 mM and incubated at RT for 30 min before equivalent amounts of protein were loaded onto NuPAGE 4-12% bis-tris or 3-8% tris-acetate gels. For protein samples modified with mPEG2K-mal, an aliquot of the sample buffer resuspended protein pellets was moved to a fresh Eppendorf and sample buffer containing 15 mM mPEG2K-mal was added for a final concentration of 5 mM mPEG2K-mal before heating at 42°C for 45 min, pelleting debris, and adding DTT.

Western protocol

Western blots were carried out by transferring whole cell lysate extracts or *in vitro* ubiquitination or binding assay samples onto 0.45 micron nitrocellulose membranes and wet transfers were carried out at 300 mA constant for 90 min at 4°C. Membranes were incubated with ponceau S, washed with TBST, blocked with 5% milk in TBST for 1 h, and incubated with 1:5000 Mouse anti-FLAG M2 antibody (Sigma, Cat#F3165), 1:5000 Mouse anti-HA(12CA5) (Roche, Ref#11583816001), 1:50,000 Rabbit anti-RPN10 (Abcam, ab98843), or 1:3000 Goat anti-Cdc53 (Santa Cruz, yC-17) in 5% milk in TBST overnight at 4°C. After discarding primary antibody, membranes were washed 3 times for 5 min each before incubation with appropriate HRP-conjugated secondary antibody for 1 h in 5% milk/TBST. Membranes were then washed 3 times for 5 min each before incubating with Pierce ECL western blotting substrate and exposing to film.

RNA Extraction and qPCR Analysis

RNA isolation of five OD_{600} units of cells under different growth conditions was carried out following the manufacture manual using MasterPure yeast RNA purification kit (epicentre). RNA concentration was determined by absorption spectrometer. 5 µg RNA was reverse transcribed to cDNA using Superscript III Reverse Transcriptase from Invitrogen. cDNA was diluted 1:100 and real-time PCR was performed in triplicate with iQ SYBR Green Supermix from BioRad. Transcripts levels of genes were normalized to ACT1. All the primers used in RT-qPCR have efficiency close to 100%, and their sequences are listed below.

ACT1_RT_F TCCGGTGATGGTGTTACTCA

ACT1_RT_R GGCCAAATCGATTCTCAAAA

MET17_RT_F CGGTTTCGGTGGTGTCTTAT

MET17_RT_R CAACAACTTGAGCACCAGAAAG

GSH1_RT_F CACCGATGTGGAAACTGAAGA

GSH1_RT_R GGCATAGGATTGGCGTAACA

SAM1_RT_F CAGAGGGTTTGCCTTTGACTA

SAM1_RT_R CTGGTCTCAACCACGCTAAA

Metabolite extraction and quantitation

Intracellular metabolites were extracted from yeast using a previous established method (Tu et al., 2007). Briefly, at each time point, ~12.5 OD_{600} units of cells were rapidly quenched to stop metabolism by addition into 37.5 mL quenching buffer containing 60% methanol and 10 mM Tricine, pH 7.4. After holding at -40°C for at least 3 min, cells were spun at 5,000xg for 2 min at 0°C, washed with 1 mL of the same buffer, and then resuspended in 1 mL extraction buffer containing 75% ethanol and 0.1% formic acid. Intracellular metabolites were extracted by incubating at 75°C for 3 min, followed by incubation at 4°C for 5 min. Samples were spun at 20,000xg for 1 min to pellet cell debris, and 0.9 mL of the supernatant was transferred to a new tube. After a second spin at 20,000xg for 10 min, 0.8 mL of the supernatant was transferred to a new tube. Metabolites in the

extraction buffer were dried using SpeedVac and stored at −80°C until analysis. Methionine, SAM, SAH, cysteine, GSH and other cellular metabolites were quantitated by LC-MS/MS with a triple quadrupole mass spectrometer (3200 QTRAP, AB SCIEX) using previously established methods (Tu et al., 2007). Briefly, metabolites were separated chromatographically on a C18-based column with polar embedded groups (Synergi Fusion-RP, 150 3 2.00 mm 4 micron, Phenomenex), using a Shimadzu Prominence LC20/SIL-20AC HPLC-autosampler coupled to the mass spectrometer. Flow rate was 0.5 ml/min using the following method: Buffer A: 99.9% H2O/0.1% formic acid, Buffer B: 99.9% methanol /0.1% formic acid. T = 0 min, 0% B; T = 4 min, 0% B; T = 11 min, 50% B; T = 13 min, 100% B; T = 15 min, 100% B, T = 16 min, 0% B; T = 20 min, stop. For each metabolite, a 1 mM standard solution was infused into a Applied Biosystems 3200 QTRAP triple quadrupole-linear ion trap mass spectrometer for quantitative optimization detection of daughter ions upon collision-induced fragmentation of the parent ion [multiple reaction monitoring (MRM)]. The parent ion mass was scanned for first in positive mode (usually MW + 1). For each metabolite, the optimized parameters for quantitation of the two most abundant daughter ions (i.e., two MRMs per metabolite) were selected for inclusion in further method development. For running samples, dried extracts (typically 12.5 OD units) were resuspended in 150 mL 0.1% formic acid, spun at 21,000xg for 5 min at 4°C, and 125 µL was moved to a fresh Eppendorf. The 125 µL was spun again at 21,000xg for 5 min at 4°C, and 100 µL was moved to mass-spec vials for injection (typically 50 µL injection volume). The retention time for each MRM peak was compared to an appropriate standard. The area

under each peak was then quantitated by using Analyst® 1.6.3, and were re-inspected for accuracy. Normalization was done by normalizing total spectral counts of a given metabolite by OD_{600} units of the sample. Data represent the mean and SD of two biological replicates.

Protein purifications

Uba1

6xHis-Uba1 (E1) was purified as previously described (Petroski and Deshaies, 2005), with the exception that the strain was made in the CEN.PK background and the His6-tag was appended to the N-terminus of Uba1. Additionally, lysis was performed by cryomilling frozen yeast pellets by adding the pellet to a pre-cooled 50 ml milling jar containing a 20 mm stainless steel ball. Yeast cell lysis was performed by milling in 3 cycles at 25 Hrz for 3 min and chilling in liquid nitrogen for 1 min. Lysate was made by adding 4 ml of buffer for every gram of cryomilled yeast powder, and clarification was performed at 35,000xg instead of 50,000xg. Further detail on cryomilling can be found below.

Cdc34

Cdc34-6xHis (E2) similarly was purified according to previously described protocols (Petroski and Deshaies, 2005), with the following exceptions; the CDC34 ORF was cloned into pHIS parallel vector such that the N-terminal His tag was eliminated from the vector while incorporating a C-terminal 6xHis tag by PCR. BL21 transformants were grown in LB medium and expression was induced by addition of 0.1 mM IPTG. Cells were lysed by

sonication and clarification was done by spinning at 35,000xg for 20 min at 4°C before the Ni-NTA purification was performed as previously described (Petroski and Deshaies, 2005).

Met4

His6-SUMO-Met4-Strep-tagII-HA was purified by cloning the MET4 ORF into pET His6 Sumo vector while incorporating a C-terminal Strep-tagII and a single HA tag by PCR. BL21 transformants were grown in 2 liters LB medium and induced by addition of 0.1 mM IPTG O/N at 16°C at 200 rpm. Cell pellets were collected and lysed by sonication in buffer containing 50 mM Tris pH 7.5, 300 mM NaCl, 10% glycerol, 20 mM imidazole, 1 mM PMSF, 10 μM leupeptin, 50 mM NaF, 5 μM pepstatin, 0.5% NP-40, and 2x roche EDTA-free protease inhibitor cocktail tablet. Lysate was clarified by centrifugation at 35,000xg for 20 min at 4°C and the supernatant was transferred to a 50 ml conical and Met4 was batch purified with 1.5 ml of Ni-NTA agarose by incubating for 30 min at 4°C. After spinning down the Ni-NTA agarose, the supernatant was removed and the agarose was resuspended in the same buffer and moved to a gravity flow column and washed 3 times with 50 mM Tris pH 7.5, 300 mM NaCl, 10% glycerol, and 20 mM imidazole before elution with the same buffer containing 200 mM imidazole. Eluted Met4 was then run over 2 ml of Strep-Tactin Sepharose in a 10 ml gravity flow column, washed with 5 CVs Strep-Tactin wash buffer (100 mM Tris pH 8.0, 150 mM NaCl), and eluted by diluting 1 ml 10X Strep-Tactin Elution buffer in 9 ml Strep-Tactin wash buffer and collecting 1.5 ml fractions. Fractions containing pure, full-length Met4 were pooled and concentrated while exchanging the buffer with buffer

containing 30 mM Tris pH 7.6, 100 mM NaCl, 5 mM MgCl2, 15% glycerol, and 2 mM DTT. Protein concentration was measured and 1 mg/ml aliquots were made and stored at −80°C.

Cryomill protocol

A Retsch Cryomill was used for yeast cell lysis for E1 purification as well as large-scale Met30-Flag immunopurifications. Typically 3000 OD_{600} units of yeast were pelleted in a 50 ml conical before flash-freezing in liquid nitrogen and long term storage in the −80°C. Before retrieving the frozen cell pellets for lysis, a (completely dry) cryomill chamber/milling jar containing one large stainless steel ball would be cooled in liquid nitrogen. Once the cell pellet was retrieved and cooled in liquid nitrogen, the 50 ml conical was wrapped with a paper towel and cracked by firmly hitting the sides of the plastic tube with a large wrench to free the cell pellet. After removing any small plastic pieces, cell pellet chunks would quickly be moved to the milling jar containing the large stainless steel ball before sealing and submerging the chamber in liquid nitrogen again. Once cooled, the milling jar was placed in the cryomill and subjected to three cycles of milling at 25 Hrz for 3 min with 1-2 min in liquid nitrogen between cycles. I would often not count the first round, as it takes some time for the cell pellet chunks to break up and the cryomill "rattling" to sound right. The procedure also seems to work better if between cycles you hit the jar against the counter a few times (using a buffer of folded paper towels) to dislodge any powder frozen against the sides or ends of the jar. Once the last cycle was complete, the top of the jar was hit against the counter to remove powder on the lid of the jar and collect powder in the main chamber, and a cooled spatula was used to move powder from the

chamber to a new 50 ml conical partly submerged in liquid nitrogen. The 50 ml conical would then be weighed and the weight of the powder would be noted. As a general rule, 4 ml of buffer would be added for every gram of yeast cryomill powder, and after a few rounds of vortexing, would be briefly sonicated at 50 mA for 10 seconds to shear DNA and reduce the viscosity of the lysate.

$SCF^{Met30\text{-}Flag}$ IP and *in vitro* assays

In vitro ubiquitination assay

The E3 ligase activity of SCF^{Met30} was assessed by immunopurification of Flag-tagged Met30 and its binding partners and using the concentrated IP in *in vitro* ubiquitination reactions with purified E1, E2, yeast ubiquitin (Boston Biochem, U-100sc), and ATP. First ~3000 OD_{600} units of yeast grown in either rich YPL media or −Sulfur (SFL) minimal media (15 min after switch from YPL) were pelleted, frozen in liquid nitrogen, and cryomilled as described above. Cryomilled yeast powder (~ 3-4 grams) was moved to a 50 ml conical and resuspended in 12-16 ml SCF IP buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM NaF, 1% NP-40, 1 mM EDTA, 5% glycerol) containing 10 μM leupeptin, 1 mM PMSF, 5 μM pepstatin, 100 μM sodium orthovanadate, 2 mM 1, 10-phenanthroline, 1 μM MLN4924, 1X Roche EDTA-free protease inhibitor cocktail tablet, and 1 mM DTT when specified. Small molecule inhibitors of neddylation and deneddylation were included, and along with a short IP time, intended to minimize exchange and preserve F-box protein/Skp1 substrate recognition modules (Reitsma et al., 2017). The lysate was then briefly sonicated to sheer

DNA and subsequently clarified at 35,000xg for 20 min and the supernatant was incubated with with 50 μL of Thermo Fisher protein G dynabeads (Cat# 10004D) DMP crosslinked to 25 μL of Mouse anti-FLAG M2 antibody (Sigma, Cat#F3165) for 30 min at 4°C. The magnetic beads were pelleted at 500xg for 5 min, the supernatant was aspirated, and the magnetic beads transferred to an Eppendorf tube. The beads were washed 5 times with 1 ml SCF IP buffer with or without DTT before elution with 1 mg/ml Flag peptide in PBS. The eluent was concentrated in Amicon Ultra-0.5 centrifugal filter units with 10 kDa MW cutoffs to a final volume of ~ 40 μL. Silver stains of the IPs were carried out using the Pierce Silver Stain for Mass Spectrometry kit (Cat#24600) according to the manufacturers protocol. The *in vitro* ubiquitination assay was performed by placing a PCR tube on ice and adding to it 29 μL of water, 8 μL of 5X ubiquitination assay buffer (250 mM Tris pH 7.5, 5 mM ATP, 25 mM MgCl2, 25% glycerol), 1.2 μL Uba1 (FC = 220 nM), 1.2 μL Cdc34 (FC = 880 nM), 0.5 μL yeast ubiquitin (Boston Biochem, FC = 15.5 μM) and incubating at RT for 20 min. The PCR tubes were then placed back on ice and 20 μL of water, 8 μL of 5X ubiquitination assay buffer, 10 μL of concentrated $SCF^{Met30\text{-}Flag}$ IP, and 2 μL of purified Met4 (FC = 200 nM) were added, the tubes were moved back to RT, and 20 μL aliquots of the reaction were removed, mixed with 2X sample buffer, and frozen in liquid nitrogen over the time course.

In vitro binding assay

For the Met4 binding assay, yeast cell lysate was prepared as described for the ubiquitination experiment, except that the lysate was split three ways, with 1 mM DTT, 1 mM tetramethylazodicarboxamide (Diamide) (Sigma, Cat#D3648), or nothing added to the

lysate prior to centrifugation at 21,000xg for 30 min at 4°C. The supernatant was transferred to new tubes and 100 µL of Thermo Fisher protein G dynabeads (Cat# 10004D) DMP crosslinked to 50 µL of Mouse anti-FLAG M2 antibody (Sigma, Cat#F3165) was divided evenly between the six Met30-Flag IP conditions and incubated for 2 h at 4°C while rotating end over end. After incubation, the beads were washed with IP buffer containing 1 mM DTT, 1 mM Diamide, or nothing twice before a final wash with plain IP buffer. Each set of Met30-Flag bound beads prepared in the different IP conditions was brought up to 80 µL with plain IP buffer, and 40 µL was dispensed to new tubes containing 1 mL of IP buffer ± 1 mM DTT and 1 µg of purified recombinant Met4, and were incubated for 2 h at 4°C while rotating end over end for a total of twelve (eighteen when including Met30 cysteine mutants) Met4 co-IP conditions. The beads were then collected, washed 3 times with IP buffer ± 1 mM DTT, resuspended in 60 µL 2X sample buffer, and heated at 70°C for 10 min before Western blotting for both Met4 and Met30.

DMP crosslinking protocol

Dimethyl pimelimidate dihydrochloride (Sigma, Cat# D8388) was used to crosslink mouse Flag M2 antibody (Sigma, Cat#F3165) to Thermo Fisher protein G dynabeads (Cat# 10004D) for use in immunopurification. For every 3000 OD_{600} units used in the *in vitro* ubiquitination or bindings assay, 100 µL of dynabead slurry were transferred to an Eppendorf and the beads were washed twice with 0.1 M sodium phosphate pH 8.0 before aspirating, then resuspending the beads in 100 µL of 0.1 M sodium phosphate buffer, adding 25 µL of 1 mg/ml mouse Flag M2 antibody, then shaking at room temperature for 1 h. Beads were then

concentrated on a magnetic particle concentrator (MPC), washed twice with 0.5 ml of 0.1 M sodium phosphate buffer containing 0.01% Tween-20, then washed twice more with 0.5 ml of 0.2 M triethanolamine pH 8.2. After aspirating off remaining buffer, the beads were then resuspended in 0.5 ml of 0.2 M triethanolamine pH 8.2 containing 20 mM DMP and shaken at room temperature for 30 min. Crosslinked beads were then collected on an MPC and 0.5 ml of 50 mM Tris pH 7.5 was added and beads were shaken 15 min at room temperature. Crosslinked beads were collected again on an MPC and Tris buffer aspirated before the beads were washed three times with 1 ml TBST, resuspended in 200 μL of TBST, and stored at 4°C until use in immunopurification.

Software and analysis

Metabolites were quantified using Analyst® 1.6.3, and graphs were prepared using GraphPad PRISM 9.0. Metabolite data represent the mean and SD of two biological replicates. RT-qPCR was performed on a Bio-Rad CFX384 Real-Time System and values were exported to Excel 16.0 and put into PRISM 9.0 to prepare graphs. Expression data represent the mean and SEM of technical triplicates. Western blot quantitation was performed in ImageJ (version 1.53) and graphs prepared in PRISM 9.0.

CHAPTER THREE

Results

THE SULFUR STARVATION ISOFORM OF MET30

Dependencies of the Met30 "short" isoform

As previously mentioned, Met4 activation in response to sulfur starvation results in the emergence of a second, faster-migrating "short" proteoform of Met30, which disappears after repletion of sulfur metabolites (Sadhu et al., 2014). Sadhu et al. go to some length to analyze the 5' end of the Met30 transcript, and discovered a hairpin structure they hypothesized might be involved in regulating the Met30 short form synthesis. Although disruption of this hairpin had no effect on the ability to produce this proteoform during sulfur starvation, I also suspected focus on the transcriptional basis of this proteoform would be revealing.

Because *MET30* transcription is under the control of Met4, and the short isoform is only produced under conditions of Met4 transcriptional activation, I sought to determine if this isoform of Met30 was *MET4* dependent. I found that the appearance of this proteoform is dependent on *MET4*, as it was not observed in *met4*Δ cells during sulfur starvation (Figure 1A). Additionally, this proteoform is dependent on new mRNA translation as it is not synthesized when cells are treated with cycloheximide during sulfur-limitation, and persists after rescuing cells with a sulfur source in the presence of a proteasome inhibitor (Figure 1B

and C). Taken together these results imply that this proteoform is newly synthesized Met30 protein, which cannot be derived from or converted to the "long" proteoform during sulfur starvation or after rescuing cells with a sulfur source, respectively.

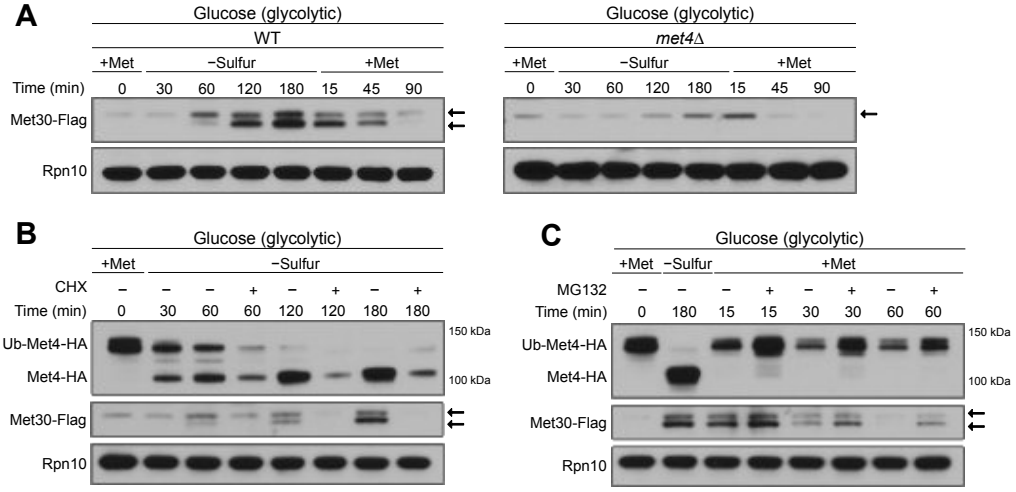

Figure 1: The short isoform of Met30.

A) The faster-migrating proteoform of Met30 is dependent on *MET4*. The *met4*Δ yeast strain does not produce the second proteoform of Met30 when starved of sulfur.
B) Western blot of yeast treated with 200 μg/ml cycloheximide during sulfur starvation demonstrates that production of the faster-migrating proteoform is dependent on new translation.
C) The faster-migrating proteoform persists after rescue from sulfur starvation when treated with a proteasome inhibitor. Cells were starved of sulfur for 3 h to accumulate the faster-migrating proteoform, and then sulfur metabolites were added back concomitantly with MG132 (50 μM).

Identification and characterization of "long" and "short" Met30

After these data were collected, I hypothesized that this short form might be the result of alternative translational initiation at an internal methionine residue of Met30. Early attempts to identify the nature of this short form were hampered by poor sequence coverage of the N-terminus by mass spectrometric methods (data not shown), preventing a thorough

comparison of peptides that might be unique to Met30 isolated from high or low sulfur conditions – a problem also encountered by Sadhu et al. in their study (Sadhu et al., 2014). The apparent difference in molecular weight between the long and short isoforms of Met30 is relatively small, certainly no more than 5 or 6 kDa, and examination of the N-terminal amino acid sequence of Met30 revealed multiple methionine residues at which translation could potentially be initiated.

I then moved on to a bioinformatic approach to find conserved methionine residues that could be responsible, and multiple sequence alignments of Met30 in closely related *Saccharomyces* species showed near perfect conservation of methionine residues at positions 30, 35, and 36 in the Met30 protein (Figure 2). To generate strains that expressed only the long or short proteoform of Met30, I created a strain that would express Met30 from the HO locus and mutated methionine residues 30, 35 and 36 to alanine, and in parallel created a strain that would have the first twenty amino acids of Met30 deleted. The rationale was to prevent translational initiation from the first methionine of Met30, as well as the less-well conserved methionines at positions 8 and 9, while also retaining the translational initiation context of methionines 30, 35, and 36 by preserving the immediate upstream nucleotide sequences.

In support of this hypothesis, mutation of methionine residues 30, 35, and 36 to alanine blocked the appearance of a short form during sulfur starvation (Figure 3A). Conversely, deletion of the first 20 amino acids containing the first three methionine residues of Met30 resulted in expression of a Met30 proteoform that migrated at the apparent

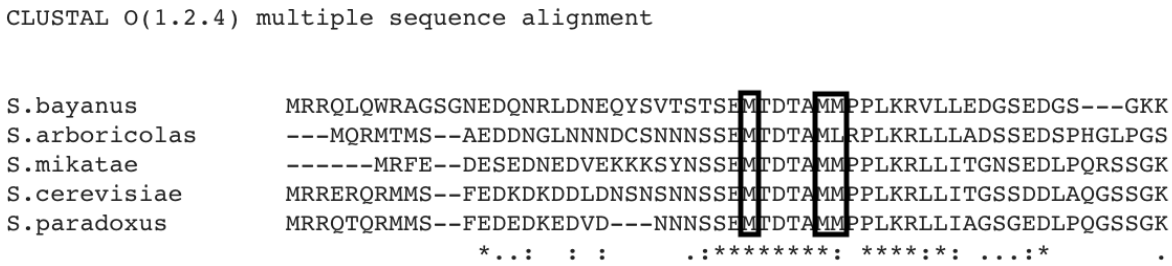

Figure 2: Multiple sequence alignment of Met30 in closely related *Saccharomyces* species.

Uniprot Met30 protein sequences for the above species were aligned using CLUSTAL O and highly conserved methionine residues boxed in black.

molecular weight of the wild type short form and did not generate a new, even-faster migrating proteoform under sulfur starvation (Figure 3A). Moreover, the Met30 M30/35/36A and Met30 Δ1-20 strains expressing either solely the long or short form of the Met30 protein had no obvious phenotype with respect to Met4 ubiquitination or growth in high or low sulfur media (Figure 3B). It could be reasonably argued that there is a phenotype in the M30/35/36A mutant strain in terms of the re-ubiquitination of Met4 after rescuing with a sulfur source, however this appears inconsequential for the recovery from transient sulfur starvation as it produced no growth defect compared to the WT or Δ1-20 strains (Figure 3B, last panel).

While I am still to a degree investigating the purpose of this proteoform, it appears dispensable for regulating sulfur metabolism in the context of sulfur starvation. To test the possibility that this proteoform might instead be important in the context of heavy metal toxicity, I treated these same Met30 strains with 0.5 mM $CdCl_2$ and blotted for Met30 and Met4. These data also suggest that this proteoform has no effect on Met4 ubiquitination during cadmium-specific heavy metal stress (Figure 4).

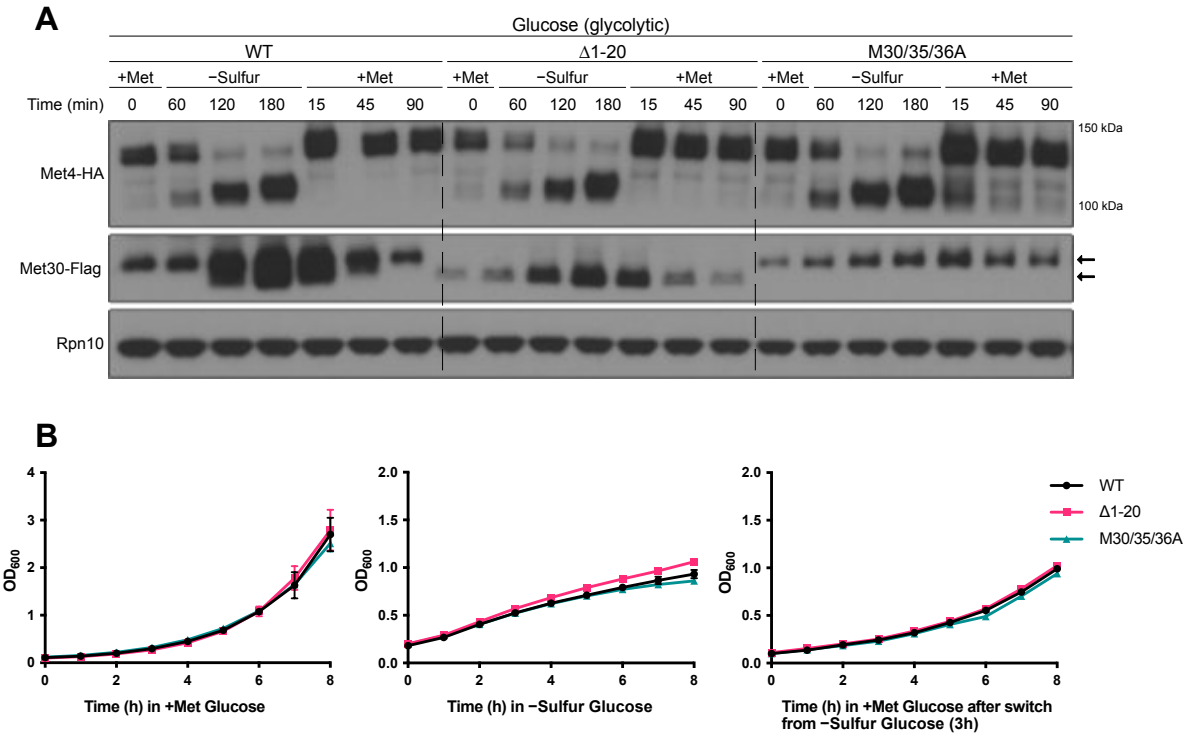

Figure 3: The Met30 long and short isoforms appear to be inconsequential in the context of sulfur starvation.

A) Western blot analysis of strains expressing either wild type Met30, Met30 Δ1-20aa, or Met30 M30/35/36A. Yeast cells harboring the N-terminal deletion of the first twenty amino acids of Met30 (which contain the first three methionine residues) or have the subsequent three methionine residues (M30/35/36) mutated to alanine do not create faster-migrating proteoforms.
B) Met30 Δ1-20 and Met30 M30/35/36A strains do not exhibit any growth phenotypes in −sulfur glucose media with or without supplemented methionine. There are also no defects in growth rate following repletion of methionine. Data represent mean and SD of technical triplicates.

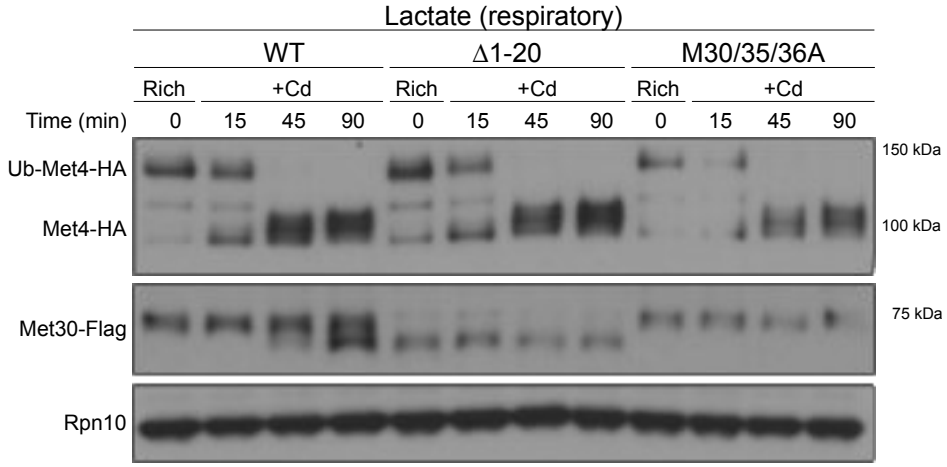

Figure 4: The Met30 long and short isoforms have little effect during cadmium-induced heavy metal stress.

Western blot analysis of strains expressing either wild type Met30, Met30 Δ1-20aa, or Met30 M30/35/36A were challenged with 0.5 mM $CdCl_2$ in rich lactate media. Note that similar results are observed in glucose media (data not shown).

MET30 IS A REDOX SENSOR: IN VIVO EVIDENCE

Using respiratory growth conditions to study acute sulfur depletion

The first three years of my work I utilized sulfate-free media containing glucose to study Met30 and Met4 dynamics, which proved to be too slow a response to reveal Met30's sulfur-sensing mechanism. A much more rapid and acute depletion of sulfur metabolites, I hypothesized, would provide a better system to study the mechanistic details of Met30. Previous work in our lab has characterized the metabolic and cellular response of yeast cells grown in respiratory conditions following switch from rich lactate media (YPL) to minimal lactate media (SL) media (Kato et al., 2019, Laxman et al., 2013, Sutter et al., 2013, Wu and Tu, 2011, Yang et al., 2019, Ye et al., 2017, Ye et al., 2019). Under such respiratory conditions, yeast cells engage regulatory mechanisms that might otherwise be subject to glucose repression. Among other phenotypes, this switch results in the acute depletion of sulfur metabolites and the activation of the *MET* gene regulon (Sutter et al., 2013, Ye et al., 2019). To better study the response of yeast cells to sulfur starvation specifically, I replaced the glucose carbon source in my sulfate-free media (SFD) with lactate (SFL, referred to in figures as "−Sulfur") and used this instead of our normal minimal lactate media (SL), as prototrophic CEN.PK yeast strain can assimilate sulfur in the form of inorganic sulfate into reduced sulfur metabolites (See Chapter 1 for more detail).

After switching cells from YP lactate media (Rich) to the new minimal sulfur-free lactate media (−Sulfur), it became clear that Met30 and Met4 indeed responded quickly to sulfur starvation through the extensively studied ubiquitin-dependent mechanisms regulating

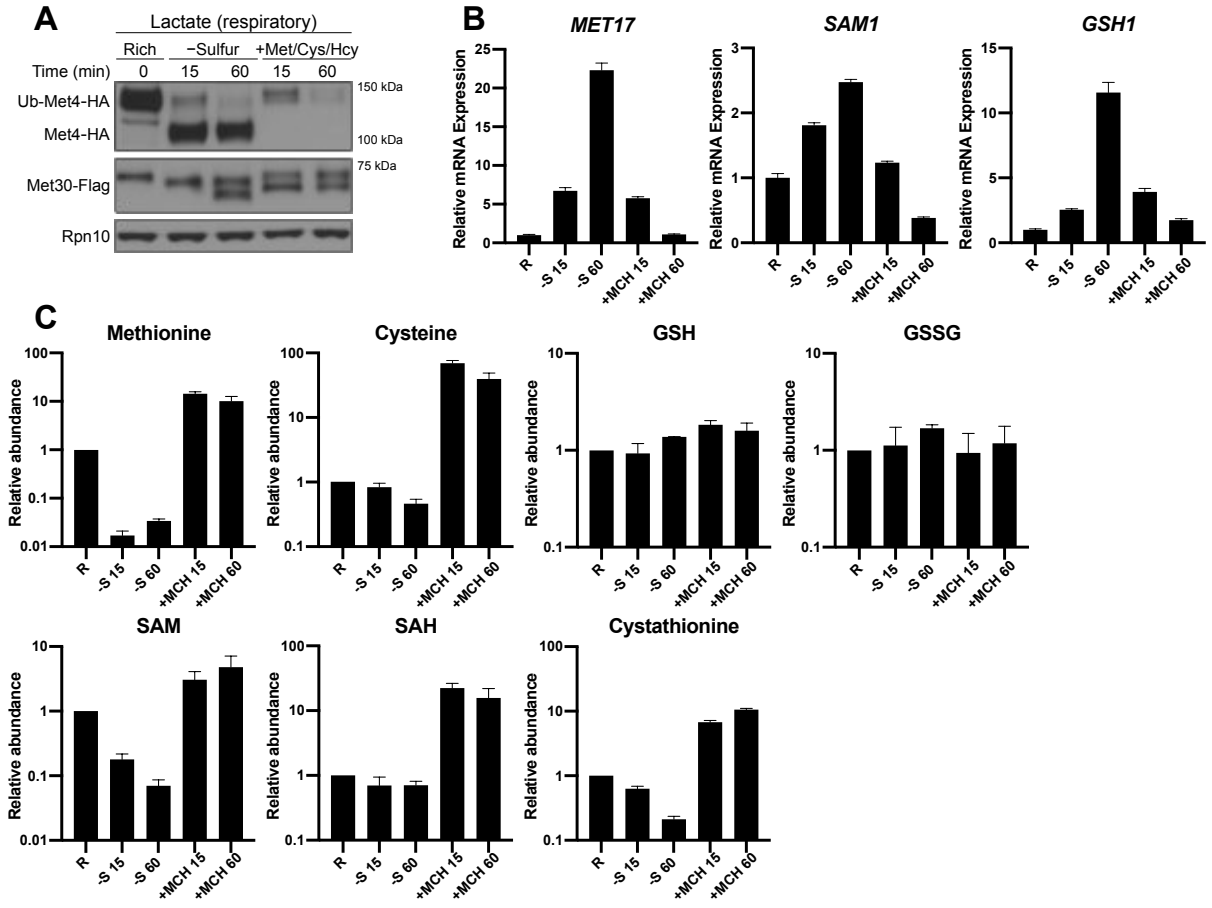

Figure 5: Met30 and Met4 response to sulfur starvation in respiratory conditions.

(A) Western blot analysis of a time course performed with yeast containing endogenously tagged Met30 and Met4 that were cultured in rich lactate media (Rich) overnight to mid log phase before switching cells to sulfur-free lactate media (−sulfur) for 1 h, followed by the addition of a mix of the sulfur containing metabolites methionine, homocysteine, and cysteine at 0.5 mM each (+Met/Cys/Hcy).
(B) The relative expression of *MET* gene transcript levels was assessed by qPCR over the time course shown in (A). Data are presented as mean and SEM of technical triplicates.
(C) Levels of key sulfur metabolites were measured over the same time course as in (A) and (B), as determined by LC-MS/MS. Data represent the mean and SD of two biological replicates.

Met4 activity (Figure 5A) (Barbey et al., 2005, Flick et al., 2004, Flick et al., 2006, Kaiser et al., 2000, Yen et al., 2005). As previously observed, the deubiquitination of Met4 resulted in the activation of the *MET* genes (Figure 5B) and corresponded well with changes in observed sulfur metabolite levels (Figure 5C). Addition of sulfur metabolites to sulfur-starved cells quickly rescued Met30 activity and resulted in the re-ubiquitination of Met4 and the repression of the *MET* genes.

With a more robust system in place, I next moved on to determining which branch of sulfur metabolism Met30 responds to. By genetically blocking key points in the sulfur metabolic pathway (Figure 6A), it can be determined which branch is necessary to stimulate Met30's E3 ligase activity after cells are depleted of sulfur by the addition of different sulfur metabolites either in front of or behind the genetic block to the media. To determine whether the synthesis of methionine is requisite to rescue Met30 activity, cells lacking methionine synthase (*met6*Δ) were fed either homocysteine or methionine after switching to sulfur-free lactate (−Sulfur) media. Interestingly, cells fed homocysteine were still able to ubiquitinate and degrade Met4, while methionine-fed cells appeared to oligo-ubiquitinate and stabilize Met4 (Figure 6B). These observations are consistent with previous reports and suggest that Met30 and Met4 interpret sulfur sufficiency through both branches of sulfur metabolism to a degree (Flick et al., 2004, Hansen and Johannesen, 2000, Kaiser et al., 2000, Kuras et al., 2002, Menant et al., 2006, Sadhu et al., 2014), with the stability of Met4, but not the E3 ligase activity of Met30, apparently dependent on the methionine branch.

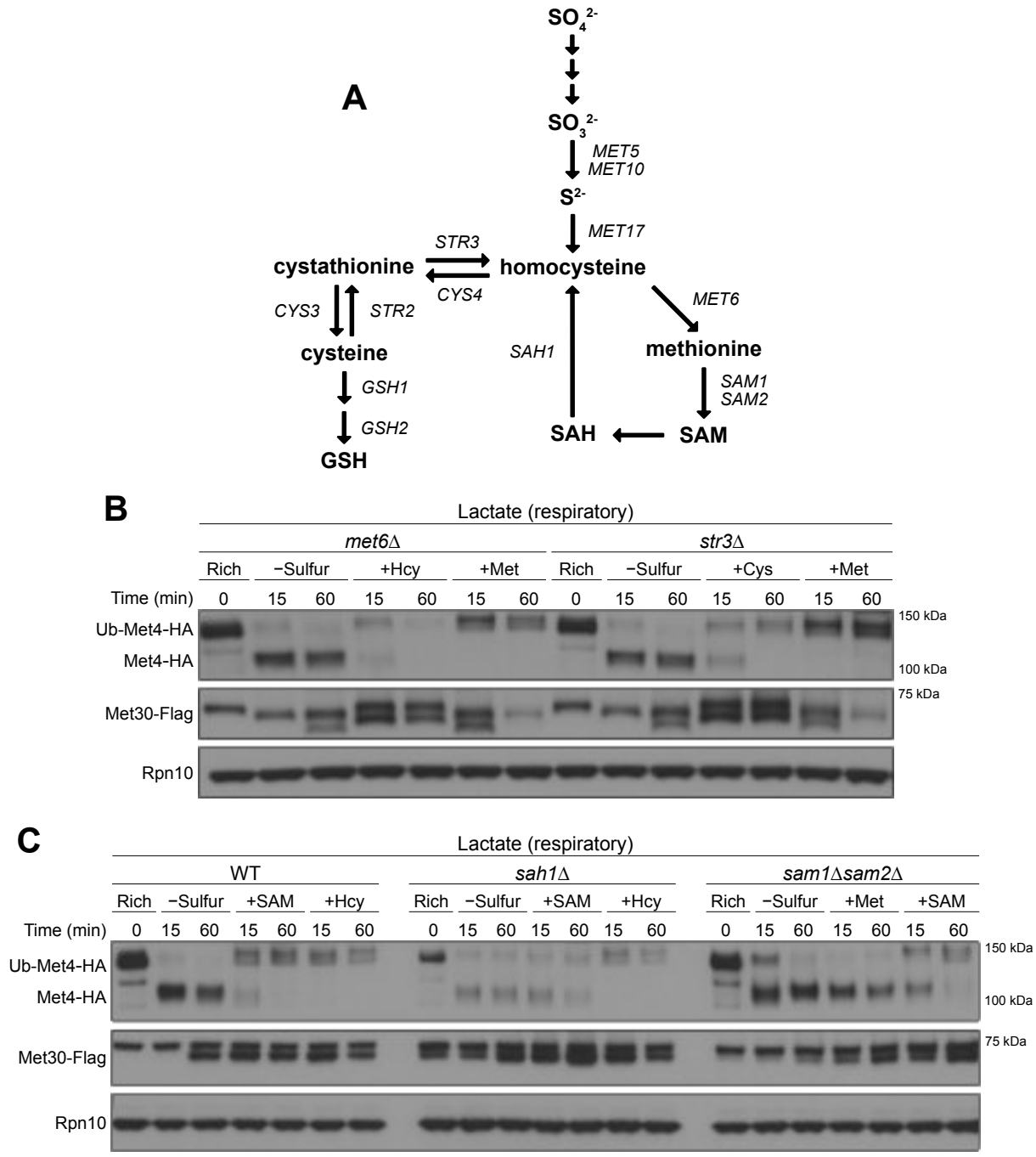

Figure 6: Met30 responds to the GSH branch of sulfur metabolism.

(A) Simplified diagram of the sulfur metabolic pathway in yeast.
(B) *met6*Δ or *str3*Δ strains were grown in "Rich" YPL and switched to "−sulfur" SFL for 1 h to induce sulfur starvation before the addition of either 0.5 mM homocysteine (+HCY), 0.5 mM methionine (+MET), or 0.5 mM cysteine (+CYS).
(C) WT, *sah1*Δ, and *sam1*Δ *sam2*Δ strains were grown as in (B) with the exception that the *sah1*Δ strain was supplemented with 0.2 mM homocysteine and *sam1*Δ *sam2*Δ cells were supplemented with 0.5 mM SAM in the YPL media before switching to −sulfur media for one hour before the addition of the indicated sulfur metabolites (0.5 mM each).

To determine whether Met30 specifically responds to cysteine, cells lacking cystathionine beta-lyase (*str3*Δ), the enzyme responsible for the conversion of cystathionine to homocysteine, were starved of sulfur and fed either cysteine or methionine. This mutant is incapable of synthesizing methionine from cysteine via the two-step conversion of cysteine into the common precursor metabolite homocysteine. The data show cysteine was able to rescue Met30 activity even in a *str3*Δ mutant, further suggesting cysteine or a downstream metabolite, and not methionine, as the signal of sulfur sufficiency for Met30 (Figure 6B).

Further demonstrating the necessity of the cysteine branch, a strain lacking *S*-adenosyl homocysteine lyase (*sah1*Δ) was constructed to examine the importance of transulfuration and the recycling of SAM. Cells lacking *SAH1* are unable to recycle SAH, the byproduct of SAM-dependent methyltransferase reactions, into homocysteine and adenosine – and consequently cannot utilize to a significant extent the transulfuration pathway when SAM or methionine is the sole sulfur source. Consistent with my hypothesis, sulfur-starved *sah1*Δ cells did not re-ubiquitinate Met4 when given SAM, but were however able to ubiquitinate Met4 when provided homocysteine (Figure 6C). To eliminate the possibility that Met30 could be responding directly to methionine levels, a *sam1*Δ *sam2*Δ double knockout was constructed to make a completely SAM auxotrophic yeast strain. After starving this strain for sulfur for one hour, the addition of methionine was insufficient to rescue Met4 ubiquitination status, while SAM was able to slowly restore Met4 to a fully ubiquitinated state (Figure 6C). It should be noted that WT yeast also displayed this delayed response when rescued with SAM as the sulfur source (Figure 6C, fourth lane).

Met30 cysteine residues are oxidized *in vivo*

The synthesis of cysteine from homocysteine contributes to the production of the downstream tripeptide metabolite glutathione (GSH), which exists at millimolar concentrations in cells and is the major cellular reductant for buffering against oxidative stress (Cuozzo and Kaiser, 1999, Wu et al., 2004). Specifically, glutathione serves to neutralize reactive oxygen species such as peroxides and free radicals, detoxify heavy metals, and preserve the reduced state of protein thiols (Penninckx, 2000, Pompella et al., 2003). Considering the relatively high number of cysteine residues in Met30 (Figure 7A), I sought to determine if these residues might become oxidized during acute sulfur starvation. Utilizing the thiol-modifying agent methoxy-PEG-maleimide (mPEG2K-mal), which adds ~2 kDa per reduced cysteine residue, I assessed Met30 cysteine oxidation *in vivo* by Western blot. Theoretically, full modification of the 23 cysteines in Met30 by mPEG2K-mal should significantly shift the apparent molecular weight of Met30 by ~45-50 kDa. As expected, Met30 in sulfur-replete rich media migrates at ~140 kDa (Figure 7B, first lane), nicely corresponding to the modification of most if not all of its 23 cysteine residues, implying they are all in the reduced state while sulfur levels are high and Met4 is repressed. However, after shifting into sulfur-free lactate media, Met30 migrates at ~80 kDa – suggesting the majority of its cysteine residues are rapidly becoming oxidized *in vivo* following acute sulfur starvation (Figure 7B, second and third lane). In contrast, the loading control Rpn10 contains a single cysteine residue, and did not exhibit significant oxidation within the same time period. As expected, repletion of sulfur metabolites led to the reduction and modification of

Met30's cysteine residues by mPEG2K-mal to the extent seen in rich media. Such oxidation and re-reduction of Met30 cysteines corresponds well with Met4 ubiquitination status (Figure 7B). Additionally, when cells were grown in sulfur-free media containing glucose as the carbon source (SFD), Met30 also becomes oxidized — although on a slower timescale — suggesting this mechanism is not specific to yeast grown under non-fermentable conditions (Figure 7C).

Considering the link between sulfur starvation and oxidative stress, I next assessed whether simply changing the redox state of sulfur-starved cells could mimic sulfur repletion with respect to Met30 E3 ligase activity. Addition of the potent, membrane-permeable reducing agent DTT to yeast cells starved of sulfur readily reversed Met30 cysteine oxidation. DTT also resulted in the partial re-ubiquitination of Met4, suggesting that Met30 cysteine redox status influences its ubiquitination activity against Met4 (Figures 7D, and 8, third lane).

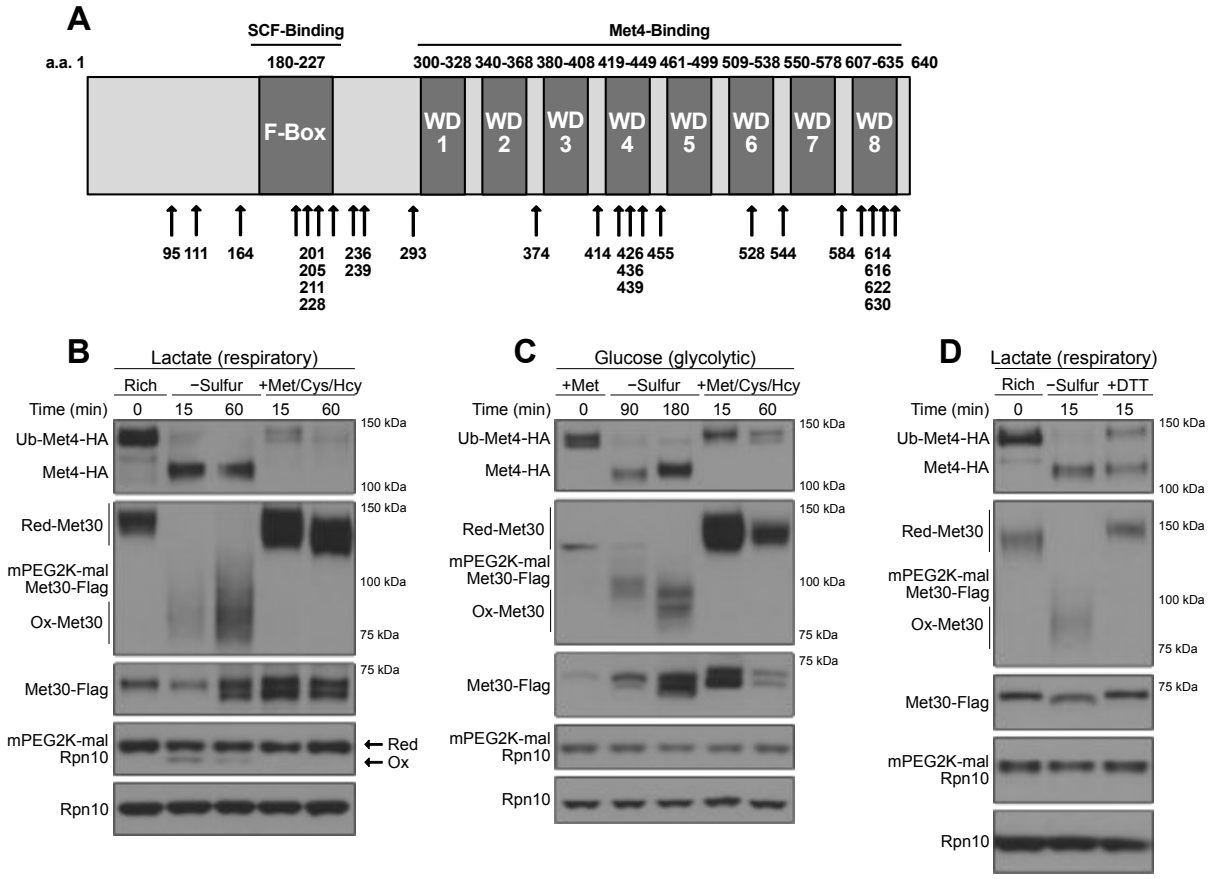

Figure 7: Met30 cysteine residues are oxidized during sulfur starvation.

A) Schematic of Met30 protein architecture and cysteine residue location.
B) Western blot analysis of Met30 cysteine redox state in lactate media as determined by methoxy-PEG-maleimide (mPEG2K-mal) modification of reduced protein thiols. For every reduced cysteine thiol in a protein, mPEG2K-mal adds ~ 2 kDa in apparent molecular weight.
C) Same Western blot analysis as in (B), except that yeast were cultured in sulfur-free glucose media (SFD) for 3 h before the addition of 0.5 mM each of the sulfur metabolites homocysteine, methionine, and cysteine (+Met/Cys/Hcy).
D) Yeast were subjected to the same rich to −sulfur media switch as in (B), except that following the 15 min time point, 5 mM DTT was added to the culture for 15 min and Met30 cysteine residue redox state and Met4 ubiquitination were assessed by Western blot.

Met30 cysteine mutants display aberrant sulfur-sensing *in vivo*

After establishing Met30 cysteine redox status as an important factor in sensing sulfur starvation, I sought to determine whether specific residues played key roles in the sensing mechanism. In principle, mutation of any cysteine to serine should mimic the reduced state of the Met30 protein, and result in the constitutive ubiquitination of Met4 even when cells are depleted of sulfur metabolites. Through site-directed mutagenesis of Met30 cysteines individually (Figure 8), I observed that mutation C414S resulted in dysregulated Met4 ubiquitination in both high and low sulfur conditions. Although it was generated on accident by mutating cysteine 622 in the C584S plasmid instead of the wildtype plasmid, the double cysteine to serine mutation of C584/622S produces a similar but more subtle phenotype relative to C414S. These data led me to hypothesize that it was the cysteine residues clustered in the WD-40 repeat regions of Met30 with the highest density of cysteine residues (WD-40 repeats 4 and 8, see Figure 7A) that are responsible for sulfur-sensing.

I then began mutating these cysteines as two separate groups. Strains containing the mutations C414/426/436/439S (data not shown) and C614/616/622/630S phenocopy the C414S single mutant with respect to Met4 ubiquitination status (Figure 9A). The mixed population of ubiquitinated and deubiquitinated Met4 in the C414S and C614/616/622/630S strains resulted in reduced induction of *SAM1* and *GSH1*, while *MET17* appears to be upregulated in the mutants but is largely insensitive to the changes in the sulfur status of the cell (Figure 9B). These mutants also exhibit slight growth phenotypes when cultured in both rich and −sulfur lactate media supplemented with homocysteine (Figure 9C).

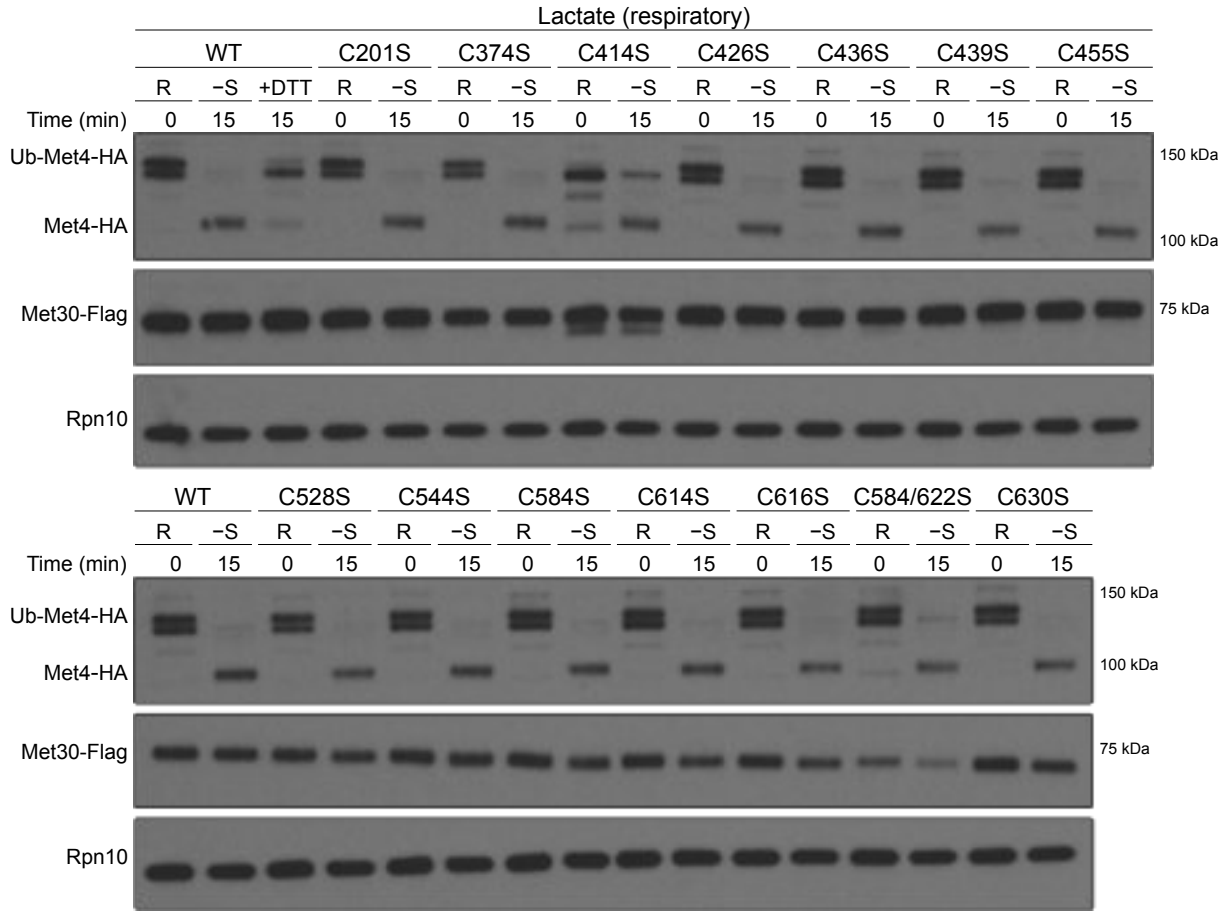

Figure 8: Met30 cysteine 414 plays a key role in sulfur sensing.

Met30 cysteines were mutated individually (C584/622S was accidental) and resulting yeast strains screened for sulfur-sensing defects. Data in this figure was produced by Benjamin Sutter and is presented with his permission.

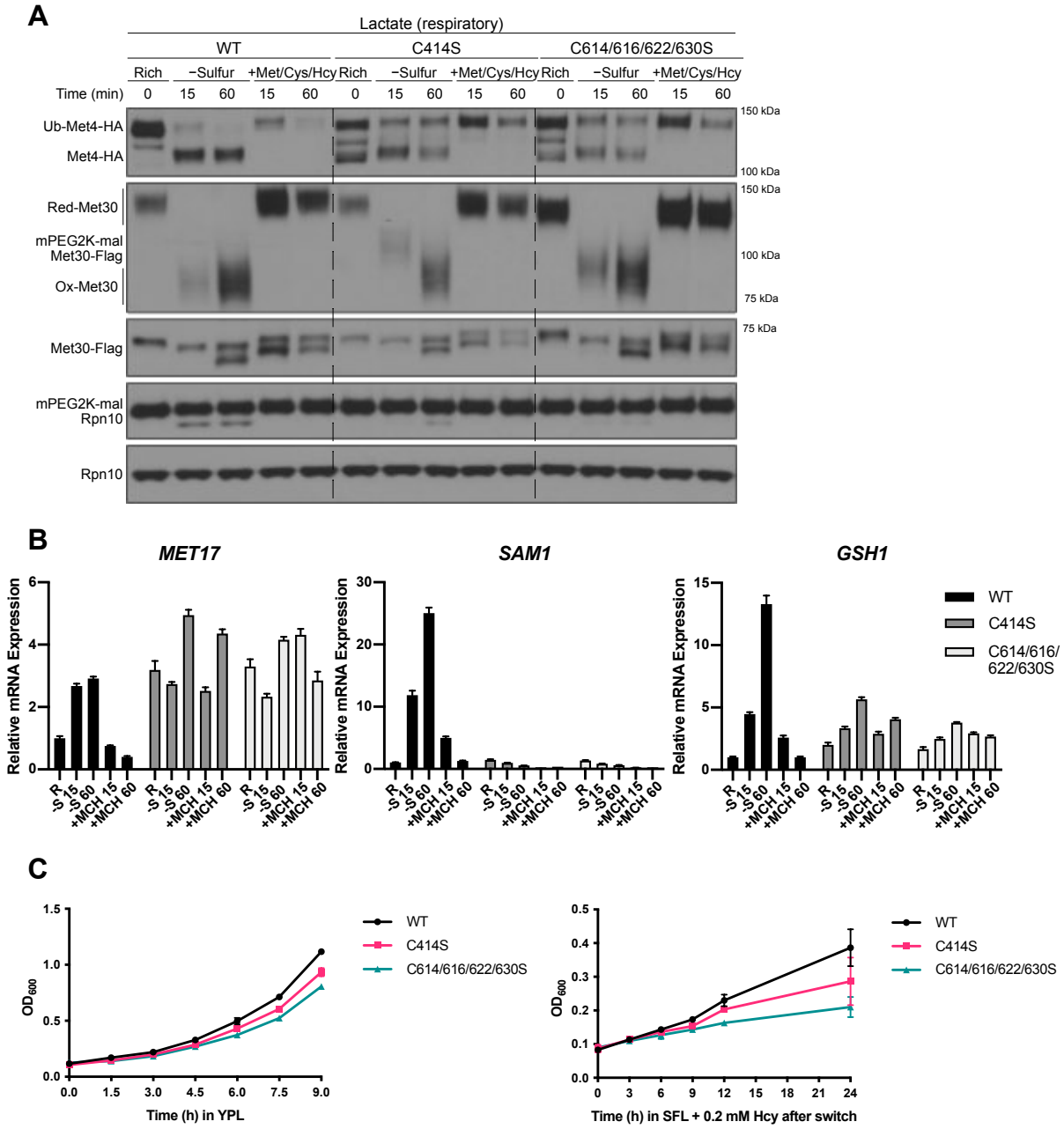

Figure 9: Met30 cysteine mutants are defective in regulating sulfur metabolism.

A) Western blot analysis of Met30 cysteine redox state and Met4 ubiquitination status in WT and two cysteine to serine mutants, C414S and C614/616/622/630S.
B) MET gene transcript levels over the same time course as (A) for the three strains, as assessed by qPCR. Data are presented as mean and SEM of technical triplicates.
C) Growth curves of the three yeast strains used in (A) and (B) in sulfur-rich YPL media or −sulfur SFL media supplemented with 0.2 mM homocysteine.

Met30's sulfur starvation and cadmium sensing mechanisms are not redundant

As cadmium detoxification depends on the consumption of GSH, and cadmium itself is thought to exert its toxic effects by sensitizing cells to oxidative stress, it is plausible that Met30 senses both through cysteine redox status (Li et al., 1997, Stohs et al., 2001). However, treatment of Met30 C414S and C614/616/622/630S strains did not result in the same constitutive Met4 ubiquitination phenotype that is seen when these strains are starved of sulfur (Figure 10). While this does not necessarily rule out the possibility that Met30 uses cysteine residues to sense the presence of cadmium ions, it is striking that there is no detectable oxidation of Met30 cysteine residues upon treatment with cadmium, particularly given the conventional understanding of cadmium toxicity (Figure 10, mPEG2K-mal Met30-Flag blot).

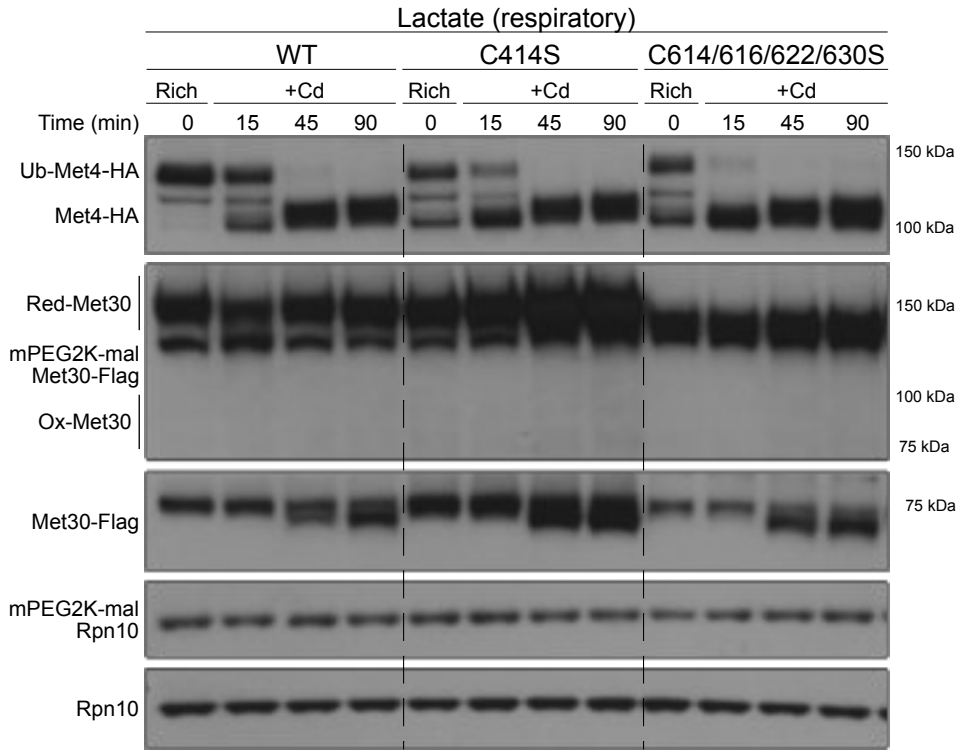

Figure 10: Met30 sulfur and cadmium-sensing mechanisms are not redundant.

Western blot analysis of Met30 cysteine redox state and Met4 ubiquitination status in WT and two cysteine to serine mutants, C414S and C614/616/622/630S, following treatment with 500 µM CdCl2.

MET30 IS A REDOX SENSOR: IN VITRO EVIDENCE

Reconstitution of Met4 ubiquitination by SCF^{Met30}

Having observed that Met30 cysteine redox status is correlated with Met4 ubiquitination status *in vivo*, I sought to determine whether the sulfur/redox-sensing ability of SCF^{Met30} E3 ligase activity could be reconstituted *in vitro*. Inspired by work on the phosphate-sensing kinase regulating the *PHO* regulon (Lee et al., 2007), I performed large scale immuno-purifications of $SCF^{Met30\text{-}Flag}$ to pull down Met30 and its interacting partners in both high and low sulfur conditions for use in *in vitro* ubiquitination assays with recombinantly purified E1, E2, and Met4 protein. Initial *in vitro* ubiquitination experiments showed little to no difference in activity between the high and low sulfur conditions, mirroring prior efforts to demonstrate differential activity of the Met30 E3 ligase in response to stimuli that effect its activity *in vivo* (Figure 11A) (Barbey et al., 2005).

Since the cysteine residues within Met30 became rapidly oxidized in sulfur-free media conditions, the addition of 1 mM DTT as a standard component in my IP and wash buffers could potentially reduce oxidized Met30 cysteines and alter its ubiquitination activity towards Met4. To test this possibility, I then performed the Met30 IP and *in vitro* assay in the complete absence of reducing agent from both high and low sulfur conditions. These data show little to no ubiquitination activity in either condition (Fig. 11B), suggesting that oxidized Met30 exhibits significantly reduced ubiquitination activity. Additionally, the absence of reducing agent in the *in vitro* reactions did not interfere with E1 and E2 thioester transfer of ubiquitin (data not shown).

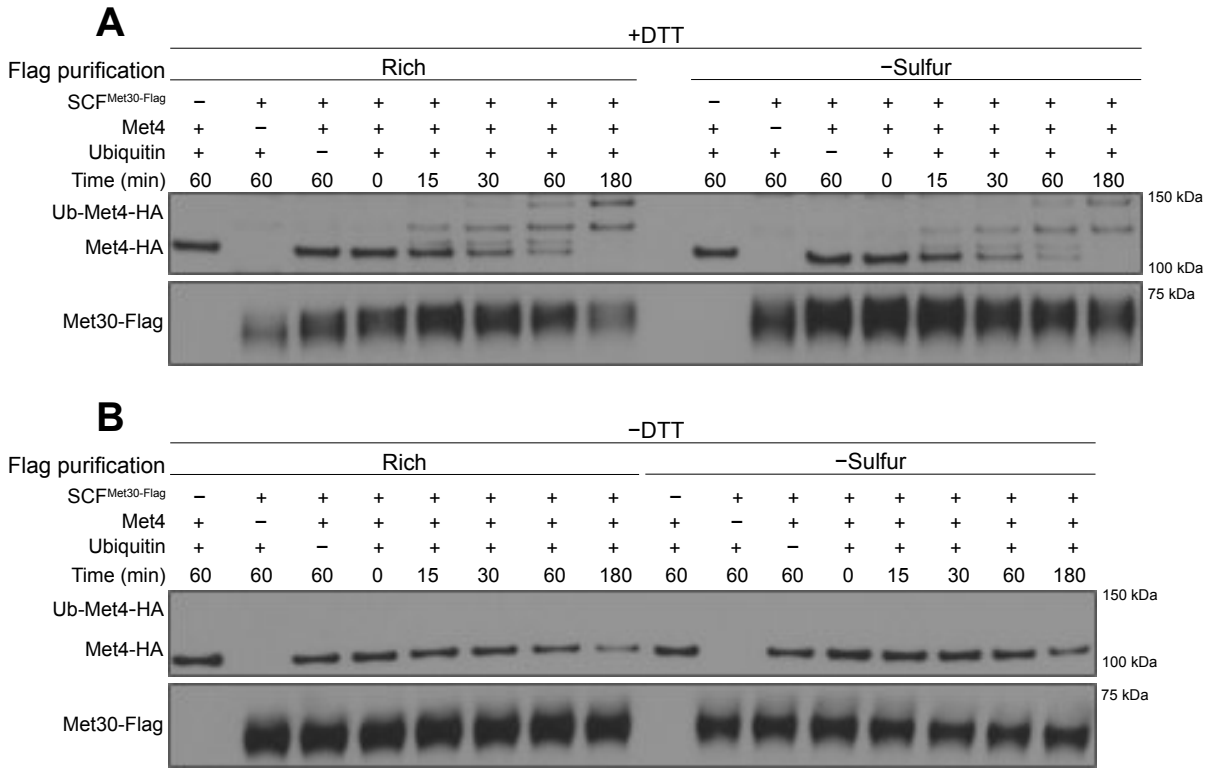

Figure 11: Met30 cysteine mutants are defective in regulating sulfur metabolism.

A) Initial IPs for $SCF^{Met30\text{-}Flag}$ complex were performed in the presence of 1 mM DTT prior to Flag peptide elution and concentration. No DTT was used in the *in vitro* ubiquitination assay itself, yet the E3 ligase activities for the E3 complex were indistinguishable between complex isolated from high sulfur versus low sulfur cells.
B) The same IP/*in vitro* assay as in (A), with the sole exception that DTT was not included during the IP and wash steps.

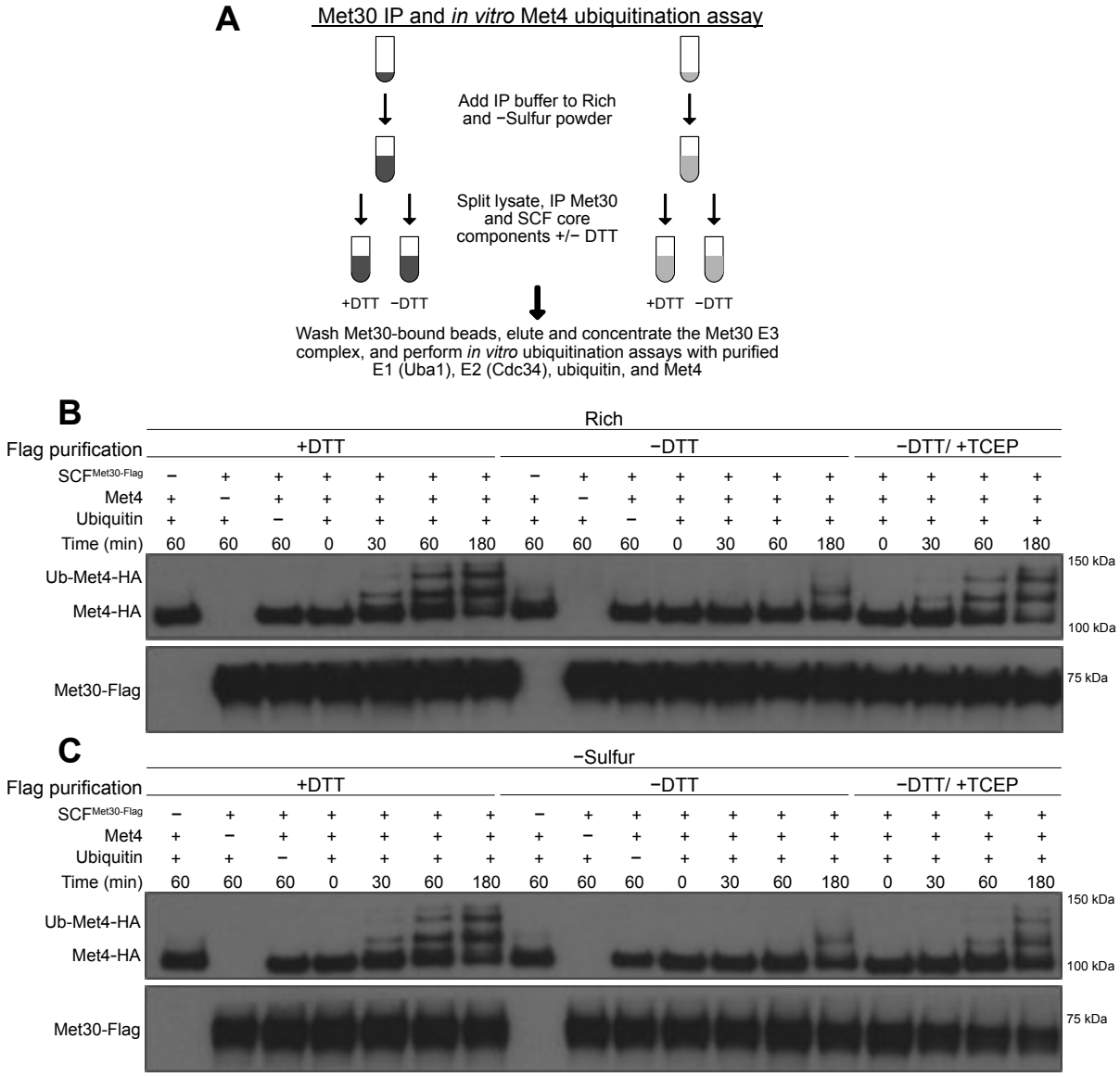

Figure 12: Met30 cysteine oxidation disrupts Met4 ubiquitination *in vitro* .

A) Schematic for the large-scale $SCF^{Met30\text{-}Flag}$ immunopurification from rich high sulfur and −sulfur conditions for use in *in vitro* ubiquitination assays with recombinant Met4 protein.
(B) Western blot analysis of Met4 *in vitro* ubiquitination by $SCF^{Met30\text{-}Flag}$ immunopurifications from cells cultured in sulfur-replete rich media. Cryomilled YPL yeast powder was divided evenly for two Flag IPs performed identically with the exception that one was done in the presence of 1 mM DTT (+DTT) and the other was performed without reducing agent present (−DTT). To test if the addition of reducing agent could rescue the activity of the "−DTT" IP, a small aliquot of the "−DTT" $SCF^{Met30\text{-}Flag}$ complex was transferred to a new tube and was treated briefly with 5 mM TCEP while the *in vitro* ubiquitination reaction was set up (−DTT/+TCEP).
(C) The same Western blot analysis of Met4 *in vitro* ubiquitination as in (B), except that the $SCF^{Met30\text{-}Flag}$ complex was produced from −sulfur cells.

To more rigorously test the effect of reducing agents on the activity of immunopurified SCF^{Met30}, I performed in parallel the Met30-Flag IP with cells grown in both high and low sulfur conditions, with and without reducing agent in the IP (Figure 12A). After performing the initial IP and washing the beads in buffer with and without reducing agent, the final wash step and Flag peptide elution were done without reducing agent in the buffer for all four IP conditions in order to remove any residual reducing agent from the final ubiquitination reaction, which was also performed without reducing agent. A small aliquot of the rich and −sulfur "−DTT" immunopurified SCF^{Met30} was transferred to a new tube and treated with 5 mM TCEP, a non-thiol, phosphine-based reducing agent, for approximately 30 min while the *in vitro* ubiquitination assays were set up to test if the low activity of the oxidized SCF^{Met30} complex could be rescued by treating with another reducing agent before addition to the final reaction. The data clearly demonstrate that the presence of reducing agent in the IP and wash buffer, but not in the elution or final reaction, significantly increased the E3 ligase activity of SCF^{Met30} *in vitro* regardless of whether the cells were grown in high (Figure 12B) or low sulfur media (Figure 12C). Further supporting my hypothesis, brief treatment of the oxidized −DTT IP complex with TCEP (−DTT/+TCEP) rescued the activity of the E3 complex *in vitro* (Figures 12B and C).

Silver stains of the eluted co-IP Met30 complexes showed similar levels of total protein overall and little apparent difference in the abundance of major binding partners between the four conditions (Figure 13A). Western blots of the co-IP samples for the Cdc53/cullin scaffold showed similar binding between the samples with the exception of the

−sulfur, −DTT sample which had approximately a third of the amount of Cdc53 bound to Met30 (Figure 13B). I would speculate that this difference is due to the canonical regulation of SCF E3 ligases, which uses cyclic changes in the affinity of Skp1/F-box protein heterodimers to the cullin scaffold based on binding between the F-box protein and its substrate (Reitsma et al., 2017).

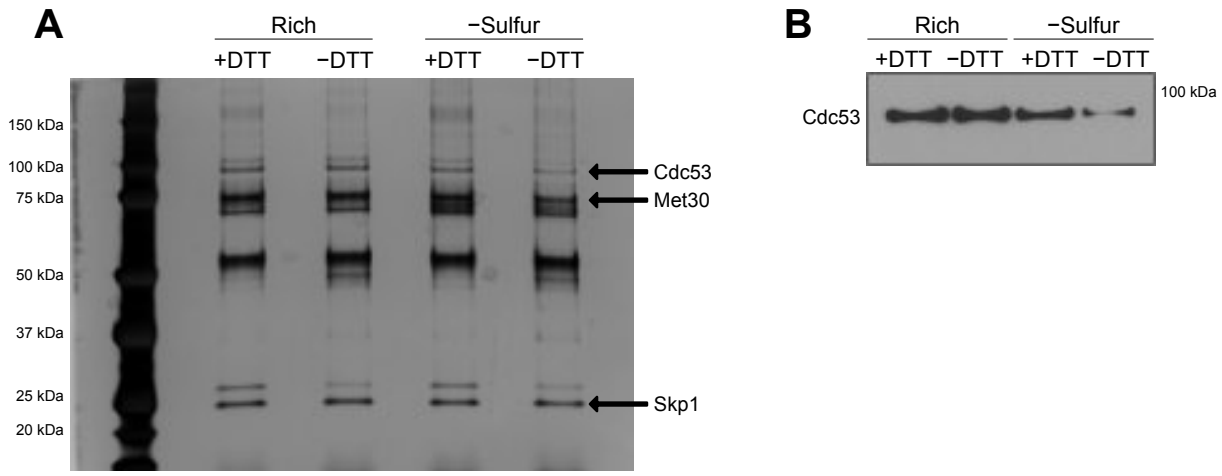

Figure 13: Met30 IP complexes are relatively stable across IP conditions.

A) Silver stains of immunopurified $SCF^{Met30\text{-}Flag}$ complex isolated from rich and −sulfur cells prepared in the presence or absence of DTT used in Figures 12B and C.
B) Western blot for Cdc53 from immunopurified $SCF^{Met30\text{-}Flag}$ complex shown in Figure 13A and used in Figures 12B and C.

The same +/ − DTT *in vitro* ubiquitination experiment described in Figure 10A was performed with the C414S and C614/616/622/630S Met30 mutants, and showed lower E3 ligase activity overall relative to wild type Met30, but smaller differences between the plus and minus reducing agent conditions (Figure 14). The increased abundance of the C414S mutant relative to the other two conditions likely reflects a difference in cryomill lysis efficiency, as equal amounts of powder were used for all three strains in the experiment.

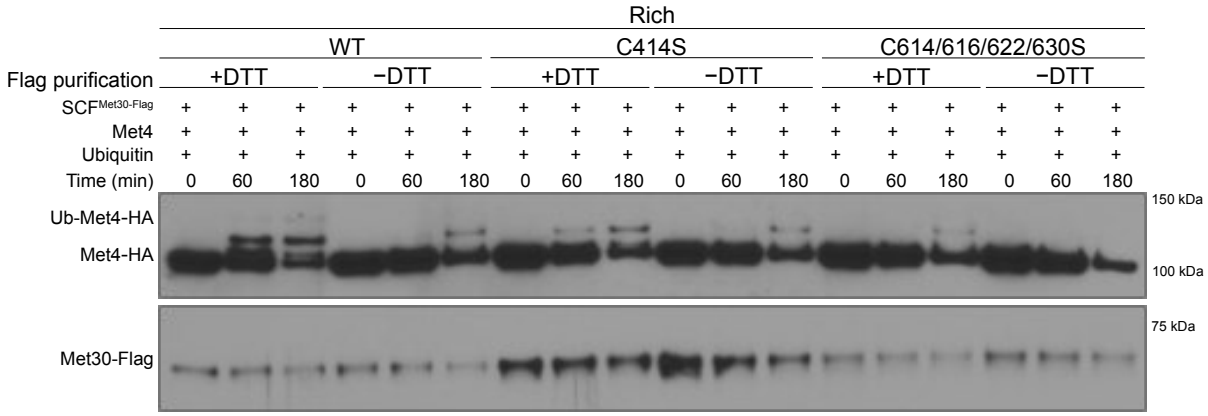

Figure 14: SCF^{Met30} cysteine mutants are less active but also less sensitive to reducing agents relative to wildtype SCF^{Met30}.

Western blots of *in vitro* ubiquitination assays were carried out as described in Figure 12A with cell lysate powder from WT, C414S, and C614/616/622/630S Met30 strains grown in rich media. The greater abundance of the C414S mutant is likely due to a difference in cryomill lysis efficiency, and is not a difference in the amount of starting material used.

Met30 cysteine oxidation disrupts binding to Met4 *in vitro*

As SCF^{Met30} E3 ligase activity *in vitro* is independent of the sulfur-replete or starved state of the cells from which the co-IP concentrate is produced, and that the activity of the SCF^{Met30} co-IP concentrate purified in the absence of reducing agent can be rescued by treatment with another reducing agent, I hypothesized that the low E3 ligase activity of SCF^{Met30} purified in the absence of reducing agent is due to decreased binding between Met30 and Met4, and not decreased binding between Met30 and the other core SCF components. This hypothesis is also supported by Met30 cysteine point mutant data that demonstrates that mutation of cysteine residues in the WD-40 repeat region of Met30 responsible for Met30's interaction with Met4 have the largest effect *in vivo* (Figures 7, 8, and 9).

To test this possibility, lysate for "rich" and "−sulfur" cells was prepared and each was split into three groups, with either reducing agent (+DTT), the thiol-specific oxidizing agent tetramethylazodicarboxamide (+Diamide), or control (−DTT) (Figure 15A). Met30-Flag IPs were performed as previously described for the *in vitro* ubiquitination assay, except instead of eluting Met30 off of the beads, the +DTT, −DTT, and +Diamide beads were each split into two tubes containing IP buffer ±DTT and bacterially purified Met4. The beads were incubated with purified Met4 prior to washing with IP buffer with or without DTT. The data demonstrate a clear, DTT-dependent increase in the fraction of Met4 bound to the Met30-Flag beads, with the "+DTT" Met30 IP showing a larger initial amount of bound Met4 compared to the "−DTT" Met30 IP, with even less Met4 bound to the "+Diamide" Met30-Flag beads (Figure 13B). Consistent with my hypothesis, the addition of DTT to the Met4 co-IP with "−DTT" or "+Diamide" Met30-Flag beads restored the Met30/Met4 interaction to the degree seen in the "+DTT" Met30-Flag beads (Figure 15B, lanes 3, 5, 9, and 11).

I then performed the same experiment with the Met30 cysteine point mutant strains. The amount of Met4 bound to these mutants was less sensitive to the presence or absence of reducing agent relative to wildtype, particularly in the "−DTT" or "+Diamide" IPs (Figure 16). Collectively, these data suggest that the reduced form of key cysteine residues in Met30 enables it to engage its Met4 substrate and facilitate ubiquitination.

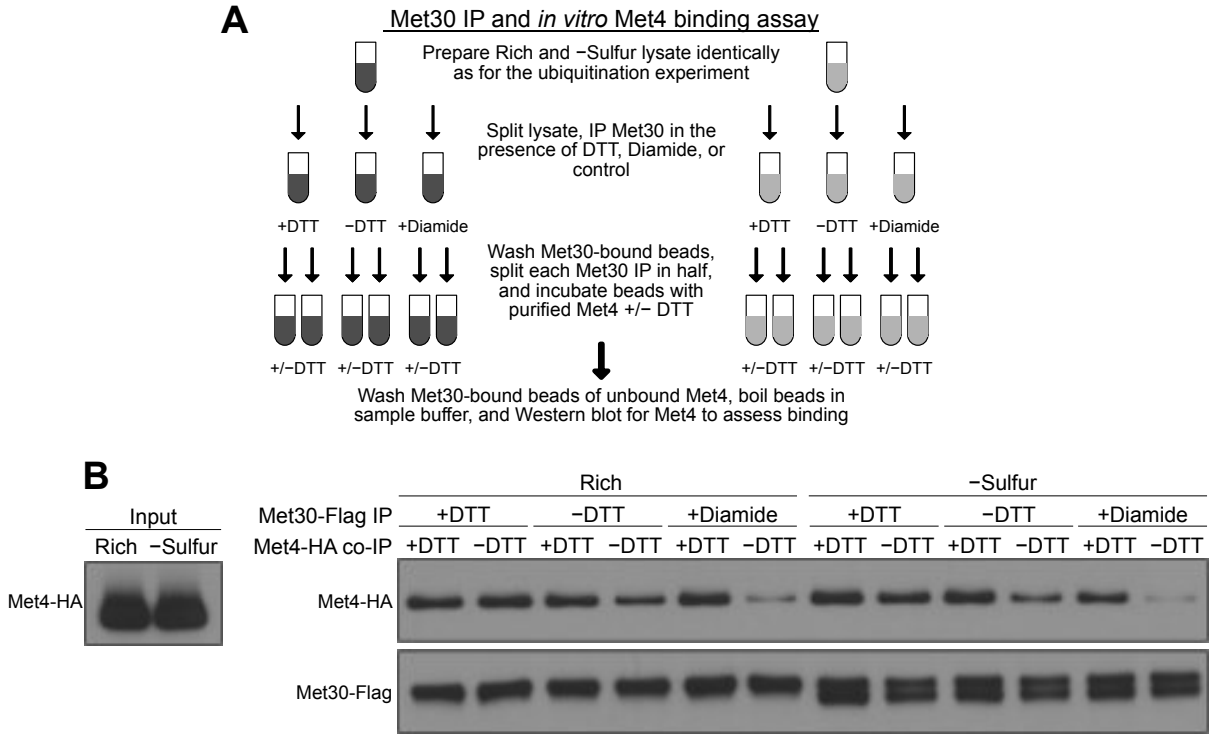

Figure 15: Binding to Met4 is regulated by Met30 cysteine redox state.

A) Schematic for the large-scale $SCF^{Met30\text{-}Flag}$ immunopurification from rich high sulfur and −sulfur conditions for use in *in vitro* binding assays with recombinant Met4 protein.
B) Western blot analysis of the Met4 binding assay illustrated in (A). Rich and −sulfur lysate were both split three ways, and lysate with 1 mM DTT (+DTT), 1 mM diamide (+Diamide), or control (−DTT) were incubated with anti-Flag magnetic beads to isolate Met30-Flag complex. The Met30-Flag bound beads from each condition were then split in half and distributed into tubes containing IP buffer ± 1 mM DTT and purified recombinant Met4. The mixture was allowed to incubate for 2 h before the beads were washed, boiled in sample buffer, and bound proteins were separated on SDS-PAGE gels and Western blots were performed for both Met30 and Met4.

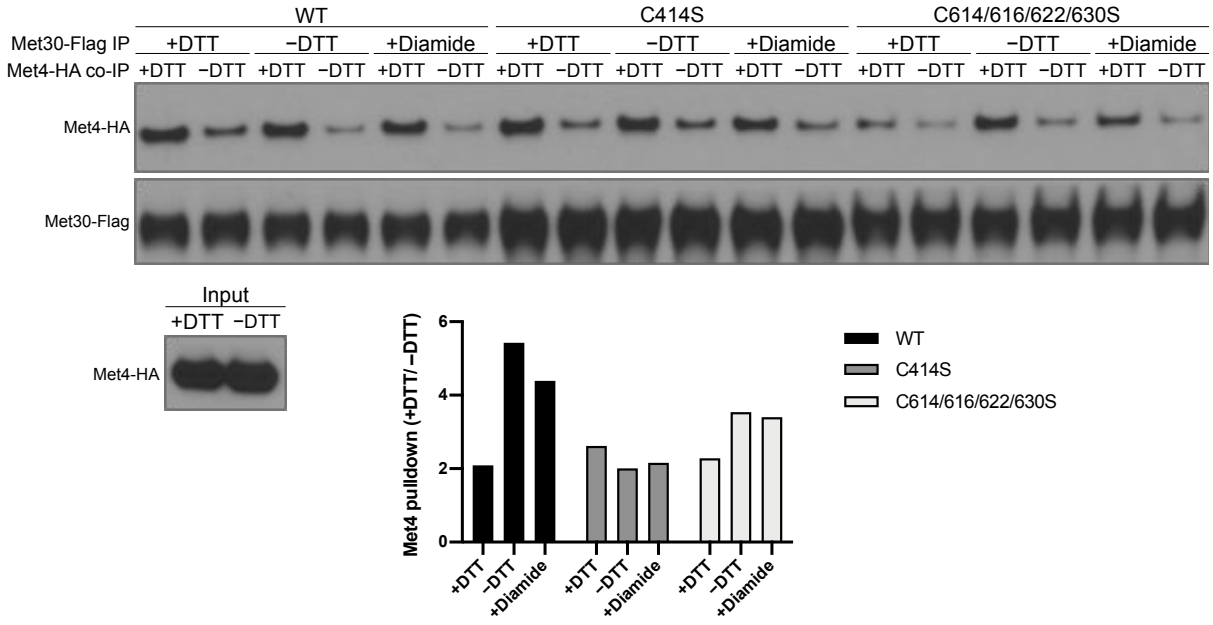

Figure 16: Met30 and Met4 binding is less sensitive to redox state in the Met30 cysteine mutants.

Met4 binding was assessed in WT and the C414S and C614/616/622/630S mutants as described in Figure 15A using cell lysate powder from cells grown in rich media. The fold change in Met4 binding in the presence and absence of DTT was quantified for each strain and for each Met30 immunopurification condition using ImageJ (version 1.53).

MET30 AND SULFIDE

Sulfide as signal of sulfur-sufficiency

A late discovery in my research involves the possibility that Met30 may utilize sulfide itself as a sensing mechanism. Of the small family of signal-transducing biological gases known as gasotransmitters, hydrogen sulfide may be the most peculiar. Mice exposed to 80 ppm hydrogen sulfide quickly enter a state of "suspended animation" that is freely

reversible upon return to normal atmospheric conditions, and results in no harm to the mouse (Blackstone et al., 2005). In fact, at non-toxic biologically-relevant levels, hydrogen sulfide has been shown to be cytoprotective in multiple tissues and cell types, and it's therapeutic potential is an active area of research (Szabó, 2007). The beneficial effect of sulfide does not seem to be limited to multicellular eukaryotes either, as the treatment of stationary-phase yeast with sodium sulfide has recently been demonstrated to increase their chronological lifespan (Shah et al., 2021).

As illustrated in Figure 6, the signal for sulfur-sufficiency for Met30 must stem from some part of the transsulfuration pathway, as neither methionine, SAM, nor SAH rescue Met30's E3 activity. Recent work on hydrogen sulfide production and signaling has shown the enzymes cystathionine beta-synthase (CBS), and to a greater extent cystathionine gamma-lyase (CSE), are responsible the generation of biologically relevant amounts of sulfide for the purpose of signal transduction (Singh et al., 2009). Directly testing the role of these enzymes with respect to Met30 is impossible, as both are essential in our prototrophic yeast strain background even when supplemented with cysteine. However, both sulfite reductase (*met10*) and homocysteine synthase (*met17*) knockout strains are viable, and permit the testing of Met30's ability to sense exogenous sodium sulfide when sulfate assimilation and sulfide incorporation into organic sulfur compounds are compromised. Interestingly, this strain responds like wildtype when rescued with 20 μM sodium sulfide as a sulfur source with respect to Met4 ubiquitination (Figure 17A). Additionally, this occurs while maintaining similar (cysteine and GSH) or lower (homocysteine and cystathionine) levels of

sulfur metabolites relative to wildtype (Figure 17B). While it is odd that some sulfur metabolites like methionine, SAM, and SAH increase upon rescue with sulfide in the *met10*Δ *met17*Δ, these metabolites have already been shown not to be the signal for sulfur sufficiency for Met30 (Figure 6). These data suggest that sulfide alone may be sufficient for Met30 to sense sulfur and ubiquitinate Met4.

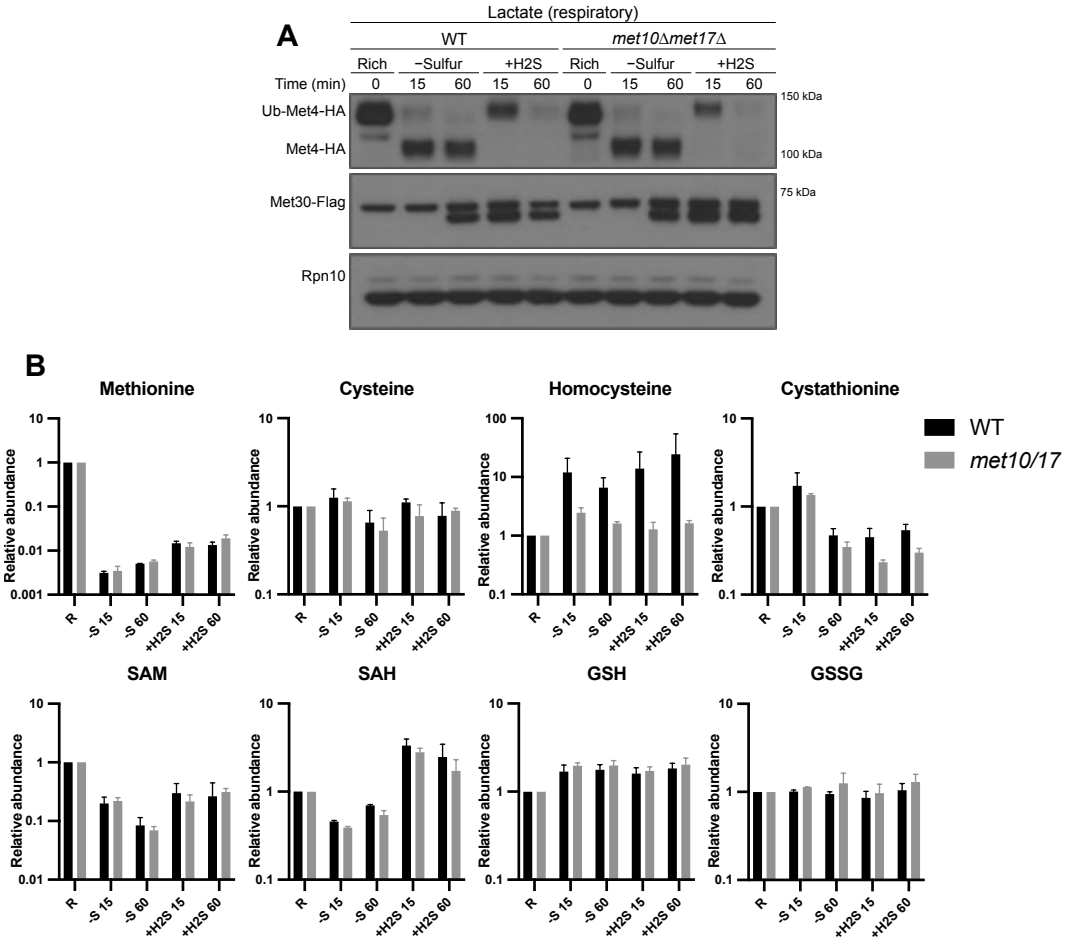

Figure 17: Met30 senses sulfide in a *met10*Δ*met17*Δ double mutant.

A) Western blot analysis of WT and *met10*Δ*met17*Δ strains after rescuing with 20 µM sodium sulfide. Note that the media was spiked with 5 mM tris pH 7.5 prior to sulfide addition to normalize the pH of the media.
B) Sulfur metabolite levels measured over the same time course as in (A).

CHAPTER FOUR

Discussion

MET30 CYSTEINE REDOX AS A PROXY FOR MEASURING SULFUR LEVELS

Significance

The unique redox chemistry and reactivity offered by sulfur and sulfur-containing metabolites renders many of the biochemical reactions required for life possible. The ability to carefully regulate the levels of these sulfur-containing metabolites is of critical importance to cells as evidenced by an exquisite sulfur-sparing response. In yeast, sulfur starvation induces the transcription of *MET* genes and specific isozymes, which themselves contain few methionine and cysteine residues (Fauchon et al., 2002). Furthermore, along with the dedicated cell cycle F-box protein Cdc4, Met30 is the only other essential F-box protein in yeast, thus linking sulfur metabolite levels to cell cycle progression (Su et al., 2005, Su et al., 2008). My findings further highlight the intimate relationship between sulfur metabolism and redox chemistry in cellular biology, revealing that the key sensor of sulfur metabolite levels in yeast, Met30, is regulated by reversible cysteine oxidation. Such oxidation of Met30 cysteines in turn influences the ubiquitination status and transcriptional activity of the master sulfur metabolism transcription factor Met4. While much work has been done to characterize the molecular basis of sulfur metabolic regulation in yeast between Met30 and Met4, this work describes the biochemical basis for sulfur-sensing by the Met30 E3 ligase (Figure 18).

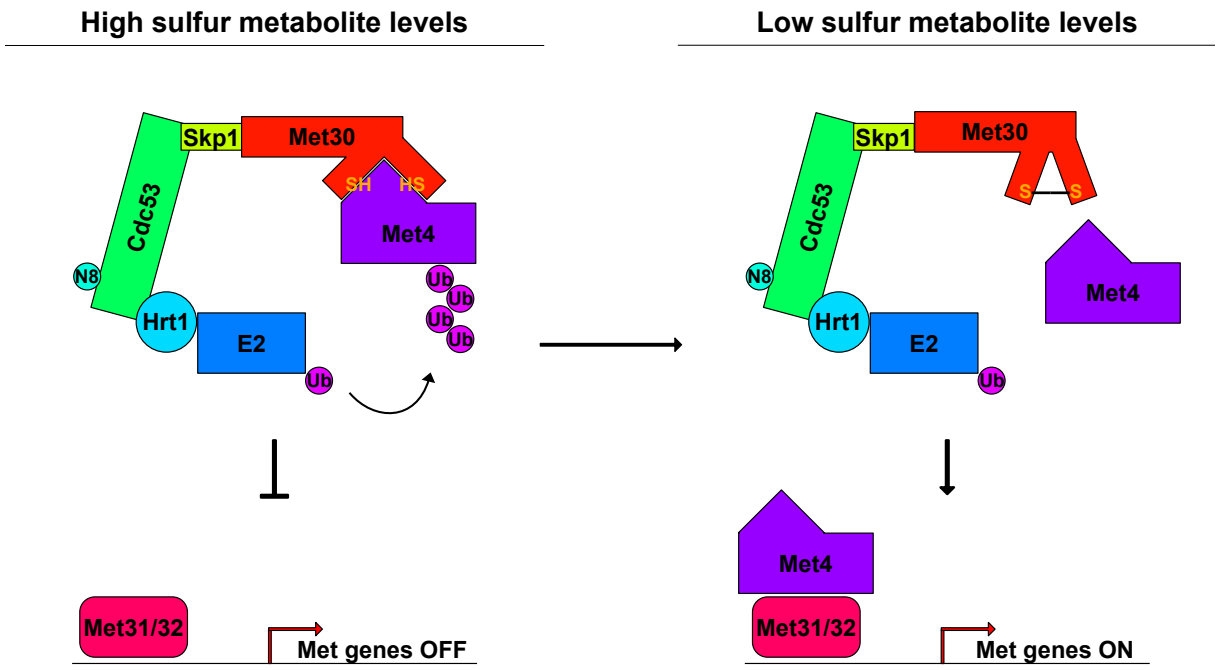

Figure 18: Model for *MET* gene regulation and sulfur sensing by the SCF^{Met30} E3 ligase.

In conditions of high sulfur metabolite levels, cysteine residues in the WD-40 repeat region of Met30 are reduced, allowing Met30 to bind and facilitate ubiquitination of Met4 in order to negatively regulate the transcriptional activation of the *MET* regulon. Upon sulfur starvation, Met30 cysteine residues become oxidized, resulting in conformational changes in Met30 that allow Met4 to be released from the SCF^{Met30} complex, deubiquitinated, and transcriptionally active.

Ubiquitin-dependent redox rheostats

The ability of Met30 to act as a cysteine redox-responsive E3 ligase is unique in *Saccharomyces cerevisiae*, but is reminiscent of the redox-responsive KEAP1 E3 ligase in humans. In humans, KEAP1 ubiquitinates and degrades its NRF2 substrate to regulate the cellular response to oxidative stress. When cells are exposed to electrophilic metabolites or oxidative stress, key cysteine residues are either alkylated or oxidized into disulfides, resulting in conformational changes that, in turn, either disrupt KEAP1 association with CUL3 or NRF2, both leading to NRF2 activation (Yamamoto et al., 2018). My data suggest that in response to sulfur starvation, Met30 can still maintain its association with the SCF E3 ligase cullin scaffold, but that treatment of the oxidized complex with reducing agent is sufficient to stimulate ubiquitination of Met4 *in vitro*. This, along with the *in vivo* and *in vitro* Met30 cysteine point mutant data, leads me to conclude that it is the ability of Met30 to bind its substrate Met4 that is being disrupted by cysteine oxidation.

Previous work on the yeast response to cadmium toxicity demonstrated that Met30 is stripped from SCF complexes by the p97/CDC48 segregase upon treatment with cadmium, suggesting that like KEAP1, Met30 can utilize both dissociation from SCF complexes and disrupted interaction with Met4 to modulate Met4 transcriptional activation (Barbey et al., 2005, Yen et al., 2012). Recent work on the sensing of oxidative stress by KEAP1 has found that multiple cysteines in KEAP1 can act cooperatively to form disulfides, and that the use of multiple cysteines to form different disulfide bridges creates an "elaborate fail-safe mechanism" to sense oxidative stress (Suzuki et al., 2019). In light of my findings, I suspect

Met30 might similarly use multiple cysteine residues in a cooperative disulfide formation mechanism to disrupt the binding interface between Met30 and Met4, but more work will be needed to demonstrate this definitively. It is worth noting the curious spacing and clustering of cysteine residues in Met30, with the highest density and closest spacing of cysteines found in two WD-40 repeats that are expected to be directly across from each other in the 3D structure (Figure 7A). That the mutation of these cysteine clusters to serine have the largest *in vivo* effect, but mutation of any one cysteine to serine (with the notable exception of Cys414) has no effect, implies some built-in redundancy in the cysteine-based redox-sensing mechanism (Figure 8). I speculate that the oxidation of the cysteines in the WD-40 repeat region of Met30 work cooperatively to produce structural changes that position Cys414 to make a key disulfide linkage that disrupts the interaction with Met4.

A similar but opposing system to KEAP1 in humans to handle "reductive stress" was recently described that utilizes precisely the opposite mechanism, using reduction of a key cysteine disulfide linkage in the E3 substrate protein FNIP1 as the biochemical mechanism regulating substrate binding and ubiquitination by the $CUL2^{FEM1B}$ E3 ligase (Manford et al., 2020). Degradation of reduced FNIP1 results in the activation of mitochondria and increases production of ROS, which in turn alleviates the reductive stress and restores redox homeostasis. I would be unsurprised if a similar system were to be found in yeast, especially considering that yeast are facultative anaerobes and must dispense of excess reducing equivalents generated from glucose oxidation via alcohol fermentation under typical growth conditions. In summary, it would appear that eukaryotic evolution has arrived at a consensus

on the use of ubiquitin-dependent redox rheostats, and Met30 seems to be no exception (Manford et al., 2020, Salahudeen et al., 2009, Wang et al., 2020, Wei and Kenyon, 2016, Yamamoto et al., 2018, Zhao et al., 2016).

Redox, sulfur, and anabolism

What makes Met30 and Met4 appear to stand out is that they regulate sulfur metabolism, and not a general response to redox stress. A peculiar finding made by Thomas and Surdin-Kerjan in the early 1990s was the discovery that the organic sulfur requirement of *met19*Δ mutants was a result of deletion of the gene encoding glucose-6-phosphate dehydrogenase (now referred to as *ZWF1*), the rate-limiting enzyme of the pentose phosphate pathway (PPP) (Thomas et al., 1991). It is widely understood that a major function of this pathway is to produce reducing equivalents in the form of NADPH, but why would this result in organic-sulfur auxotrophy? It does not seem to be a result of critically low NADPH levels preventing sulfate assimilation, as sulfide does not rescue growth of *zwf1*Δ cells (Thomas et al., 1991). Supplementation of wildtype or *met15*Δ cells with methionine increases both PPP enzyme and metabolite levels, and appears to directly affect resistance to oxidative stress via this pathway in a *ZWF1*-dependent manner (Campbell et al., 2016). A better understanding this phenotype would be an excellent avenue for future research on the connection between these metabolic pathways.

In addition to the PPP, sulfur metabolism is also tightly linked to folate/one carbon metabolism. The methyl group of methionine originates from 5-methyl tetrahydrofolate, an

important intermediate in one-carbon metabolism. More deeply, the link between these pathways can also be seen in the allosteric inhibition of methylenetetrahydrofolate reductase (MTHFR), the enzyme responsible for 5-methyl tetrahydrofolate synthesis, by SAM (Kutzbach and Stokstad, 1967). The importance of this is illustrated by recent research demonstrating this feedback inhibition is required for the proper maintenance of nucleotide levels – the other major function of one carbon metabolism (Bhatia et al., 2020). Combining the metabolic cost of sulfate assimilation and maintenance of the folate cycle makes methionine (and by extension cysteine) biosynthesis one of the largest sinks of NADPH in the cell (Walvekar and Laxman, 2019).

The interlinkage of these pathways makes sulfur metabolites a good proxy for overall reductive biosynthetic capacity. The PPP provides ribose sugars and NADPH, one carbon metabolism supports nucleotide biosynthesis, and sulfur metabolism signals translational capacity while also producing SAM (a prerequisite for most methylation reactions and polyamine biosynthesis) and GSH (the quintessential cellular redox buffer). Stated another way, sulfur metabolites biochemically link different indicators of anabolic capacity. Simply adding methionine to nutrient-starved yeast cells triggers the synthesis of all other amino acids and nucleotides, inhibits autophagy, and promotes growth (Sutter et al., 2013, Walvekar et al., 2018, Wu and Tu, 2011). In retrospect, it seems fitting that methionine was first discovered as a pro-growth cue (Mueller, 1923).

Concluding remarks

The utilization of reactive cysteine residues in Met30 to sense sulfur levels draws interesting comparisons to the regulation of Met4 via ubiquitination in that both mechanisms are rapid and readily reversible, require no new RNA or protein synthesis, and there is no requirement for the consumption of sulfur equivalents so as to spare them for use in *MET* gene translation under conditions of sulfur scarcity. It is also striking that while Met30 contains many cysteine residues, Met4 contains none – which has the consequence that as Met30 cysteines are oxidized, there is no possibility that Met4 can make an intermolecular disulfide linkage that might interfere with its release and recruitment to the promoters of *MET* genes. Upon repletion of sulfur metabolites, cellular reducing capacity is restored, and Met30 cysteine reduction couples the regulation of *MET* gene activation to sulfur assimilation, both of which require significant reducing equivalents.

Lastly, it should be noted that there is considerable overlap between the redox and sulfide hypotheses. When dissolved in solution, sulfide exists in the form of the bisulfide anion (HS^-), which itself is nucleophilic and reductive in nature. It is conceivable that Met30 may actually sense the flux through the transsulfuration pathway, using H_2S generated from cystathionine beta-synthase (*CYS4*) and cystathionine gamma-lyase (*CYS3*) — as well as H_2S generated from sulfite reductase (*MET5* and *MET10*) during sulfate assimilation — to quickly gauge the levels and activity of these enzymes. Once sulfur metabolism enzyme levels are sufficiently high, and flux through the sulfur metabolic pathway is sufficiently great, the transcriptional program that boosts the levels of those enzymes can be shut down without requiring a true end-product inhibition feedback loop. Reduction of an internal

disulfide or persulfidation of a key cysteine residue by reaction with sulfide, while not mutually exclusive mechanisms, may both be capable of producing structural changes in Met30's WD-40 repeat region that permit binding and ubiquitination of Met4. Future structural characterization of SCF^{Met30} in its reduced, oxidized, or potentially persulfidated states will likely be necessary for a more complete understanding of Met30's sulfur sensing mechanism. Nonetheless, along with SoxR and OxyR transcription factors in E. coli (Imlay, 2013) the Yap1 transcription factor in yeast (Herrero et al., 2008), and KEAP1 in mammalian cells (Yamamoto et al., 2018), our studies add the F-box protein Met30 to the exclusive list of bona fide cellular redox sensors that can initiate a transcriptional response. While it is known that sulfide gas acts on potassium channels and can interact with certain metalloproteins, as well as signal through other mechanisms, a true sulfide sensor has never been reported in the literature (Murphy et al., 2019). It is an exciting possibility that Met30 may be the first one, but more work will be necessary to demonstrate this definitively.

APPENDIX A

Yeast strains and media composition

Strains used in this study.

BACKGROUND	GENOTYPE	SOURCE
CEN.PK	MATa	(van Dijken et al., 2000)
CEN.PK	MATα	(van Dijken et al., 2000)
CEN.PK	MATa; MET30-FLAG::KanMX	This study
CEN.PK	MATa; MET30-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; MET30-FLAG::KanMX MET4-HA::Hyg met6Δ::Nat	This study
CEN.PK	MATa; MET30-FLAG::KanMX MET4-HA::Hyg sah1Δ::Nat	This study
CEN.PK	MATa; MET30-FLAG::KanMX MET4-HA::Hyg sam1Δ::Nat sam2Δ::Phleo	This study
CEN.PK	MATa; MET30-FLAG::KanMX MET4-HA::Hyg str3Δ::Nat	This study
CEN.PK	MATa; met30::MET30-C414S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C614/616/622/630S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30Δ::Phleo HO::MET30-FLAG::Nat MET4-HA::Hyg	This study
CEN.PK	MATa; met30Δ::Phleo HO::MET30Δaa1-20-FLAG::Nat Met4-HA::Hyg	This study
CEN.PK	MATa; met30Δ::Phleo HO::MET30-M30/35/36A-FLAG::Nat Met4-HA::Hyg	This study
CEN.PK	MATa; MET30-FLAG::KanMX MET4-HA::Hyg pdr5Δ::Nat	This study
CEN.PK	MATa; met4Δ::KanMX MET30-FLAG::Hyg	This study
CEN.PK	MATa; cup1p-6xHis-TEV-UBA1::Hyg	This study

CEN.PK	MATa; met30::MET30-C201S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C374S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C426S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C436S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C439S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C455S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C528S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C544S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C584S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C614S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C616S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C584/622S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; met30::MET30-C630S-FLAG::KanMX MET4-HA::Hyg	This study
CEN.PK	MATa; MET30-FLAG::KanMX MET4-HA::Hyg met17Δ::Nat met10Δ::Phleo	This study

Recipe for sulfur-free media.

salts (g L^{-1})	
$CaCl_2•2H_2O$	0.1
NaCl	0.1
$MgCl_2•6H_2O$	0.412
NH_4Cl	4.05
KH_2PO_4	1
metals (mg L^{-1})	
boric acid	0.5
$CuCl_2•2H_2O$	0.0273
KI	0.1
$FeCl_3•6H_2O$	0.2
$MnCl_2•4H_2O$	0.4684
$Na_2MoO_4•2H_2O$	0.2
$ZnCl_2•H_2O$	0.1895
vitamins (mg L^{-1})	
biotin	0.002
calcium pantothenate	0.4
folic acid	0.002
inositol	2

niacin	0.4
4-aminobenzoic acid	0.2
pyridoxine HCl	0.4
riboflavin	0.2
thiamine-HCl	0.4

ADDENDUM

A note on TCA

Near the end of doctoral studies, after generating all of the maleimide-PEG western blots to assess Met30 cysteine redox status *in vivo*, our lab ran out of the trichloroacetic acid (TCA) from Alfa Aesar that we had been ordering and reliably using for years, and were forced to order from another vendor (Sigma) while the Alfa Aesar TCA was on backorder. In the process of conducting control experiments for reviewers, I found I could no longer see changes in the redox status of Met30's cysteine residues, as they appeared constitutively reduced over the sulfur starvation time course. After two months of experimenting I was able to trace the cause back to the new TCA we had purchased. I then tested nearly a dozen different TCAs from different vendors, different catalogue numbers from the same vendor, and different lots from the same catalogue number. I found that the majority of TCAs did not show Met30 to be oxidized during sulfur starvation, and only one or two that did. This was also variable between different lots of TCA under the same catalogue number and vendor. These companies refused to disclose the suppliers for the different catalogues and lots of TCA that I tested, so I was unable to trace the issue back to a particular manufacturer. At this time I still do not know whether it is the minority or the majority of these TCAs that are "contaminated", so caution should be used when interpreting these results or conducting similar experiments in the future.

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