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UNIVERSITY OF CALIFORNIA

Los Angeles

Identification of Methylated Mitochondrial
Proteins in *Saccharomyces cerevisiae*

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Biochemistry and Molecular Biology

by

Katelyn Elizabeth Caslavka Zempel

2017

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2017

ABSTRACT OF THE DISSERTATION

Identification of Methylated Mitochondrial

Proteins in *Saccharomyces cerevisiae*

by

Katelyn Elizabeth Caslavka Zempel

Doctor of Philosophy in Biochemistry and Molecular Biology

University of California, Los Angeles, 2017

Professor Steven G. Clarke, Chair

Protein methylation has recently emerged as an abundant and important posttranslational modification, affecting many cellular functions including transcription and translation. The budding yeast *Saccharomyces cerevisiae* has become an important model organism for studying methylation reactions and the enzymes responsible for catalyzing the reaction (methyltransferases). Generally, either unfractionated lysates from cells grown under fermentation or specific protein substrates of well-characterized methyltransferases have been investigated for protein methylation. As a result of this, the methylation state of proteins inside the mitochondria remains largely unknown. The goal of this dissertation was two-fold: 1.) determine the experimental requirements for thoroughly identifying the methylation state of proteins in yeast mitochondria and 2.) identify methylated proteins in mitochondria that may have been previously overlooked.

Only one example in the scientific literature has investigated the global level of protein methylation in the mitochondria of any organism and only two proteins in the yeast mitochondria

are known to be methylated. I set out to determine mitochondrial protein methylation by detecting differences between wild type and methyltransferase gene deletion yeast strains using a combination of commonly used biochemical and molecular biology assays. When I found that these methods were ill-suited for identification of mitochondrial protein methylation, I turned to mass spectrometry approaches.

From my initial mass spectrometric analyses of proteolytic digests of yeast proteins, I learned that it was not sufficient to simply rely on computer algorithms to identify novel methylated peptides. It is important to use isotopic labeling to validate the mass shifts that occur as a result of methylation on identified peptides. This led me to use to adapt heavy methyl stable isotope labeling with amino acids in cell culture (SILAC) for use in yeast with my identification of a strain better suited for these experiments. From this approach, I have been able to curate a list of proteins comprising the mitochondrial methyl proteome. Using this method, I have also compared the cytosolic methyl proteome between yeast cells grown under fermentative or respiratory conditions.

I conclude with future directions for the work included in this dissertation. The functional implications of the mitochondrial methylation reactions have yet to be discovered and the methyltransferases responsible remain to be identified.

This dissertation contains three supplemental Excel spreadsheets. These materials are available on ProQuest.com. The first supplemental Excel table is an expanded version of Table 2-1 with a list of the known and putative methyltransferases in yeast, their confirmed and predicted substrate type, the substrate and residue methylated if known, and descriptions of the methyltransferase. The second Excel table contains data from nine separate mass spectrometry experiments, attempting to identify the methylation state of mitochondrial ribosomal proteins

from wild type and methyltransferase gene deletion strains, including peptides identified and descriptions. The third supplemental Excel table contains the peptides identified as methylated in cytosol isolated from fermenting and respiring yeast cells.

The dissertation of Katelyn Elizabeth Caslavka Zempel is approved.

Catherine F. Clarke

James A. Wohlschlegel

Steven G. Clarke, Committee Chair

University of California, Los Angeles

2017

This work is dedicated to my best friend and partner, Christian Zempel.

Thank you for tirelessly supporting my dreams no matter what.

You supported me through long distance and long hours.

Even though you knew nothing about mitochondria, you listened to countless ideas, problems, presentations, and proposals. I could not have done this without you.

I am looking forward to what this new chapter of life will bring us.

To my parents, Steve and Lisa Caslavka, thank you for valuing my education and making the sacrifices you did to give me the best opportunities possible.

Thanks to you, I became the life-long learner I am today.

To my sister, Chelsea Caslavka, thank you for making sure my life is not all serious with your humor and energy. Even though you are the little sister, I look up to and admire you.

To my teachers and advisors, thank you for showing me this path, challenging me to reach my full potential, and encouraging me along the way.

TABLE OF CONTENTS

Abstract of the Dissertation	ii
Committee Page	v
Dedication Page	vi
Table of Contents	vii
List of Figures	ix
List of Tables	xi
List of Supplementary Materials.....	xiii
Commonly Used Abbreviations.....	xiv
Acknowledgements.....	xv
Vita.....	xviii
CHAPTER 1 Introduction to the Mitochondrial Methyl Proteome and Plan of the Dissertation	1
References.....	12
CHAPTER 2 Characterizing Potential Mitochondrial Methylation Reactions in <i>Saccharomyces cerevisiae</i>	20
References.....	50
CHAPTER 3 Preliminary Characterization of Putative Mitochondrial Protein Methyltransferases	56
References.....	76
CHAPTER 4 Determining the Mitochondrial Methyl Proteome in <i>Saccharomyces cerevisiae</i> using Heavy Methyl SILAC	82
References for Introduction to the Chapter.....	84

References.....	98
CHAPTER 5 Physiological Implications of Mitochondrial Protein Methylation	102
References.....	123
CHAPTER 6 Comparing Methylated Proteins in <i>Saccharomyces cerevisiae</i> under	
Fermentative and Respiratory Growth Conditions	127
References.....	149
CHAPTER 7 Future Goals and Perspectives.....	152
References.....	160
APPENDICES	
Appendix 1 Yeast Mitochondrial Ribosomal Proteins (MRPs) and their N-terminal	
Mitochondrial Presequences	166
Appendix 2 Representative fragmentation patterns, isotope distribution, and peak areas for	
heavy and light peptides identified as methylated under fermentation and	
respiration	171
References.....	229

LIST OF FIGURES

2-1	Immunoblot of B4741 mitochondrial purification step	37
2-2	Mitochondrial lysates purified from <i>in vivo</i> radiolabeled wild type and methyltransferase gene deletion strains	39
2-3	<i>In organello</i> methylation reactions	41
2-4	Mitochondrial ribosomal proteins containing dimethylated lysine residues isolated from wild type and methyltransferase gene deletion yeast strains	42
2-5	<i>In vitro</i> methylation reactions with whole cell lysates and mitochondrial lysates of putative and known methyltransferase gene deletion strains.....	44
3-1	Example of a spot test assay for growth of <i>Saccharomyces cerevisiae</i>	67
3-2	Growth of null mutants of putative mitochondrial methyltransferases in <i>Saccharomyces cerevisiae</i> in semisynthetic lactate liquid media.....	68
3-3	Mitochondrial localization of TAP-tagged Yk1162c in <i>Saccharomyces cerevisiae</i>	69
4-1	Improved enrichment of the deuterated methionine label into proteins of <i>S. cerevisiae</i> strains with a deletion in the MET6 gene encoding the cobalamin-independent methionine synthase.....	89
4-2	Examples of good and poor methylated peptide identifications in MS1 ion chromatograms.....	90
4-3	Overlap of methylated proteins between cytoplasmic ribosomal proteins, mitochondrial ribosomal proteins, and mitochondrial protein fractions	90
4-4	Confirmation of known methylation sites on EF1A.....	92
4-5	Novel methylated peptides found in mitochondrial ribosomal protein fraction	93
4-6	Novel methylation of Ssa2/4 in all three protein fractions	95

4-7	Sequence similarity between identified methylated residues	95
4-8	Overlap of methylated proteins identified from the literature and curated from UniProt.....	96
5-1	Determination of successful tandem affinity purification of methylated protein candidates.....	113
5-2	Immunoblotting of tandem affinity purification fractions for methyl lysine.....	115
5-3	Gene Ontology term fold enrichment	117
5-4	Structural positioning of methyl groups within the context of the mitoribosome	118
5-5	Overlap of yeast mitochondrially localized methylated proteins with human mitochondrially localized methylated proteins	120
5-6	Alignment of hPDHB to scPDB1	121
6-1	Rpl23a trimethyllysine 106 fragmentation	139
6-2	Comparison of identified methylated proteins identified under respiration and fermentation conditions	141

LIST OF TABLES

2-1	Known and putative methyltransferases in <i>Saccharomyces cerevisiae</i> with predicted substrates and confirmed substrate as well as the MitoPROT probability	45
3-1	Protein BLAST of mitochondrially localized putative methyltransferases	71
3-2	Known respiration and stress associated phenotypes of putative and known methyltransferases with mitochondrial substrates	72
3-3	Experimentally determined growth phenotypes of null mutants of known and putative methyltransferases.....	74
4-1	Validating Methods for Cytoplasmic Ribosomal Fraction.....	91
4-2	Methylated Sites Found in the Mitochondrial Ribosomal Fraction	94
4-3	Methylated Peptides Found in the Mitochondrial Fraction	94
5-1	Common methylated mitochondrial proteins in humans and yeast.....	122
6-1	Methylated peptides identified in cytosols isolated from fermenting yeast cells that were found in both biological replicates.....	142
6-2	Methylated peptides identified in cytosols isolated from fermenting yeast cells that were found in only one biological replicate	143
6-3	Methylated peptides identified in cytosols isolated from respiring yeast cells that were found in both biological replicates.....	144
6-4	Methylated peptides identified in cytosols isolated from respiring yeast cells that were found in only one biological replicate	145
6-5	Proteins identified as methylated under fermentation and not respiration	146
6-6	Proteins identified as methylated under respiration and not fermentation	147

6-7	Novel methylated proteins identified in cytosols isolated from both respiring and fermenting yeast cells	148
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LIST OF SUPPLEMENTARY MATERIALS

- Supplemental Table 1 Expanded version of Table 2-1: Known and putative (in italics and bold) methyltransferases in *Saccharomyces cerevisiae* with predicted substrates and confirmed substrates
- Supplemental Table 2 Raw data from mass spectrometry analysis of mitochondrial ribosomal proteins isolated from wild type and methyltransferase gene deletion yeast strains
- Supplemental Table 3 Raw data from mass spectrometry analysis of cytosols isolated from fermenting and respiring yeast cells

These materials are available on ProQuest.com.

COMMONLY USED ABBREVIATIONS IN THIS DISSERTATION

[³ H]AdoMet	<i>S</i> - adenosyl-L-[<i>methyl</i> - ³ H]methionine
AdoMet	<i>S</i> - adenosyl-L-methionine
mmK	ϵ - <i>N</i> -monomethyllysine
mmR	ω - <i>N</i> -monomethylarginine
dmK	ϵ - <i>N</i> -dimethyllysine
dmR	ω - <i>N</i> -dimethylarginine
tmK	ϵ - <i>N</i> -trimethyllysine
mtDNA	mitochondrial DNA
LM	semisynthetic lactate medium
MRP	mitochondrial ribosomal protein
OD	optical density
ORF	open reading frame
PTM	posttranslational modification
SILAC	stable isotope labeling with amino acids in cell culture
TAP	tandem affinity purification
TCA	trichloroacetic acid
WT	wild type
YPD	yeast extract, peptone, dextrose medium
YPEG	yeast extract, peptone, ethanol, glycerol medium
YPG	yeast extract, peptone, glycerol medium

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antibodies in this dissertation were generous gifts from Dr. Catherine Clarke and Dr. Joanna Goldberg (Emory University School of Medicine). The TAP tagged yeast strains used in Chapter Five were a gift from Dr. Michael Carey. Dr. Yong Xue from Dr. Siavash Kurdistani's laboratory helped me in streaking out the TAP tagged strains.

Table 2-1 from Chapter Two was made with the help of Dr. Maria Dzialo and a version of it appeared in her 2015 dissertation. Some of the growth assays performed in Chapter Three were done with the help of my rotation student at that time, Agape Awad, now a graduate student with Dr. Catherine Clarke at UCLA.

The work presented in Chapter Four was the result of my fruitful collaboration with Dr. James Wohlschlegel's laboratory (Department of Biological Chemistry, UCLA). His support, along with Dr. Ajay Vashisht and William Barshop from his laboratory, has made the mass spectrometry work possible. The Wohlschlegel laboratory performed all the mass spectrometry work in the chapter while I purified the samples and analyzed the data. Figure 1 was made by the Wohlschlegel laboratory and methods related to the mass spectrometry analysis were written by Dr. Wohlschlegel, Dr. Vashisht, and William Barshop. I would like to thank Dr. Wohlschlegel, Dr. Vashisht, William Barshop, and Dr. Clarke as my co-authors on the culmination of this collaborative work in the journal article Caslavka Zempel, K. E.; Vashisht, A. A.; Barshop, W. D.; Wohlschlegel, J. A.; Clarke, S. G. Determining the Mitochondrial Methyl Proteome in *Saccharomyces cerevisiae* using Heavy Methyl SILAC. *J. Proteome Res.* **2016**, *15* (12), 4436–4451 (doi:10.1021/acs.jproteome.6b00521) reprinted in Chapter Four with permission.

Chapter Six is also a result of my collaboration with the Wohlschlegel laboratory. As with Chapter Four, the mass spectrometry and initial filtering of the data were performed by the

laboratory. I prepared the samples for analysis and analyzed the data. They also wrote the methods pertaining to the mass spectrometry and data filtering.

Finally, I would like to acknowledge my dissertation committee members: Dr. Catherine Clarke, Dr. Joseph Loo, Dr. Alexander van der Blik, and Dr. James Wohlschlegel. The work presented in this dissertation was supported by UCLA's Cellular and Molecular Biology Training Grant (Ruth L. Kirschstein National Research Service Award GM007185).

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PUBLICATIONS

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PRESENTATIONS

Caslavka K.E., Vashisht A., Barshop W., Wohlschlegel J.A., Clarke S.G. *Advanced Mass Spectrometry Technologies to Detect Novel Methylated Proteins in Yeast Mitochondria.* **2015.** Poster presented at the UCLA Molecular Biology Institute Retreat, Los Angeles, CA.

Caslavka K.E., Vashisht A., Barshop W., Wohlschlegel J.A., Clarke S.G. *Methylation of Mitochondrial Ribosomal Proteins in Budding Yeast.* **2014.** Poster presented at the UCLA Molecular Biology Institute Retreat, Los Angeles, CA.

Caslavka K.E., Wohlschlegel J.A., Clarke S.G. *Methylation of mitochondrial ribosomal proteins in *Saccharomyces cerevisiae*.* **2013.** Poster presented at the Cell Biology of Yeasts Conference, Cold Spring Harbor, NY.

Caslavka K.E., Clarke S.G. *Methylation of yeast mitochondrial ribosomal proteins.* **2013.** Poster presented at the USC, Caltech, UCLA Tri-Campus NIGMS Training Program Symposium, Los Angeles, CA.

CHAPTER ONE

Introduction to the Mitochondrial Methyl Proteome and Plan of the Dissertation

Organisms rely on proteomic diversity. The estimated number of protein coding genes does not correspond to the proteome. Proteomes can be two to three orders of magnitude more complex than the predicted proteomes based on protein-coding genes¹. Diversification can occur after transcription and after translation. Extending the range of proteins on the transcriptional level occurs through RNA metabolism². To diversify proteins posttranslationally, organisms rely on covalent posttranslational modifications (PTMs)¹. These reactions represent a significant commitment of cellular resources and substantial portion of the genomes of higher eukaryotes (about 5%) is devoted to encoding enzymes that perform posttranslational modification reactions¹. Furthermore, 15 of the 20 amino acids used in building proteins have side chains that can be modified by over 200 known covalent PTMs¹. Much effort has been placed on cataloging these modifications, understanding their biological significance, and determining how multiple modifications on a single polypeptide may work together¹.

Methylation and methyltransferases

A PTM that has gained significance as common and important is methylation. Of putative PTMs, methylation ranks as the 4th most abundant, behind glycosylation, phosphorylation, and acetylation,³ and these reactions are widespread in nature. While carbon, oxygen, and sulfur methylations can occur, methylations of the nitrogen containing side chains (*N*-methylation) of Lys and Arg residues are the most studied of these modifications¹. *N*-methylation is perhaps most commonly seen in the histone code, where methylation reactions individually and in tandem with other methylations and modifications affect gene expression⁴. Non-histone protein methylation is becoming just as well-characterized, given the role of methylation in ribosome biogenesis, translation, transcription, RNA splicing, and protein-protein interactions⁵⁻⁷. The

functions of the enzymes responsible for these modifications, methyltransferases, have been implicated in a variety of diseases⁸, indicating that the methylation reactions they perform are of importance to cell function.

Genes that encode methyltransferases make up 1-2% of the genome in a variety of organisms⁹, with a large portion dedicated to methylating proteins⁵. Determining the methyltransferasomes has been largely performed based on sequence homology and multiple motif scanning^{9,10}. The budding yeast *Saccharomyces cerevisiae* has one of the most fully characterized methyltransferasomes^{10,11}. Coupled with the fact that a large fraction of the genes in yeast are homologous or orthologous to humans¹², this yeast is an ideal organism to study these enzymes and the reactions they catalyze. There are about 87 methyltransferases in *S. cerevisiae*^{10,11} and a large majority of them (over 80%) have confirmed substrates, as summarized in Chapter Two.

Identification of possible enzymes and methylation sites

To better identify substrates of the remaining putative methyltransferases, approaches have generally followed one of two paths: 1) predicting substrate type and 2) identifying the possible methylation sites. Predicting substrate types for putative methyltransferases varies in success. Using homology and structural information of catalytic domains¹³ to predict substrates has its limitations due to the potential for common folds to methylate varying substrate types and the possibility for proteins to methylate substrates that deviate from their domain specificity. One such example is Sfm1, a SPOUT domain protein in *S. cerevisiae* that methylates a ribosomal protein substrate¹⁴. In predictions, Sfm1 was classified as an RNA methyltransferase¹³ due to the fact that all known SPOUT methyltransferases in yeast have RNA substrates. The known

members of the SPOUT family methyltransferases perform *O*- or *N*-methylation on rRNA and tRNA¹⁴. Sfm1 is a unique SPOUT family member in that it methylates the arginine of Rps3 instead of RNA¹⁴, being the only SPOUT protein identified so far to modify a protein substrate.

Other substrate predications have casted a broader net, looking at the combination of characteristics each methyltransferase of a particular substrate type has including its isoelectric point (pI), cellular localization, structural fold, expression pattern within the yeast metabolic and cell cycle, and time of expression onset¹⁵. These predictions, improved from structural information alone, are still only correct for 67% of known proteins¹⁵. This is partly due to the difficulty in predicting protein substrates. The significance of pI can skew this probability, given that 67% of methyltransferases with a high pI methylate RNA and 65% of methyltransferases with a low pI methylate proteins¹⁵. The remaining 30% in each group have no correlation between pI and substrate type. This can cause misidentification for any DNA or RNA binding proteins, such as Mgt1, which automethylates using a DNA methylation site as a methyl donor¹⁵, or histone methyltransferases that have a high pI for methylating proteins, such as Dot1¹⁵. Further clouding predictions is the localization of the methyltransferases in organelles where the methylation of proteins has been largely unstudied, such as the mitochondria. In such analyses, many putative protein methyltransferases may be incorrectly identified as RNA methyltransferases as there are fewer examples of known protein methylation in the mitochondria than known RNA methylation events¹³. Additionally, predicting putative methylation sites on putative substrates, as opposed to substrate types of methyltransferases, computationally is correct a little over 60% of the time at best at this time¹⁶.

The other approach, identifying methylation sites, also does not provide a clear picture of the extent of methylation in the cell. The major problem with cataloging modifications, including

methylation, is that experimental results present a snapshot of what might actually be occurring in the cell at any given time¹. Most modifications are reversible¹ and vary given the changes in cellular conditions. What is present and modified in one experiment might not be modified in the next. As a result, there is no complete inventory of PTMs in any given cell type.

Cataloguing the methyl proteome by mass spectrometry

The sub-field of “modificomics” has sought to remedy the issues with cataloguing modifications¹⁷. The most common method for cataloging modifications has been mass spectrometry. The basic strategy to these large scale proteomic studies of modifications is to extract proteins, digest them into peptides using specific proteases, enrich for a specific modification, perform peptide level analysis (using mass spectrometry), and quantify using bioinformatic analyses¹⁸. With searching for modifications, the more that is known about the sample being analyzed and the clearer the aim or the approach allows for the most success in determining the modification¹⁷.

There has been a significant effort to characterize the methyl proteome in many organisms by mass spectrometry. The majority of studies have used some kind of enrichment strategy, such as immunoprecipitation using a methylation-specific antibody, coupled to liquid chromatography with tandem mass spectrometry (LC-MS/MS) on whole cell lysates, tissue extracts, or fractionations¹⁹. However, these proteomic strategies are not without their pitfalls. Out of all the peptides identified, modified peptides represent only a small fraction and can be missed given the limitations of the dynamic range of detection in mass spectrometry²⁰. Additionally, like all PTMs, methylation can be dynamic and labile. This makes it difficult to detect specific methylation events in any given cell and to ensure methylation is retained during

sample preparation and ionization²⁰. Recently, the validity of large-scale proteomics to identify methylation has come into question given the discovery that they are subject to high false discovery rates²¹. This can be overcome by using methods that allow for further peptide validation, such as heavy methyl stable isotope labeling with amino acids in cell culture (SILAC)²².

Obtaining a complete inventory of protein methylation sites in yeast is further complicated by the intracellular compartmentation of proteins. In whole cell lysates, organellar proteins may be in lower abundance than their cytoplasmic counterparts, depending on growth conditions²³. If proteins are lower in abundance, this increases the difficulty of identifying whether or not they are methylated as there is already a lower abundance of modified to unmodified peptides in large scale analyses¹⁸. While there has been an increasing interest in mitochondrial protein methylation in higher eukaryotes²⁴⁻²⁷, these have largely resulted in studies of individual proteins and not the entire methyl proteome. To date, there have only been two global studies on the mitochondrial methyl proteome^{28,29}, one of which is presented in this dissertation. The other was a global look at arginine methylation in the mitochondria of the parasite *Trypanosoma brucei*²⁸. Mitochondrial protein methylation has largely been overlooked, rendering the current inventory of the methyl proteome as incomplete.

Mitochondrial protein methylation in higher eukaryotes

Only two mitochondrial protein methyltransferases responsible are known in yeast. This is in contrast to the multiple mitochondrial protein methyltransferases that have been identified in human cell lines. Much of the mammalian mitochondrial methyltransferasome characterization has been undertaken by John Walker and his laboratory²⁵⁻²⁷. There are two

known lysine methyltransferases in human mitochondria: ETF-KMT and METTL12. The electron transfer flavoprotein β -subunit (ETF β) is trimethylated by ETF-KMT (METTL20) on lysine 199 and nearby lysine 202²⁶, affecting the protein-protein interactions ETF β can make^{26,30}. METTL12 has been shown to be responsible for the trimethylation of K366 near the active site of citrate synthase²⁷. Removal of this methyl mark does not affect citrate synthase activity and Rhein *et al.* postulated that this mark also may be required for protein-protein interactions²⁷. All three of the lysine methylation sites characterized in mammalian mitochondria are substoichiometric, varying with growth conditions and appearing as partially methylated²⁷.

A recently described mammalian novel arginine methyltransferase (NDUFAF7) localizes to the mitochondria to symmetrically dimethylate R86 of NDUFS2 in Complex I²⁵. This methylation event is not only required for Complex I assembly, but for embryogenesis as well³¹. Lastly, HEMK1 methylates a conserved glutamine on the mitochondrial release factor³². The methyltransferases responsible for methylating the other methylated proteins in mammalian mitochondria, such as the ADP/ATP translocase³³ and ATP synthase²⁴, have not been identified.

Many of the substrates described here are not homologous in yeast or do not have conservation of the methyl mark. ATP synthase methylation is common and conserved in metazoans, but not lower eukaryotes³⁴. Additionally, only HEMK1 is conserved in yeast³². NDUFAF7 is homologous to yeast Yk1162c²⁵, but the substrate is not present in yeast mitochondria. Yeast do not have a Complex I, but rather rely on Nde1, Nde2, and Ndi1 for a similar function^{35,36}. Due to the family of proteins METTL20 and METTL12 belong to, they are more similar to the lysine methyltransferases that methylate eukaryotic elongation factor 1a (eEF1A)^{27,37} and not to mitochondrially localized methyltransferases.

Methylation in Saccharomyces cerevisiae mitochondria

Much of what we know about mitochondrial function and disease is the direct result of experiments in *S. cerevisiae*³⁸. Additionally, many yeast proteins have a homolog or ortholog in humans¹². Not much is known about the overall methylation state in yeast mitochondria. The common methyl donor used by methyltransferases *S*-adenosyl-L-methionine (AdoMet) has a carrier (Sam5) that allows for transport into the mitochondria³⁹. The absence of Sam5 causes multiple phenotypes, including growth defects on fermentable and non-fermentable carbon sources³⁹. Yeast mitochondrial DNA (mtDNA) has been demonstrated to be unmethylated^{40,41}. While the mitochondrial tRNAs are methylated similar to their cytoplasmic partners⁴²⁻⁴⁵, mitochondrial rRNA is minimally modified⁴⁶⁻⁴⁸. Mrm1 and Mrm2 are responsible for the two methyl marks on the 21S rRNA⁴⁹. Small molecule methylation in mitochondria has been well-characterized due to its involvement in coenzyme Q (CoQ) biosynthesis. Methyltransferases Coq3 and Coq5 are both required for biosynthesis and part of the CoQ synthome⁵⁰. While there is no evidence thus far for mitochondrially localized lipid methyltransferases, lack of methylation of phospholipid membrane components affects mitophagy⁵¹.

As mentioned previously, only two yeast methyltransferases are known to methylate mitochondrial protein substrates, M tq1 and C tm1. M tq1 is mitochondrially localized and the ortholog to HEMK1.³² It methylates the mitochondrial release factor M rf1 on a conserved glutamine⁵². Like many of the other mitochondrial protein methyl marks, this modification is substoichiometric and not required for function⁵². On the other hand, C tm1 is cytoplasmically localized and trimethylates its substrate cytochrome *c* on a lysine residue pre-import into the mitochondria⁵³. Like other examples, this methylation is not required for function, but rather appears to help facilitate import⁵⁴⁻⁵⁶, apoptosis⁵⁷, and protein-protein interactions⁷. Additionally,

four putative methyltransferases^{10,11} with unknown substrates localize to the mitochondria: Rsm22⁵⁸, Oms1⁵⁹, Mtf1⁶⁰, and Ykl162c⁶¹.

Plan of the dissertation

The original trajectory for my dissertation was to aid in the completion of the characterization of the methyltransferasome in yeast by identifying the substrates for this handful of putative methyltransferases that localize to the mitochondria. Given how much was unknown about mitochondrial protein methylation in general, I changed course to better inventory the methyl proteome of the yeast mitochondria.

Mitochondrial specific methods for determining methylation sites had not been developed previously. In Chapter Two, I outline the experimental strategies I used to determine if protein methylation was occurring in the mitochondria of budding yeast. I used the ability for yeast cells to take up radiolabeled methyl donor *S*-adenosyl-L-[methyl-³H]methionine to allow for the incorporation of tritiated methyl groups on putative substrates that could be detected by autoradiography. I also adapted methods for radiolabeling the mitochondrial translated proteins (*in organello* translation) to instead target methyl proteins in whole mitochondria (*in organello* methylation). As a result of these experiments, I was able to confirm the presence of mitochondrial protein methylation. However, it remains unclear if the methyltransferases responsible are mitochondrial or cytoplasmic. Methyltransferases acting on mitochondrial protein substrates could be either mitochondrially localized (like Mtf1) or cytoplasmically localized (like Ctm1).

To determine if putative methyltransferases act on mitochondrial protein substrates, I performed growth screens on fermentative and respiratory media of strains with deletions in the

genes encoding known and putative protein methyltransferases. The results of these screens are described in detail in Chapter Three. Only methyltransferases whose absence is associated with known mitochondrial defects displayed any phenotype and some mitochondrially localized methyltransferases, such as Ykl162c and Mtt1, displayed no growth phenotype.

In Chapter Four, I collaborated with Dr. James Wohlschlegel (UCLA) and his laboratory to use heavy methyl SILAC to determine the methylation state of mitochondrial proteins. Here, we corroborated the previous findings indicating a high level of false discovery in large-scale methyl proteomic studies. We also demonstrated that using the wild type strain (BY4741), employed in other yeast heavy methyl SILAC analyses, results in dilution of the label complicating the identification of truly methylated species. In our work, we made use of an alternative yeast strain (*met6Δ*) for heavy methyl SILAC studies so that such dilution does not occur.

I further explore the physiological implications of the methyl proteins identified in Chapter Five. I conclude that mass spectrometry should be used to validate immunoblotting experiments rather than the other way around. Using tandem affinity purification (and a tandem affinity tag), I purified methyl candidate proteins identified from Chapter Four. These TAP tagged constructs showed cross-reactivity with methylation specific antibodies, regardless of methylation state. I hypothesized that the Protein A portion of the TAP tag was responsible and suggested this domain was recognized by the IgG antibodies. This was confirmed as cleavage with tobacco etch virus (TEV) protease, which removes the Protein A portion of the tag, resulted in a decreased signal. In this chapter, I also confirm the methylation of Aco1 using the methylation specific antibodies.

Additionally, the methyl proteins found in the three protein fractions can be grouped by

the Gene Ontology (GO) terms⁶² and the GO terms related to the fractions confirm the localizations and functions of the proteins identified. Structurally, I investigated the position of the methyl groups on the mitoribosome. Using the recently determined three-dimensional structure⁶³, I found that the methylation sites on Mrpl40 and Mrpl4 are surface exposed. Additionally, the arginine that is methylated on Mrpl4 (R204) appears to be involved in protein-protein interactions with neighboring Mrpl27. This is in contrast with the cytoplasmic ribosome, where some of the methylation sites are buried deep within the ribosome, facilitating assembly and protein-RNA interactions⁵. Using human cell data that has been recently published⁶⁴, I found that many methylated mitochondrial proteins may be similarly methylated in humans. Interestingly, Pdb1 and its human homolog (PDHB) are similar in sequence at the methylation site determined in Chapter Four. Larsen *et al.* confirm that this site is also methylated in the human homolog⁶⁴. This site appears to be conserved, suggesting the existence of a novel protein arginine methyltransferase in the mitochondria.

Chapter Six is a further collaboration with the Wohlschlegel laboratory. In Chapter Four, many cytoplasmic ribosomal proteins that were previously identified as methylation were not confirmed as methylated. In that study, it was unclear if this was due to growth conditions (in that, those proteins are not methylated in respiring yeast cells) or due to the method used. Chapter Six compares the methyl protein content of cytosols isolated from fermenting and respiring yeast cells. While many proteins were identified as methylated in both conditions, respiring yeast had increased mitochondrial proteins identified as methylated.

Finally, Chapter Seven will summarize the results of the dissertation, the significance of the findings, and future directions.

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CHAPTER TWO

Characterizing Potential Mitochondrial Methylation Reactions in *Saccharomyces cerevisiae*

Introduction

To investigate whether or not protein methylation occurs in the mitochondria of the model eukaryotic organism *Saccharomyces cerevisiae*, I started by looking at the possible methyltransferases that might catalyze these reactions. *S. cerevisiae* has one of the most highly characterized methyltransferasomes^{1,2} and an extensive gene deletion library^{3,4}. This means that methyltransferase gene deletions are readily available and can be used to screen for methylation defects. There are 87 methyltransferases identified to date in the yeast methyltransferasome (Table 2-1). Of those, 16 do not have confirmed substrates and remain putative. These putative methyltransferases can potentially methylate protein, RNA, or lipids/small molecules. Much effort has been put into predicting methyltransferase substrates^{5,6} (as described in detail in Chapter One). These efforts have been informative in some cases (Table 2-1); however, many putative methyltransferases may be categorized incorrectly because of the limitations of the predictive models. Specifically, predicting protein methylation reactions by the criterion of isoelectric point can be particularly problematic since any methyltransferase with a high pI would be classified as an RNA methyltransferase, regardless of its substrate⁶. This is an issue for some histone methyltransferases with high pI values as well as any protein that may bind RNA or DNA irrespective of its enzymatic ability.

In this study, putative methyltransferases were selected for analysis based on known mitochondrial localization or a predicted high probability of import into the mitochondria⁷ (Table 2-1). Notable candidates are Rsm22, Oms1, Mtf1, and Ykl162c as they have known functions or known localization in the mitochondria⁸⁻¹¹. While it is possible any of the candidates in the mitochondria may be methylating other substrates, there is a high likelihood that these are protein methyltransferases. Previous reports have shown that yeast mitochondrial rRNA is

minimally modified¹² and the major tRNA methylating species in the mitochondria is likely to have dual localization in the cytoplasm^{13,14}. While there is DNA methylating activity in the mitochondria, observed via incubation of radiolabeled methyl donor in mitochondrial extracts¹³, the corresponding nuclear DNA is not enzymatically methylated¹⁵. Methylation of mtDNA has been hotly debated. Originally, it was determined that yeast mtDNA was not methylated¹⁶. However, as methods have grown more sensitive, increasing evidence has demonstrated the presence of methylation on mtDNA in mammalian cells^{17,18}, even though it is at low levels (at most 5% of the mtDNA)¹⁹. This has yet to be investigated in yeast once more. Furthermore, the methyltransferases that show similarity to DNA methyltransferases appear to be RNA methyltransferases (Dim1, Trm11) and are not mitochondrial²⁰. There has been minimal evidence that mitochondrial extracts can methylate proteins¹³. I will expand on this evidence using results presented in this chapter.

Identifying methylation reactions in mitochondria is possible due to the mitochondrial *S*-adenosyl-L-methionine (AdoMet) carrier²¹. AdoMet is the most common methyl donor used by methyltransferases. The fact that this molecule is imported into the mitochondria allows for radiolabeled AdoMet to be used to compare differences in methylation levels between wild type and methyltransferase gene deletion strains. Standard radiolabeling methods can then be used to determine which methyltransferases may be acting upon mitochondrial substrates. As outlined in this chapter, this may be more of a challenge than previously thought as levels of methylation in the mitochondria seem to be low in comparison to the more abundant methylated protein and RNA species that have been identified in the cytosol^{12,22-26}. I will also describe standard methods that have been used in the identification of protein methyltransferases, but appear to vary in their success for application in the mitochondria.

Methods

Mitochondrial preparation of BY4741

Mitochondrial ribosomal proteins were purified from wild type yeast BY4741 as described previously²⁷. 20 µg of protein fractions were mixed with 2X SDS sample buffer heated for 5 minutes at 100 °C. Samples were loaded onto a pre-cast NUPAGE Novex 4-12% BisTris gel (ThermoFisher Scientific NP0321). The gel was run with the NUPAGE MES running buffer system (ThermoFisher Scientific NP0002) for 1 hour and 15 minutes at 150 V. Proteins were transferred from the gel to a PVDF membrane (Amersham Hybond, GE Healthcare Life Sciences, Cat 10600023) using an XCell II Blot Module (ThermoFisher Scientific EI9051) with Tris-glycine-methanol transfer buffer run at 30 V for 1 hour. The membrane was blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% (v/v) Tween 20 (TSBT) for an hour at room temperature.

The membrane was incubated overnight at 4 °C in anti-mouse Rip1 methyl lysine antibody that was 1:1000 diluted in 1% non-fat dry milk and 0.02% (w/v) sodium azide (a gift from Dr. Catherine Clarke, UCLA). The membrane was incubated for 1 hour with a 1:100000 dilution of anti-mouse IgG conjugated to horseradish peroxidase (HRP) in 1% BSA in TBST (Cell Signaling 7076). After visualization using chemiluminescence with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, RPN2232), the membrane was exposed to autoradiography film (Hybond CL, Danville Scientific, 1001364). The amount of polypeptides transferred to the membrane was determined qualitatively by staining with 0.5% (w/v) Ponceau S in 1% acetic acid.

In vivo methylation

The *in vivo* labeling protocol was adapted from previous reports^{28,29}. 5 mL of semisynthetic lactate medium (LM; 3 g/L yeast extract, 0.5 g/L dextrose, 0.5 g/L CaCl₂-2H₂O, 0.5 g/L NaCl, 0.6 g/L MgCl₂-6H₂O, 1 g/L KH₂PO₄, 1 g/L NH₄Cl, 2% (v/v) lactic acid, 8 g/L NaOH, brought to pH 5.5 with NaOH)³⁰ overnight cultures of each strain were inoculated into 10 mL of LM and grown at 30 °C with shaking at 250 rpm until an optical density at 600 nm of 0.7-0.9. 7 optical density units for each strain were harvested by centrifugation at 5000 x g at 4 °C for 5 minutes. Pellets were resuspended in 1 mL LM and transferred to a 1.5 mL microcentrifuge tube. Cells were centrifuged for 5 minutes at 10,600 x g at room temperature. Cells were washed once more with 1 mL of LM. The resulting pellet was resuspended in 848 µL LM and 152 µL of S-adenosyl-L-[methyl-³H]methionine ([³H]AdoMet, Perkin Elmer, 83.3 Ci/mmol, 0.55 mCi/ml in 10 mM H₂SO₄-ethanol (9:1), NET 155H001M). The cells were incubated at 30 °C with shaking at 250 rpm for 30 minutes. Labeling was stopped by centrifugation at 5000 x g at 4 °C for 5 minutes. Cells were washed twice with 1 mL of sterile water.

Mitochondrial lysates from labeled wild type and methyltransferase gene deletion cells were prepared as previously described^{30,31}, but in a scaled-down protocol. The labeled pellet was resuspended with 200 µL 0.1 M Tris-SO₄, pH 9.4 with 10 mM dithiothreitol. This suspension was incubated at 30 °C with shaking at 250 rpm for 15 minutes. Cells were pelleted by centrifugation for 5 minutes at 2000 x g and at room temperature. The pellet was resuspended in 200 µL 1.4 M sorbitol, 20 mM phosphate, pH 7.4. Cells were pelleted by centrifugation for 5 minutes at 2000 x g and at room temperature. Cells were converted to spheroplasts by incubation with 100 µL of 1.25 mg/mL zymolyase 20T for 30 minutes at 30 °C with shaking at 250 rpm. Spheroplasts were centrifuged at 4000 x g for 5 minutes at room temperature. Spheroplasts were

washed twice in 200 μ L 1.4 M sorbitol, 20 mM phosphate, pH 7.4. Washed spheroplasts were resuspended in 200 μ L 0.6 M sorbitol, 20 mM HEPES, pH 7.4 with 0.5 mM phenylmethylsulfonyl fluoride. Spheroplasts were dounce homogenized with 15 strokes of a plastic pestle. Homogenized spheroplasts were brought to a volume of 500 μ L with 0.6 M sorbitol, 20 mM HEPES, pH 7.4 with 0.5 mM phenylmethylsulfonyl fluoride. The lysate was cleared by centrifugation at 1500 x g for 5 minutes. The supernatant was reserved and the homogenization step was repeated using the pellet. The combined supernatants were centrifuged for 10 minutes at 12,000 x g. Mitochondrial pellets were resuspended in 240 μ L 0.6 M sorbitol, 20 mM HEPES, pH 7.4 and centrifuged for 5 minutes at 1500 x g. The supernatant was transferred to a fresh tube and centrifuged again for 10 minutes at 12,000 x g. The supernatant was removed and the mitochondrial pellet was resuspended in 100 μ L lysis buffer (350 mM ammonium chloride, 20 mM magnesium acetate, 1 mM EDTA, 2 mM β -mercaptoethanol, 20 mM Tris, pH 7.5). Mitochondria were then lysed by addition of 0.5 μ L 26% (v/v) Triton X-100 in lysis buffer. The lysate was cleared by centrifugation for 10 minutes at 20,000 x g. Radiolabeled mitochondrial lysates were dried in a vacuum concentrator and stored at -20 $^{\circ}$ C.

Dried radiolabeled mitochondrial lysates were resuspended with SDS sample buffer and heated for 5 minutes at 100 $^{\circ}$ C. Radiolabeled samples were loaded onto NUPAGE Novex 4-12% BisTris gel (ThermoFisher Scientific NP0321). The gel was run with the NUPAGE MES running buffer system (ThermoFisher Scientific NP0002) for 35-40 minutes at 200 V. The gel was stained with Coomassie Brilliant Blue for 1 hour and destained overnight. The gel was washed with water twice for 30 minutes. To increase the signal of the tritium label, the gel was incubated with EN³HANCE (PerkinElmer, 6NE9701) for 1 hour. The gel was washed with water for 1 hour then dried in a gel dryer for 2 hours. The dried gel was placed in an X-ray cassette with a

piece of autoradiography film shielded from light at -80 °C until the film was developed after 1-5 months.

In organello methylation

Highly purified mitochondria were obtained from wild type strain B4742 through differential centrifugation and separation using a nycodenz gradient as previously described³⁰. Mitochondria were resuspended in 500 µL 0.6 M sorbitol, 20 mM potassium HEPES, pH 7.4 and flash frozen in 80 µL aliquots in liquid nitrogen, each approximately 40 µg. The aliquots were then stored at -80°C. Using 0.6% SDS, one aliquot was lysed to determine the protein concentration via absorbance at 280 nm and the Lowry method³².

The *in organello* methylation reaction was adapted from *in vivo* methylation labeling²⁹ and *in organello* translation³³ in addition to the experiments identifying the mitochondrial S-adenosyl-L-methionine (AdoMet) carrier²¹. Aliquots of mitochondria were rapidly thawed in a water bath. For each experiment, 80 µg of mitochondria were used (two aliquots). Previously prepared mitochondria from W303-1a, a respiration proficient strain, were also used at 1 mg each. Mitochondria were collected by centrifugation for 10 minutes at 20,000 x g at 4 °C. The mitochondria were washed once using 100 µL 0.6 M sorbitol, 20 mM potassium HEPES, pH 7.4 and centrifuged again. The mitochondria were resuspended in 424 µL 0.7 M sorbitol, 11.8 mM PIPES, pH 7.0 (or 0.6 M sorbitol, 20 mM potassium HEPES, pH 7.4) and 76 µL [³H]AdoMet (Perkin Elmer, 83.3 Ci/mmol, 0.55 mCi/ml in 10 mM H₂SO₄-ethanol (9:1), NET 155H001M), giving a final concentration of 0.1 µM [³H]AdoMet, 0.6 M sorbitol, and 10 mM PIPES. The mitochondria were incubated with the [³H]AdoMet for 1 hour at 25 °C. Mitochondria were collected by centrifugation 10 minutes at 20,000 x g at 4 °C. The supernatant was discarded as

radioactive waste. The mitochondrial pellet was washed twice in 500 μ L 0.6 M sorbitol, 20 mM potassium HEPES, pH 7.4 and collected by centrifugation 10 minutes at 20,000 x g at 4 °C. The labeled mitochondrial pellet was resuspended in 20 μ L 2X SDS sample buffer.

Samples were loaded onto a 12.6% polyacrylamide gel. The gel was run at 35 mA for about four hours. The gel was stained for 1 hour in Coomassie Brilliant Blue stain and destained overnight. The gel was washed with water twice for 30 minutes. To increase the signal of the tritium label, the gel was incubated with EN³HANCE (PerkinElmer 6NE9701) for 1 hour. The gel was washed with water for 30 minutes then dried in a gel dryer for 2 hours. The dried gel was placed in an X-ray cassette with a piece of autoradiography film shielded from light at -80 °C until the film was developed after five weeks.

Methylation specific antibodies

Mitochondrial ribosomal proteins were purified from wild type and gene deletions strains as described previously²⁷. 20 μ g of protein were mixed with 2X SDS sample buffer were heated for 5 minutes at 100 °C. Samples were loaded onto a pre-cast NUPAGE Novex 4-12% BisTris gel (ThermoFisher Scientific NP0321). The gel was run with the NUPAGE MES running buffer system (ThermoFisher Scientific NP0002) for 40 minutes at 200 V. Proteins were transferred from the gel to a PVDF membrane as described in the methods for immunoblotting BY4741 mitochondrial fractions. The membrane was blocked in 5% non-fat dry milk in phosphate buffered saline with 0.1% (v/v) Tween 20 (PSBT) overnight at room temperature.

The membrane was incubated for 1 hour in anti-pan methyl lysine antibody that was 1:1000 diluted in 1% non-fat dry milk in PBST (Abcam, ab7315). The membrane was incubated for 1 hour with a 1:100,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase

(HRP) in 1% non-fat dry milk in phosphate buffered saline (PBST) (Abcam, ab6721). After ECL reagent exposure for 5 minutes, the gel was exposed to film.

An anti-di/trimethyllysine antibody, diluted 1:10,000 in 1% non-fat dry milk in PBST (Upstate Biotechnology, Inc., 07-756, a gift from Dr. Joanna Goldberg (Emory University School of Medicine)³⁴) was also used.

In vitro methylation

In vitro methylation reactions were adapted from previously published methods^{35,36}. Mitochondrial lysates of wild type and gene deletion strains were prepared as previously described²⁷. Whole cell extracts of wild type and gene deletion strains were prepared as follows. Cells were grown in 5 mL YPD (1% yeast extract, 2% peptone, and 2% dextrose) overnight at 30 °C with shaking at 250 rpm. Cell pellets were washed twice with 1 mL of water and centrifuged at 5000 x g at 4 °C for 5 minutes. Cell pellets were resuspended in 5 mL of LM and used to inoculate larger aliquots of LM to an optical density at 600 nm of 0.1 (100 mL were inoculated for gene deletion strains, 250 mL were inoculated for wild type). Cells grew at 30 °C with shaking at 250 rpm until an optical density at 600 nm of 0.5-0.8 was reached. 10 optical density units of each strain were harvested by centrifugation at 5000 x g at 4 °C for 5 minutes and washed once with 1 mL of water. 10 OD cell pellets were stored at -20 °C until lysis.

Cell pellets were thawed on ice and combined with 0.5 g of glass beads (Biospec Products, 11079105) with 50 µL of lysis buffer (phosphate buffered saline with 1 mM EDTA and 1% Triton X-100 with a Roche Applied Science Complete protease inhibitor tablet added to every 50 mL). This was vortexed for 30 seconds followed by 30 seconds on ice. This cycle was repeated seven times. The lysate was removed from the glass beads by puncturing the bottom of

the microcentrifuge tube with an 18 gauge needle and collected the flow through in a new microcentrifuge tube by centrifugation in a clinical centrifuge for 30 seconds on setting 6. Glass beads were washed with an additional 50 μ L of lysis buffer, flow through was collected, and this was combined with the original flow through. The resultant lysate was cleared by centrifugation at 12,000 x g for 15 minutes at 4 $^{\circ}$ C. The supernatant was transferred to a new microcentrifuge tube. Protein concentrations were determined using the Lowry method³² and whole cell extracts were stored at -20 $^{\circ}$ C until use.

To perform the *in vitro* methylation reactions, three reaction conditions of each gene deletion strains were performed: 40 μ g protein of wild type whole cell extract or mitochondrial lysate (wild type alone), 40 μ g protein of gene deletion whole cell extract or mitochondrial lysate (knockout alone), and 20 μ g protein of wild type lysate or mitochondrial lysate combined with 20 μ g of gene deletion lysate or mitochondrial lysate (wild type + knockout) for a total of 40 μ g of protein. For all conditions, 40 μ g of protein was combined with 0.2 M sodium phosphate, pH 7.0 and incubated with [³H]AdoMet (Perkin Elmer, 83.3 Ci/mmol, 0.55 mCi/ml in 10 mM H₂SO₄-ethanol (9:1), NET 155H001M). Reactions were diluted with water to bring the reaction volume to 50 μ L, final buffer concentration to 0.1 M, and the final concentration of [³H]AdoMet to 0.66 μ M. Reactions were incubated at 30 $^{\circ}$ C for 60 minutes.

The reaction was stopped with the addition of 50 μ L 2X SDS sample buffer and reactions were heated for 5 minutes at 100 $^{\circ}$ C. All 100 μ L of the reaction mixture were loaded onto a 12.6% polyacrylamide gel. Using a Tris-glycine-SDS running buffer, the gel was run at 30 mA until the dye front reached the bottom of the gel. The gel was stained with Coomassie Brilliant Blue stain for one and a half hours and destained in acetic acid and methanol overnight. The gel was rinsed in water for 30 minutes, followed by an hour incubation in EN³HANCE (PerkinElmer

6NE9701) and another 30 minute wash in water. The gel was dried for two hours in a gel dryer. The dried gel was placed in an X-ray cassette with a piece of autoradiography film shielded from light at -80 °C until the film was developed after four to six weeks.

Results

Purifying mitochondria from common wild type yeast strains

In order to use commercially available methyltransferase gene deletion strains, I first determined if mitochondria could be purified from the wild type background of those mutants, either BY4741 or BY4742. Due to polymorphisms, the parent *S. cerevisiae* strain S288C has a high propensity for the formation of petite colonies, indicating a possible defect in mitochondrial DNA (mtDNA)³⁷. As the commonly used BY4741 and BY4742 are derivatives of S288C, and selected as the wild type background for the genome deletion project³⁸, this problem exists with using those strains as well. BY4741/2 are less ideal for mitochondrial studies than strains such as W303-1a and D273-10b³⁹. However, as BY4741 and BY4742 are the parent strains of the methyltransferase gene deletion strain, I first attempted to use them for this purpose.

Mitochondrial purification from BY4741 was successful. Differences in the mitochondrial marker, Rip1, throughout the purification appear to be due to increased levels of mitochondrial protein instead of differences in protein concentration (Figure 2-1). There was no mitochondrial protein present in the cytosol and wash steps that did not contain mitochondria. The largest amount of Rip1 appears to be in the mitochondrial lysate and in the cells that did not properly lyse, which contain mitochondria as well (Figure 2-1).

Radiolabeling assays for determination of mitochondrial methylation

Once mitochondria could be obtained from the BY4741 background, I could use it as wild type in comparison to the methyltransferase gene deletion strains. There are a variety of radiolabeling experiments that can detect differences in methylation between the two strains. With the addition of radiolabeled *S*-adenosyl-L-methionine ($[^3\text{H}]\text{AdoMet}$), the main methyl donor used by methyltransferase, signal can be detected and compared by autoradiography or liquid scintillation counting. In particular, I wanted to look at the differences of methylation in the mitochondrial lysate. With these assays, I would be able to identify 1) if protein methylation occurred in the mitochondria and 2) which methyltransferases could be responsible.

The first approach was to radiolabel yeast cells and obtain mitochondrial lysates from wild type and gene deletions to identify if methylation as a whole in the mitochondria was affected by any particular gene deletion. By adapting an existing protocol for purification of mitochondria, I was able to obtain mitochondria from a 1 mL volume of radiolabeled cells. Wild type was used for comparison and *rmt1Δ* was used as a positive control. Rmt1 is the major arginine methyltransferase⁴⁰ and would cause a decrease in overall protein methylation if deleted. After the experiments were performed, both Bmt2 and Bmt5 were determined to be RNA methyltransferases^{41,42} (Table 2-1). Thus, *BMT2* and *BMT5* deletion strains can be utilized as negative controls for protein methylation.

Though mitochondrial protein methylation appears to be occurring, there were no detectable differences between the strains tested (Figure 2-2). Results were inconsistent from one experiment to the next. The material from the wild type strain had the highest amount of signal in one experiment (Figure 2-2, B), but a similar signal to gene deletion strains in another (Figure 2-2, A). The deletion of *RMT1*, the major protein arginine methyltransferase in *S. cerevisiae*, does

cause a decrease of methylation in the mitochondrial lysate (Figure 2-2, B). The small scale mitochondrial preparations did not yield protein amounts that were detectable by Coomassie stain or other standard methods for protein quantification. As a result, variations between strains and experiments may be due to differences in the amount of protein loaded, rather than anything of biological significance. Such a scenario is indicated by the overall decrease in signal in *rsm22Δ*, *oms1Δ*, and *mtf1Δ* (Figure 2-2, B). These strains have known mitochondrial phenotypes (discussed in Chapter Three) and would result in less protein obtained due to lower mitochondrial density.

Increased signal at the top of the wells of each experiment (Figure 2-2) is most likely due to hydrophobic aggregates in the mitochondrial lysate. This may have occurred due to heating of the samples in the SDS buffer, which can cause the extremely hydrophobic proteins in localized to mitochondrial membranes to associate with each other⁴³. However, this result also suggests that hydrophobic components of the mitochondria, such as membrane proteins and oxidative phosphorylation components, could be methylated. Overall, these experiments of radiolabeling whole cells and then purifying mitochondria do not distinguish between the possibilities that proteins are methylated pre- or post-import into the mitochondria.

To avoid this ambiguity, I developed an *in organello* methylation assay. *In organello* translation assays³³ are commonly used to radiolabel the mitochondrial translation products to observe defects in mitochondrial translation and differences in mitochondrially translated protein amounts. Mitochondria contain an AdoMet carrier²¹, which would allow mitochondrial proteins to be radiolabeled in a similar manner to whole cell extracts with the addition of [³H]AdoMet. I tested this method with wild type and a respiration proficient strain, W303-1a. However, no major methylated protein bands appeared after five weeks in the wild type mitochondria (Figure

2-3). There appears to be lipid or small molecule methylation, due to the signal below 6.5 kDa, just below the dye front, which indicates that there is an active methyltransferase in the wild type mitochondria.

The presence of multiple types of methyltransferases is more clearly demonstrated in the W303-1a mitochondria. In addition to the large bands below 6.5 kDa, faint bands were present from 31 kDa and below (Figure 2-3). This could be indicative of protein methylation. There is the possibility that this region contains methylated tRNA as well⁴⁴. There was no difference in methylation between reactions performed at pH 7.0 or pH 7.4 (Figure 2-3). The results here suggest that there are mitochondrially localized methyltransferases. This method was ultimately abandoned due to the faint protein signal and time of detection; neither of which made *in organello* methylation a viable option for screening for mitochondrial methyltransferase candidates.

Screening for substrates using methyl-specific antibodies

To continue to screen for differences in protein methylation signal between wild type and gene deletion strains, I moved to a more targeted approach, methyl-specific antibodies. By immunoblotting for methyllysine, differences in lysine methylation, one of the more common amino acids to be methylated, could be determined in theory.

Once again, it was difficult to determine differences in the mitochondrial ribosomal proteins (MRPs) of various strains (Figure 2-4, A). MRPs were examined as potential mitochondrial targets of methylation as the cytoplasmic translational system contains abundant protein methylation²⁴. There was very little signal present in each MRP fraction. Additionally, the bands that did appear consistently, one at 50 kDa and one at 15 kDa, were most likely

indicative of the cytoplasmic ribosome contamination that occur in the preparation of crude mitochondrial lysates³⁰. The eukaryotic elongation factor eEF1a (50 kDa) is highly methylated on lysine residues^{45,46}. The 15 kDa band appears to be Rpl23ab (14.5 kDa). Rpl23ab is dimethylated on two lysine residues by Rkm1²⁸. This is substantiated by the disappearance of the band given the gene deletion of *RKMI* (Figure 2-4). Additionally, though the antibody used was marketed as an anti-pan methyllysine, it is apparently specific for dimethyllysine³⁴.

With a longer exposure, there is methyllysine signal in two bands between 30 and 35 kDa (Figure 2-4, A). These bands appear to decrease or even disappear in the gene deletions of *YDR316W*, *BMT2*, and *BMT5* (Figure 2-4, A). However, even in lanes that seem to be evenly loaded, there is a difference in abundance of specific proteins (Figure 2-4, A, bottom panel). This may not be due to the lack of any particular methyltransferase. At least one of the protein bands between 30 and 35 kDa is a mitochondrial ribosomal protein. This is demonstrated in two separate experiments where the signal of the band between 31 and 38 kDa decreases in the lysate compared to MRPs or even mitochondrial lysates (Figure 2-4, B).

In vitro methylation reactions

Since determination of methylation sites using *in vivo* methods proved challenging, an *in vitro* approach was used. By mixing lysate from wild type with lysate from methyltransferase gene deletion strains and [³H]AdoMet, potential protein substrates can be identified by autoradiography or liquid scintillation counting. The gene deletion lysate would not contain a functioning methyltransferase and on its own would not demonstrate methylation with addition of [³H]AdoMet. The wild type lysate would have the functioning methyltransferase, but, unless the particular methyl mark is substoichiometric or regulatory, the substrate would be fully

methylated, also resulting in no perceptible signal with addition of [³H]AdoMet. Only when the two are combined will there be detectable methylation as the wild type lysate would provide the methyltransferase and the gene deletion strain lysate would provide the unmethylated substrate. This method was crucial for the identification of Efm4 as the methyltransferase for eEF1a lysine 316³⁵.

I attempted to use this for identification of mitochondrial methyltransferases and substrates. First, I used whole cell lysates of the candidate mitochondrial methyltransferases as well as selected known methyltransferases. This resulted in little to no signal via autoradiography for every combination (data not shown, except for *efm4Δ*, shown in Figure 2-5). Then, I shifted to combining wild type lysate with mitochondrial lysates from the methyltransferase gene deletions. Presumably, this would increase the likelihood of a mitochondrial protein becoming methylated and therefore, radiolabeled.

Each of the methyltransferase gene deletion mitochondrial lysate, when combined with the wild type lysate, did not yield any signal of radioactivity after 4 weeks (Figure 2-5). On the other hand, the *EFM4* deletion lysate yielded a dark band at around 50 kDa when combined with the wild type lysate, indicating strong methylation of eEF1a in the deletion lysate by the wild type Efm4. This result is consistent with previous experiments using this approach³⁵. The results from the known methyltransferase gene deletion strains also gave little to no signal. The cause of the lack of any radioactivity signal from the candidate methyltransferases could not be distinguished from the following potential reasons: 1.) putative methyltransferases did not demonstrate methylation due to the fact that they are not protein methyltransferases, 2.) the assay does not work for substoichiometric methylations, or 3.) the assay does not allow for detection of methylation that occurs on a protein that is not as highly abundant or highly methylated protein

as eEF1a. Other confounding factors include the need for cofactors. In a recent report, radiolabeled methylation increased with the addition of GTP to the *in vitro* reaction with the *efm2Δ* lysate when combined with purified Efm2⁴⁷. This assay appears to work best if there is an idea of what the substrate might be. Again, this is not ideal as I did not know what was being methylated in the mitochondria.

Discussion

The existence of mitochondrial protein methylation, and potentially a mitochondrial protein methyltransferase, has been confirmed with the *in organello* methylation assay. Which methyltransferase is responsible has yet to be determined. It appears that the traditional radioactive methods for identifying and pairing methyltransferases to their substrates are not ideal to use when dealing with two unknowns (unknown methyltransferase and unknown substrate). While these assays were informative and indicated the presence of active methyltransferases, they did not bring me any closer to identifying which proteins in the mitochondria were methylated.

FIGURES

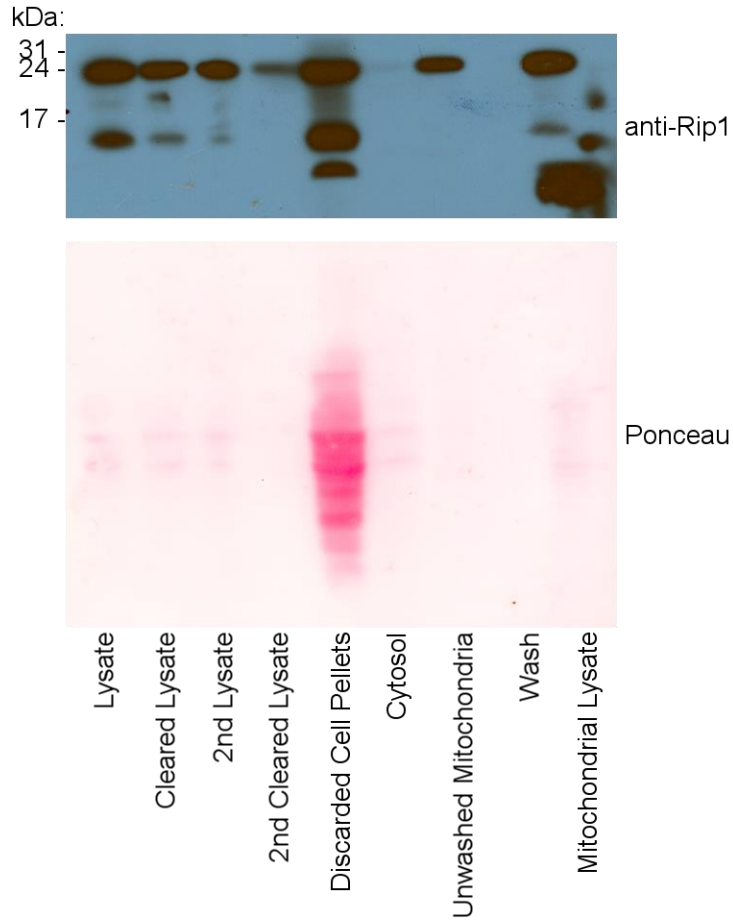


Figure 2-1. Immunoblot of B4741 mitochondrial purification steps. Mitochondria from BY4741 yeast were purified. Lysate represents the step after dounce homogenization of the spheroplasts and cleared lysate was the reserved supernatant after centrifugation of the dounce homogenized lysate. Pellets from that step were dounce homogenized again (2nd lysate) and cleared by centrifugation with the supernatant reserved (2nd cleared lysate). The pellet from that step was discarded (discarded cell pellets). Cleared lysates were combined and centrifuged to pellet the mitochondria. The supernatant from this step (cytosol) was discarded and pellets were resuspended for centrifugation again. The supernatant after centrifugation (unwashed mitochondria) contains the mitochondria. After centrifugation, the supernatant (wash) was

discarded and pelleted mitochondria were lysed (mitochondrial lysate). 20 μ g of each step of the mitochondrial purification were loaded on a pre-cast 4-12% BisTris gel. Protein bands were visualized using an anti-Rip1 antibody (1:1000) and an anti-mouse IgG antibody conjugated to HRP (1:100,000) with chemiluminescence detected using ECL reagent. Top panel is an autoradiography film, exposed to the membrane for 30 seconds. Bottom panel is the Ponceau stained membrane. Mitochondrially localized Rip1 has a molecular weight of 23.4 kDa.

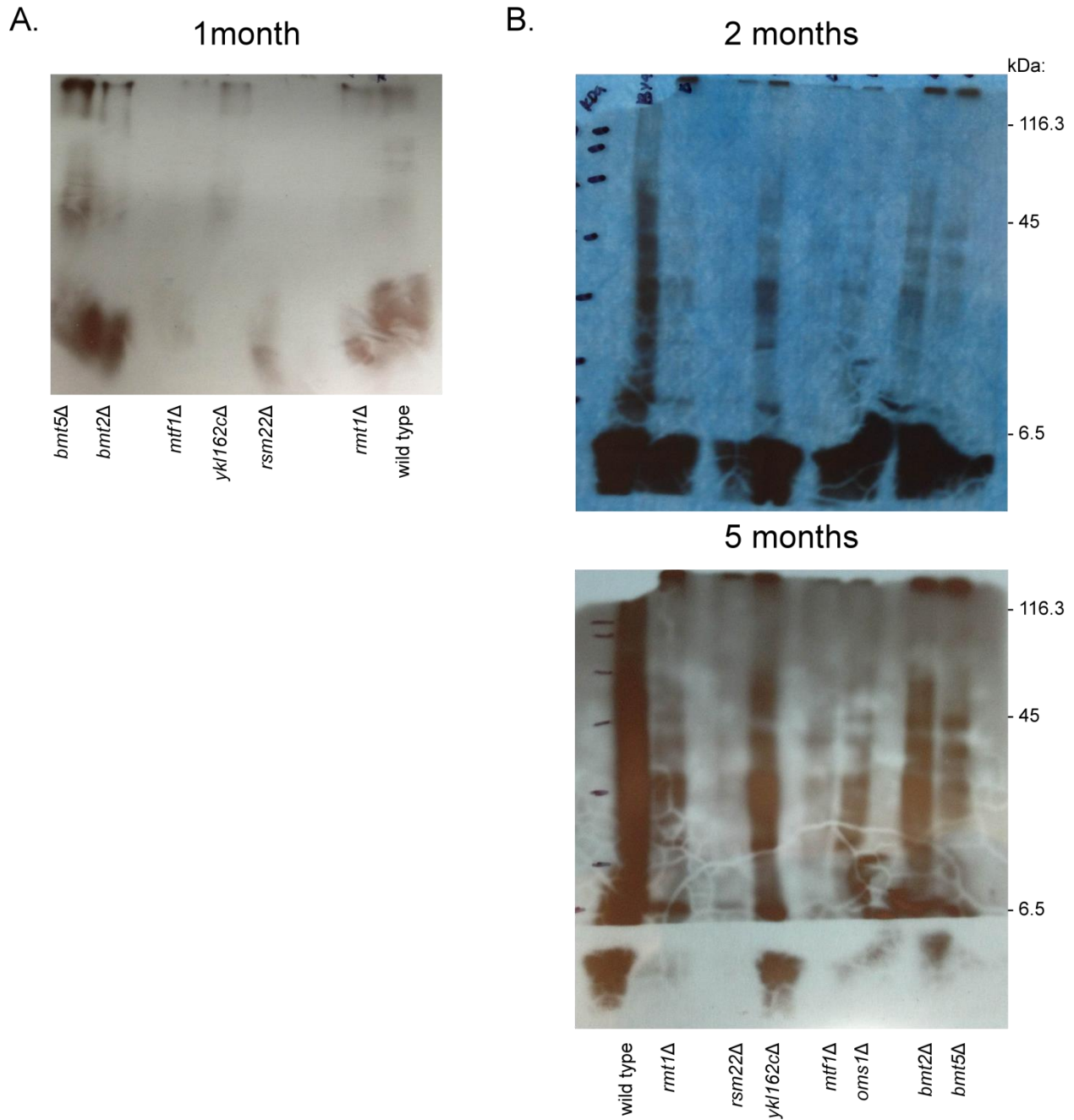


Figure 2-2. Mitochondrial lysates purified from *in vivo* radiolabeled wild type and methyltransferase gene deletion strains. Cells from wild type (BY4742) and methyltransferase gene deletion yeast strains were radiolabeled with [³H]AdoMet. Mitochondria were purified from the radiolabeled cells using a small-scale protocol. The entirety of the mitochondrial lysate was separated with SDS-PAGE on a 12.6% polyacrylamide gel. A.) Autoradiography film after one

month exposure to dried radioactive gel, replicate 1. B.) Autoradiography film after two month (top panel) and five month (bottom panel) exposure to dried radioactive gel, replicate 2. For the five month exposure, the portion of the gel below 6.5 kDa was taped over to avoid overexposure masking the signal of other bands. These images are representative of three separate experiments.

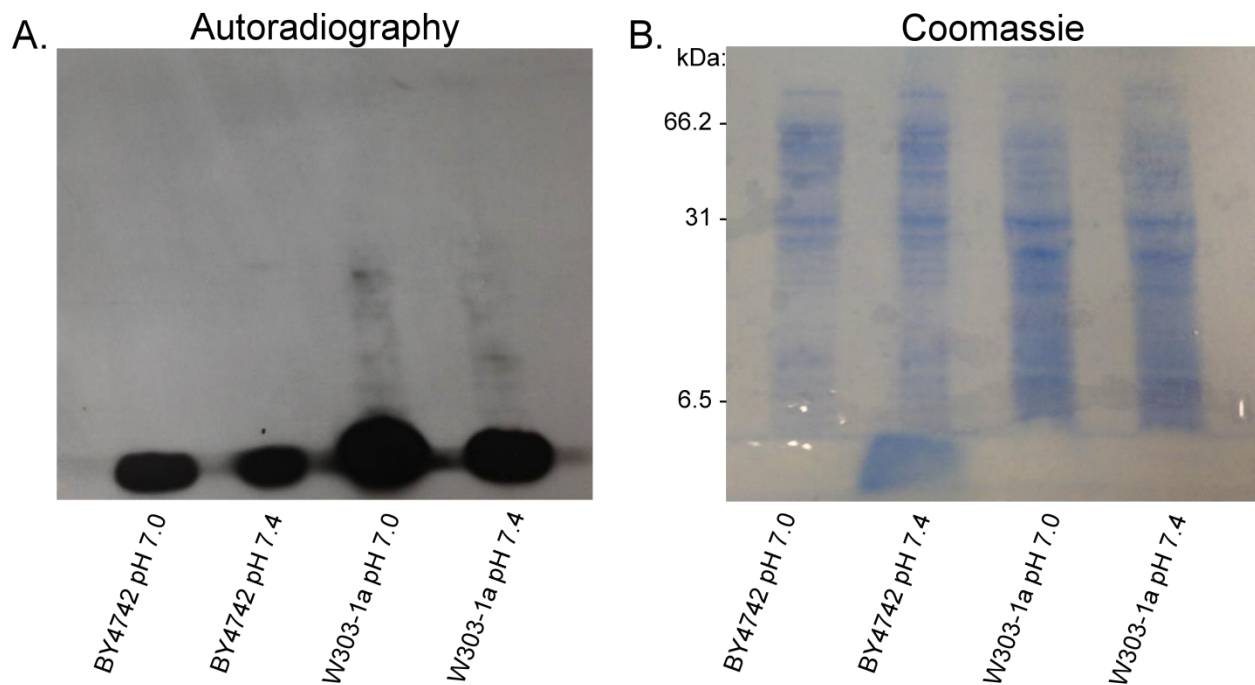


Figure 2-3. *In organello* methylation reactions. A.) Autoradiography film after five week exposure to dried radioactive gel, demonstrating two reaction conditions, pH 7.0 and 7.4, in purified mitochondria that were *in organello* methylated from either BY4742 or W303-1a. B.) Dried Coomassie stained gel of *in organello* labeled mitochondria, purified from either BY4742 (80 μ g) or W303-1a (1 mg) yeast.

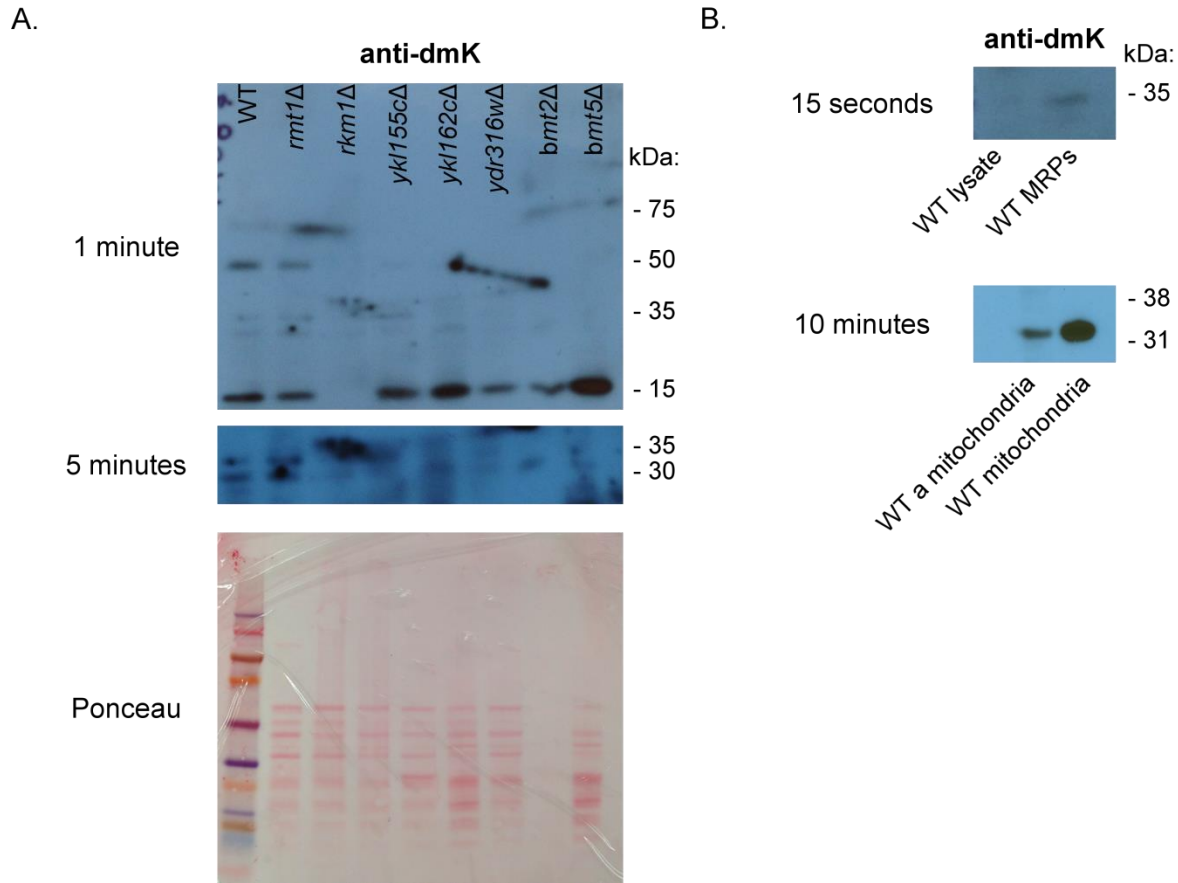


Figure 2-4. Mitochondrial ribosomal proteins containing dimethylated lysine residues isolated from wild type and methyltransferase gene deletion yeast strains. 20 μ g of protein were loaded on a pre-cast 4-12% BisTris gel. Protein bands were visualized using an anti-pan methyl lysine antibody (1:1000, which is more specific to dimethyl lysine) and an anti-rabbit IgG antibody conjugated to HRP (1:100,000) with chemiluminescence detected using ECL reagent. WT denotes the parent strain BY4742 and WT a denotes the parent strain BY4741. A.) MRPs from wild type and methyltransferase gene deletion strains were probed for the presence of dimethylated lysine. The film was exposed to the membrane for one minute and five minutes. Ponceau S was used to ensure even loading and transfer. This image is representative of three separate experiments. B.) WT lysate, mitochondria, and MRPs were probed for the presence of

dimethylated lysine. Two separate experiments with one film exposure for 15 seconds (top panel) and one film exposure for 10 minutes (bottom panel).

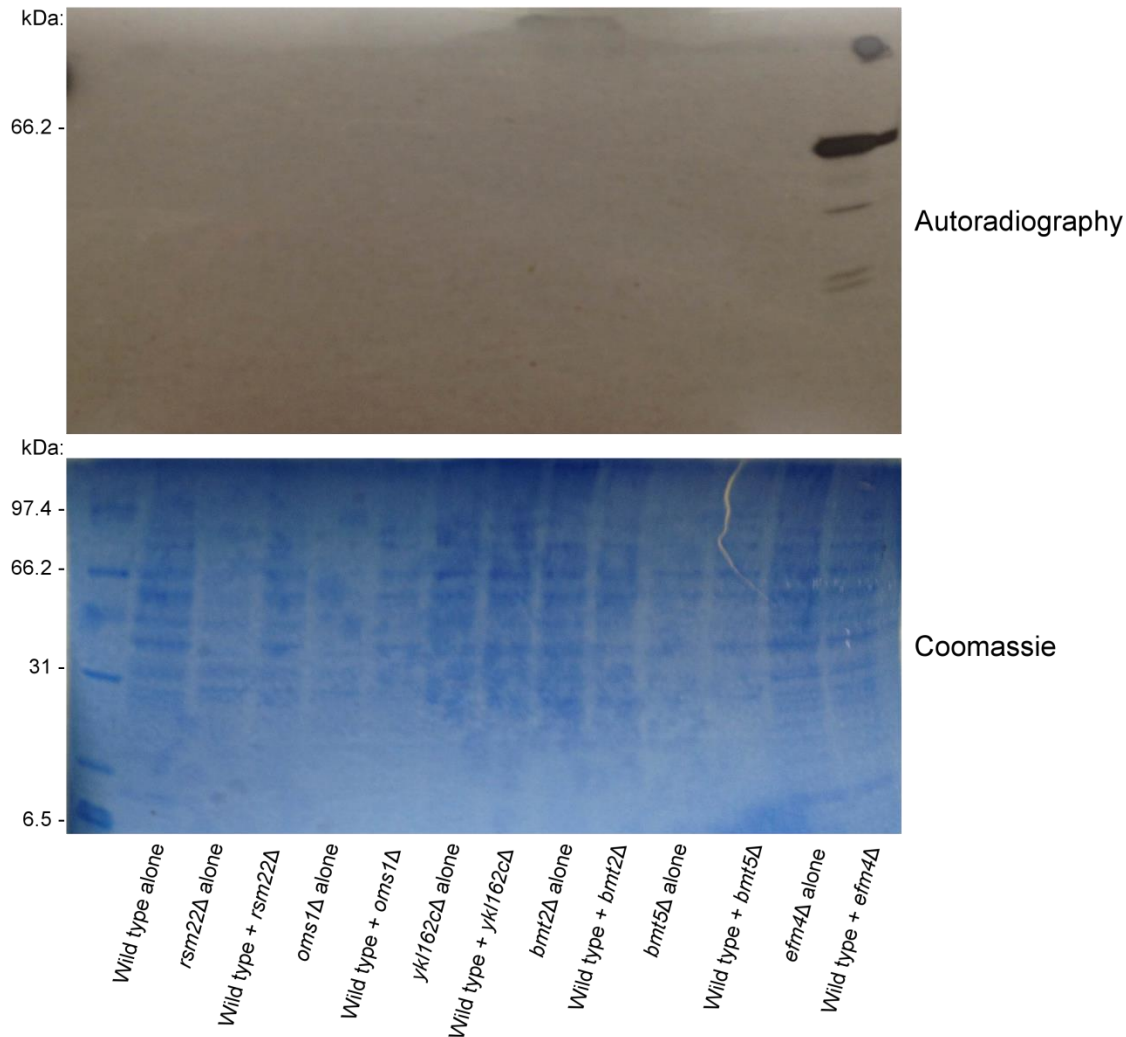


Figure 2-5. *In vitro* methylation reactions with whole cell lysates and mitochondrial lysates of putative and known methyltransferase gene deletion strains. Wild type whole cell lysate (20 μ g) was incubated with gene deletion strain whole cell lysate or mitochondrial lysate (20 μ g) and [3 H]AdoMet. All gene deletion strains here are mitochondrial lysates, except for the reaction with *efm4Δ*, which is a whole cell lysate. Reactions were loaded onto a 12.6% polyacrylamide gel. Gel was stained, destained, incubated in EN 3 HANCE and dried. Autoradiography film was exposed to dried gel for 4 weeks.

TABLES

Table 2-1. Known and putative (in italics and bold) methyltransferases in *Saccharomyces cerevisiae* with predicted substrates^{5,6} (probabilities from Szczepinska *et al.* 2014⁶), confirmed substrate (green is determined prior to 2011, blue prior to 2014, purple after 2014) and the MitoPROT probability⁷ (high probability in gold). This table has expanded on previous work⁴⁸ and the full version of this table is available as Supplemental Table 1 (available on ProQuest.com).

ORF	Protein Name	Known or Predicted Substrate Type from Wlodarski et al. 2011	Predicted Substrate Type from Szczepińska et al. 2014	Protein	RNA	Other	Confirmed Substrate Type	MitoPROT Probability
YBL024W	TRM4/NCL1	tRNA	RNA	0.29	0.66	0.04	tRNA	0.2359
YBR030W	RKM3	Protein	Protein	0.97	0.02	0.02	Protein	0.2169
YBR034C	HMT1/RMT1	Protein	Protein	0.67	0.22	0.11	Protein	0.2468
YBR061C	TRM7	tRNA	RNA	0.14	0.84	0.02	tRNA	0.0603
<i>YBR133C</i>	<i>HSL7</i>	Protein	Protein	0.67	0.22	0.11	Protein	0.0404
YBR141C	BMT2	RNA	RNA	0.14	0.84	0.02	rRNA	0.9990
<i>YBR225W</i>		Unknown	RNA	0.18	0.60	0.22		0.0948
YBR236C	ABD1	mRNA	RNA	0.14	0.84	0.02	mRNA	0.0831
YBR261C	TAE1/NTM1	Protein	Protein	0.67	0.22	0.11	Protein	0.0329
YBR271W	EFM2	Protein	Protein	0.67	0.22	0.11	Protein	0.0257
YCL054W	SPB1	rRNA	RNA	0.14	0.84	0.02	rRNA	0.0362
<i>YCL055W</i>	<i>KAR4</i>	Unknown	RNA	0.18	0.60	0.22		0.0715
YCR047C	BUD23	rRNA	RNA	0.14	0.84	0.02	rRNA	0.0588
YDL014W	NOP1	rRNA	RNA	0.14	0.84	0.02	RNA/ Protein	0.9987
YDL112W	TRM3	tRNA	Protein	0.67	0.22	0.11	tRNA	0.0390
YDL200C	MGT1	Protein	RNA	0.22	0.52	0.26	Protein	0.1124
YDL201W	TRM8	tRNA	RNA	0.14	0.84	0.02	tRNA	0.4220
YDR083W	RRP8	RNA	RNA	0.14	0.84	0.02	rRNA	0.0130
YDR120C	TRM1	tRNA	RNA	0.14	0.84	0.02	tRNA	0.1606
YDR140W	MTQ2	Protein	Protein	0.67	0.22	0.11	Protein	0.0224
YDR198C	RKM2	Protein	Protein	0.97	0.02	0.02	Protein	0.0122
YDR257C	RKM4/SET7	Protein	Protein	0.97	0.02	0.02	Protein	0.0837
<i>YDR316W</i>	<i>OMS1</i>	Protein	RNA	0.14	0.84	0.02		0.9604

ORF	Protein Name	Known or Predicted Substrate Type from Wlodarski et al. 2011	Predicted Substrate Type from Szczepińska et al. 2014	Protein	RNA	Other	Confirmed Substrate Type	MitoPROT Probability
YDR410C	STE14	Protein	RNA	0.22	0.52	0.26	Protein	0.0060
YDR435C	PPM1	Protein	Other	0.33	0.16	0.51	Protein	0.3843
YDR440W	DOT1	Protein	RNA	0.14	0.84	0.02	Protein	0.0226
YDR465C	RMT2	Protein	Protein	0.67	0.22	0.11	Protein	0.1673
YER091C	MET6	Small Molecule	Other	0.37	0.05	0.58	Small molecule	0.2035
YER175C	TMT1	Small Molecule	Other	0.36	0.07	0.57	Small Molecule	0.1952
YGL050W	TYW3	tRNA	RNA	0.22	0.52	0.26	tRNA	0.0727
YGL136C	MRM2	rRNA	RNA	0.14	0.84	0.02	rRNA	0.8861
YGL192W	IME4	mRNA	RNA	0.18	0.60	0.22	mRNA	0.365
YGR001C	AML1/EFM5	Unknown	Protein	0.67	0.22	0.11	Protein	0.0137
YGR157W	CHO2	Lipid	Other	0.07	0.09	0.84	Lipid	0.3173
<i>YGR283C</i>		RNA	RNA	0.14	0.84	0.02		0.0387
YHL039W	EFM1	Protein	Protein	0.97	0.02	0.02	Protein	0.0874
YHR070W	TRM5	tRNA	RNA	0.14	0.84	0.02	tRNA	0.9777
YHR109W	CTM1	Protein	Protein	0.97	0.02	0.02	Protein	0.2220
YHR119W	SET1	Protein	Protein	0.76	0.23	0.01	Protein	0.4881
<i>YHR207C</i>	<i>SET5</i>	Protein	Protein	0.84	0.01	0.15	Protein	0.0186
YHR209W	CRG1	Unknown	Other	0.36	0.07	0.57	Small Molecule	0.3334
YIL064W	SEE1/EFM4	Protein	Protein	0.67	0.22	0.11	Protein	0.2229
YIL096C	BMT5	RNA	RNA	0.14	0.84	0.02	rRNA	0.9956
YIL110W	HPM1	Protein	Protein	0.67	0.22	0.11	Protein	0.0211
<i>YJL105W</i>	<i>SET4</i>	Protein	Protein	0.76	0.23	0.01		0.0087

ORF	Protein Name	Known or Predicted Substrate Type from Wlodarski et al. 2011	Predicted Substrate Type from Szczepińska et al. 2014	Protein	RNA	Other	Confirmed Substrate Type	MitoPROT Probability
YJL125C	GCD14/TRM61	tRNA	RNA	0.14	0.84	0.02	tRNA	0.1186
YJL168C	SET2	Protein	Protein	0.76	0.23	0.01	Protein	0.0839
YJR073C	OPI3	Lipid	Other	0.07	0.09	0.84	Lipid	0.3210
YJR129C	EFM3	Protein	Protein	0.67	0.22	0.11	Protein	0.0666
<i>YKL155C</i>	<i>RSM22</i>	Protein	RNA	0.14	0.84	0.02		0.9670
<i>YKL162C</i>		Unknown	RNA	0.18	0.60	0.22		0.1068
<i>YKR029C</i>	<i>SET3</i>	Protein	Protein	0.77	0.13	0.11		0.0489
YKR056W	TRM2	tRNA	RNA	0.14	0.84	0.02	tRNA	0.0320
YKR069W	MET1	Small Molecule	Other	0.37	0.05	0.58	Small Molecule	0.0769
YLL062C	MHT1	Small Molecule	Other	0.37	0.05	0.58	Small Molecule	0.1795
YLR063W	BMT6	RNA	RNA	0.14	0.84	0.02	rRNA	0.8157
YLR137W	RKM5	Unknown	Other	0.36	0.07	0.57	Protein	0.041
YLR172C	DPH5	Protein	Other	0.37	0.05	0.58	PTM on a protein	0.1076
YLR186W	EMG1	rRNA	RNA	0.14	0.84	0.02	rRNA	0.0368
YLR285W	NNT1/EFM7	Protein	Protein	0.67	0.22	0.11	Protein	0.0025
YML008C	ERG6	Lipid	Other	0.36	0.07	0.57	Lipid	0.1300
YML014W	TRM9	tRNA	RNA	0.14	0.84	0.02	tRNA	0.0467
YML110C	COQ5	Small Molecule	Other	0.36	0.07	0.57	Small Molecule	0.9983
<i>YMR209C</i>		Unknown	RNA	0.14	0.84	0.02		0.8658
<i>YMR228W</i>	<i>MTF1</i>	Unknown	Protein	0.67	0.22	0.11		0.2032
<i>YMR310C</i>		RNA	RNA	0.14	0.84	0.02		0.2973

ORF	Protein Name	Known or Predicted Substrate Type from Wlodarski et al. 2011	Predicted Substrate Type from Szczepińska et al. 2014	Protein	RNA	Other	Confirmed Substrate Type	MitoPROT Probability
YNL022C	RCM1	RNA	RNA	0.14	0.84	0.02	rRNA	0.1673
YNL024C	EFM6	Protein	Protein	0.67	0.22	0.11	Protein	0.0381
YNL061W	NOP2	RNA	Protein	0.67	0.22	0.11	rRNA	0.0458
YNL063W	MTQ1	Protein	RNA	0.14	0.84	0.02	Protein	0.9486
<i>YNL092W</i>		Unknown	Other	0.36	0.07	0.57	dipeptide	0.1150
YOL093W	TRM10	tRNA	RNA	0.14	0.84	0.02	tRNA	0.0262
YOL096C	COQ3		Other	0.36	0.07	0.57	Small Molecule	0.9953
YOL124C	TRM11	tRNA	RNA	0.14	0.84	0.02	tRNA	0.2977
YOL125W	TRM13	tRNA	RNA	0.14	0.84	0.02	tRNA	0.0218
YOL141W	PPM2/TYW4	tRNA	RNA	0.67	0.22	0.11	tRNA	0.0492
YOR021C	SFM1	RNA	Protein	0.67	0.22	0.11	Protein	0.2378
YOR074C	TMP1/CDC21	Small Molecule	Other	0.35	0.12	0.53	Small Molecule	0.0058
YOR201C	MRM1/PET56	rRNA	RNA	0.14	0.84	0.02	rRNA	0.8456
YOR239W	ABP140/ TRM140	Protein	Protein	0.67	0.22	0.11	tRNA	0.0142
YPL030W	TRM44	tRNA	RNA	0.14	0.84	0.02	tRNA	0.0109
YPL157W	TGS1	telomerase/sn/ snoRNA	RNA	0.14	0.84	0.02	RNA	0.9723
<i>YPL165C</i>	<i>SET6</i>	Protein	Protein	0.76	0.23	0.01		0.0094
YPL208W	RKM1	Protein	Protein	0.97	0.02	0.02	Protein	0.1616
YPL266W	DIM1	rRNA	RNA	0.14	0.84	0.02	rRNA	0.1486
YPL273W	SAM4	Small Molecule	Other	0.37	0.05	0.58	Small Molecule	0.5904
<i>YMR321C</i>			Other					0.1220

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CHAPTER THREE

Preliminary Characterization of Putative Mitochondrial Protein Methyltransferases

Introduction

Currently, there is only one known mitochondrially localized protein methyltransferase in *Saccharomyces cerevisiae*. Mtq1 methylates Mrf1, the mitochondrial release factor, on a conserved glutamine residue. This methylation is sub-stoichiometric and loss of the enzyme results in a slight decrease in growth on non-fermentable carbon sources¹. Protein glutamine methylation, though greatly conserved among the release factors, is relatively rare². I was interested in the existence of other protein methyltransferases in the mitochondria. To do this, I began by examining putative methyltransferases that were discussed in Chapter Two, including (protein name/ORF): Rsm22/YKL155C, Oms1/YDR316W, Mtf1/YMR228W, and Ykl162c/YKL162C.

Rsm22 has been characterized as a small subunit mitochondrial ribosomal protein through purification studies³. Rsm22 is a mitochondrial specific ribosomal protein, with no homology to the ribosomal core proteins that are functionally conserved⁴. It was first identified as a putative protein methyltransferase due to structural similarity with other protein methyltransferases⁵. Later, it had been predicted and reportedly confirmed via *in vitro* methylation reactions to be an RNA methyltransferase⁶ (prediction accuracy for methyltransferase substrates is discussed extensively in Chapter Two). This assay may not be able to completely rule out other substrates as I have discussed the drawbacks to such a method in Chapter Two. As Rsm22 complexes with the mitoribosome, it is in close proximity with both mitochondrial RNA and other MRPs. However, as mitochondrial rRNA is minimally modified^{7,8}, it is possible that Rsm22 acts on a protein substrate. Interestingly, Rsm22's human homolog (Table 3-1), putative methyltransferase⁹ METT11D1, has been associated with combined

oxidative phosphorylation deficiencies when the gene encoding the protein has been disrupted¹⁰. In trypanosomes, Rsm22 is required for small subunit stability and assembly¹¹.

Recent yeast studies have suggested that Rsm22 may only be transiently associated with the mitochondrial ribosome. Even though tandem affinity purification of Rsm22 pulls down other mitochondrial ribosomal proteins³, expression patterns show that *RSM22* is expressed ahead of the mitochondrial ribosomal proteins¹². Park and Bader used this evidence to suggest that Rsm22 was an RNA methyltransferase because the gene deletions of *RSM22* cause a respiration phenotype¹². In mammalian mitochondrial ribosomes, methylation of the 3'-end of the small subunit rRNA is required for ribosome assembly and stability¹³, much like Rsm22 in some organisms. Park and Bader suggested that Rsm22 may be responsible for a similar methylation reaction¹². In mammalian mitochondria, this methylation is placed by TFB1M¹⁴, which is orthologous to Mtf1 in yeast. Mtf1, discussed later in this chapter, is also a putative methyltransferase that could be responsible for this mitochondrial rRNA methylation if such methylation occurs in yeast as well. Additionally, the recent mitochondrial ribosome structure did not include Rsm22¹⁵, further suggesting that it only transiently associates with the mitochondrial ribosome, likely due to its methyltransferase function.

OMS1 is a multicopy suppressor for mutations in the *OXA1* gene¹⁶. Oxa1 facilitates the insertion of mitochondrial proteins into the mitochondrial inner membrane. With deletion of the methyltransferase domain, overexpressed Oms1 does not suppress the phenotypes associated with *oxa1* mutations¹⁶. Therefore, Oms1's ability to suppress *oxa1Δ* phenotypes is independent of its role as a methyltransferase. Because Oms1 has a basic isoelectric point, others have hypothesized that it most likely methylates acidic substrates, such as DNA, phospholipids, or

even highly negative regions of Oxa1 (though none of these possibilities have been directly shown¹⁶).

More recently, Oms1 has been identified as being necessary for cytochrome *c* oxidase assembly through the stabilization of Cox1¹⁷. This again is independent of its function as a methyltransferase. The removal of the methyltransferase domain on Oms1 did not affect this activity¹⁷. Like Rsm22, Oms1 was originally categorized as a putative protein methyltransferase⁵, but later suggested to be a putative RNA methyltransferase⁶. This further suggests that Oms1 may bind nucleotides or acidic proteins. *In vitro* protein methylation assays showed no protein substrates⁶, but this could be due to the fact that Oms1 is a transmembrane protein¹⁶ and requires additional stabilization for *in vitro* investigations.

Mtf1 is the polymerase specificity factor for the mitochondrial RNA polymerase Rpo41¹⁸. It is homologous to TFB1M in human mitochondria (Table 3-1). TFB1M plays the same function in mammalian mitochondria, but also exists as an RNA methyltransferase¹⁴. The resultant dimethylated adenines in the 12S rRNA are required for mitochondrial ribosome biogenesis. Furthermore, TFB1M is required for mitochondrial function¹³. The cytoplasmic equivalent in yeast Dim1 is dispensable as cells can grow without the conserved dimethyl adenine marks¹⁹. It is unclear if the corresponding dimethyl adenines exist in yeast mitochondria as the rRNA is minimally methylated⁷.

Not much is known about Ykl162c. It was identified as a mitochondrial protein in a large-scale GFP-tagged protein screen²⁰. In probabilistic computational studies, it has been characterized as a protein or RNA methyltransferase^{5,6}. Ykl162c has a human homolog NDUFAF7, determined by DELTA BLAST²¹ in 2014 (though this no longer occurs in the

current version of BLAST) and Ykl162c is notated on UniProt²² as the NDUFAF7 homolog by similarity.

NDUFAF7 is the only known arginine methyltransferase in the mitochondria⁹, suggesting Ykl162c may be a protein methyltransferase. NDUFAF7 methylates NDUF52 in Complex1, aiding in assembly⁹. While this methylation reaction is vital for embryogenesis through this function²³, there does not seem to be any corresponding substrate in the yeast mitochondria as there is no Complex 1 per se- although Nde1 and Nde2 play a similar function²⁴. Given this association, it is possible for Ykl162c to be an arginine methyltransferase in the yeast mitochondria, but it would have to act on differing substrates.

Based on the data presented in Chapter Two (Table 2-1), there are other putative and known protein methyltransferases that have a high probability of localizing to the mitochondria. However, none have confirmed mitochondrial substrates or confirmed mitochondrial localization. There is also the possibility that methyltransferases can perform the methyl transfer reaction prior to import. The most well-known example of this in yeast is Ctm1. Ctm1 methylates cytochrome *c* pre-import²⁵. The trimethylated lysine on cytochrome *c* does not appear to affect its function, but rather aids in the transport into the mitochondria²⁶ and disrupts necessary protein-protein interactions²⁷.

The situation with Ctm1 may not be unique; other cytoplasmically localized methyltransferases may act on mitochondrial substrates pre-import as well. Alternatively, the methyltransferase responsible for methylating a mitochondrial substrate may be dually localized. This is the case in *Arabidopsis thaliana* where PrmA localizes to both the chloroplast and mitochondria to methylate the rpL11 equivalent in each organelle²⁸. Determining if a methyltransferase (localized in either the cytosol or mitochondria) has a mitochondrial substrate

may be identified through phenotype screens. If gene deletions of a methyltransferase cause growth defects on non-fermentable carbon sources, the methyltransferase may be acting on a mitochondrial substrate.

Of the putative mitochondrial methyltransferases, three are known to be respiration deficient with gene deletion (as summarized in Table 3-2): Rsm22, Oms1, and Mtf1. Mtf1 has a slight growth defect when grown on non-fermentable carbon sources (Table 3-2). This chapter aims to confirm the previous phenotypes and identify any new respiration deficient phenotypes for other known and putative methyltransferases.

Methods

Yeast strains

Yeast strains used in this chapter are gene deletion strains from the Yeast Knockout Collection (obtained from GE Dharmacon). The ORF of interest has been replaced with a KanMX cassette, producing the knockout. The deletion has been made in either the BY4741 (*MAT α*) or the BY4742 (*MAT α*) background. ORFs are listed in Table 3-3.

Growth curves

Overnight cultures of the strains of interest and wild type (BY4742) were made in either 5 mL of YPD (1% yeast extract, 2% peptone, 2% dextrose), LM (described in Chapter Two), or other respiratory carbon sources, such as D-raffinose or D-galactose. Overnight cultures were grown at 30 °C in an incubator shaker rotating at 250 rpm overnight. 100 mL of each medium was inoculated to an optical density at 600 nm of 0.1-0.15. Cells were grown at 30 °C in an

incubator shaker with 250 rpm. The optical density at 600 nm was taken every 1.5-2 hours and recorded over a period of 10-12 hours.

Spot test assays for respiration deficiency

Strains were cultured in 5 mL of YPD and grown at 30 °C in an incubator shaker with 250 rpm overnight. 20 mL of YPD were inoculated with the overnight culture to an optical density at 600 nm of 0.1-0.2. Cultures were grown at 30 °C in an incubator shaker with 250 rpm until an optical density at 600 nm of 0.4-0.8. From each culture, 1 mL was harvested by centrifugation at 5000 x g for 5 min. The pellet was resuspended in 1 mL of water and centrifuged again at 5000 x g for 5 minutes. Using water, the optical density was adjusted for each culture to 0.5. The OD_{600nm} 0.5 cultures were then serially diluted five-fold in water five times to a final OD_{600nm} of 0.0008. The 2.5 or 5 µL original dilutions and serial dilutions were then spotted onto an agar plate of YPD, LM, or other non-fermentable carbon sources, including YPG (1% yeast extract, 2% peptone, and 3% glycerol) and YPEG (1% yeast extract, 1% peptone, 3% glycerol, and 3% ethanol) . Cells grew at 30 °C in an incubator for three days. On the third day, plates were taken out of the incubator and imaged.

Confirmation of Ykl162c Mitochondrial Localization

Ykl162c-TAP strain (GE Dharmacon, YSC1178-202232108) was grown in LM at 30 °C until an optical density at 600 nm greater than 3. Cells were harvested by centrifugation at 5,000 x g for 5 minutes. Cells were fractionated into lysate, cytosol, nucleus, and mitochondrial lysate²⁹. Fractions were stored at -20 °C until analysis.

20 µg of protein for each fraction were mixed with 2X SDS sample buffer and heated 3 minutes at 100 °C. Mitochondrial lysate fraction was left out at room temperature for 20 minutes, instead of heated at 100 °C, giving the potential for aggregation³⁰. Samples were loaded onto a pre-cast NUPAGE Novex 4-12% BisTris gel (ThermoFisher Scientific NP0321) in addition to 10 µL of a full-range rainbow ladder (GE Healthcare Lifesciences RPN800E). The gel was run using the NUPAGE MES running buffer system (ThermoFisher Scientific NP0002) for 45 minutes at 200 V.

Proteins from the gel were transferred to a PVDF membrane (Amersham Hybond, GE Healthcare Life Sciences, Cat 10600023). The membrane was blocked in 5% non-fat dry milk in Tris buffered saline with 0.1% (v/v) Tween 20 (TBST) overnight on an orbital incubator. The membrane was incubated for 1.5 hours in rabbit anti-TAP antibody that was 1:1000 diluted in 1% non-fat dry milk in TBST (ThermoFisher Scientific, CAB1001). The membrane was incubated for 1.5 hours with a 1:6666 dilution of anti-rabbit IgG conjugated to horseradish peroxidase (HRP) in 1% non-fat dry milk in phosphate-buffered saline (PBST) (Cell Signaling, 7074). After ECL reagent (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare Life Sciences, RPN2232) exposure for 5 minutes, the gel was exposed to film.

Results

The selected methyltransferase gene deletion strains were either known protein methyltransferases or putative methyltransferases with predicted protein substrates^{5,6}. The mitochondrially localized putative methyltransferases were also included. Through screening the methyltransferase gene deletion strains, the majority of the methyltransferases are not respiration deficient, as seen in Table 3-3. This result was to be expected as most of the known protein

methyltransferases screened do not have identified mitochondrial substrates. The respiration defects of the gene deletions for three of the putative mitochondrial methyltransferase *YKL155C* (Rsm22), *YDR316W* (Oms1), and *YMR228W* (Mtf1) was confirmed, Table 3-3. The severe respiration deficiency of the gene deletion of *YKL155C* is demonstrated in Figure 3-1. Compared to the wild type strain, *ykl155cΔ* displays no growth when plated on ethanol/glycerol.

The only putative protein methyltransferase gene that did not give a growth phenotype with non-fermentable carbon sources was *YKL162C*, Figure 3-1. *ykl162cΔ* does not show any growth defect. This is measured in similarity of growth to wild and the does the negative control *ypr285wΔ*. *YLR285W* encodes Efm7. Efm7 is known to methylate the eukaryotic elongation factor eEF1a on its N-terminal glycine and adjacent lysine³¹. *EFM7* gene deletion is not expected to cause respiration deficiency.

Results were similar in liquid culture as on the spot plates. However, there is a slight defect for the *YNL063W* deletion when grown in semisynthetic lactate medium (LM) compared to wild type, Figure 3-2. This is consistent with original reports that *ynl063wΔ* has slight growth defects when provided non-fermentable carbon sources, Table 3-1. This was not observed in the spot plate assays (Table 3-3). Additionally, though comparisons are made to wild type, both negative controls, *ypl208wΔ* and *ybr034cΔ*, encoding Rkm1 and Rmt1 respectively, have similar growth to the *YNL063W* gene deletion. Neither Rkm1 nor Rmt1 has known mitochondrial substrates and strains deficient in these proteins do not have any known mitochondrial phenotypes.

The cellular localization of Ykl162c was investigated. Mitochondrial localization had only been seen in a large-scale study²⁰ and inferred through homology to NDUFAF7⁹. Given the absence of a mitochondrial phenotype, it was unclear if Ykl162C is truly mitochondrially

localized. Using the tandem affinity purification (TAP) tag³² system, Ykl162c-TAP containing cells were fractionated into lysate, nucleus, cytosol, and mitochondrial lysate. Using an anti-TAP antibody, more Ykl162c-TAP was identified in the mitochondrial lysate than in the other fractions (Figure 3-3). The increase in mitochondrial protein appears to be due to localization as opposed to the protein amount loaded, as less protein was loaded for the mitochondrial lysate fraction (Figure 3-3).

Discussion

The known respiration deficient phenotypes of the gene deletions of *YKL155C*, *YDR316W*, and *YMR228W* have been confirmed. Whether this is due to their methyltransferase activity or not is unclear. Additionally, their mitochondrial substrates could be vital proteins (e.g. mitochondrial ribosomal proteins involved in translation), RNA, or small molecules or lipids. The *YKL162C* gene deletion does not cause mitochondrial defects in either liquid or solid media. This could be due to the particular methylation reaction it performs or the substrate it methylates. While Ykl162c does not have any respiration defects, its mitochondrial localization has been confirmed more directly using immunoblotting. Additionally, based on the spot test assay results and similar growth to cytoplasmic methyltransferases, *ynl063wΔ*, though mitochondrial, does not have significant growth defects.

While the majority of known and putative methyltransferases screened did not show any respiration defects, it should be noted that Ctm1, other than having some oxidative stress sensitivity (Table 3-2), is not respiration deficient. Both Ctm1 and Mtq1 have substrates that are required for mitochondrial function, but methylation reactions do not appear to be crucial to those functions. It is possible that any of the methyltransferases screened methylate a

mitochondrial substrate pre-import and lack of that methylation reaction does not cause a respiration deficient phenotype. Additionally, many proteins localize to the mitochondria, but are not essential for mitochondrial function. If those proteins were in fact methylated, then the lack of the methyl mark would not cause a significant decrease in respiration. Phenotype screens, such as these, are useful, but if methylation occurs pre-import or if the methylation event does not affect respiration, then no phenotype will be observed. As evidenced here, this can confound the process of discovering a new methyltransferase or associating a mitochondrial methyl mark to a known or putative methyltransferase.

FIGURES

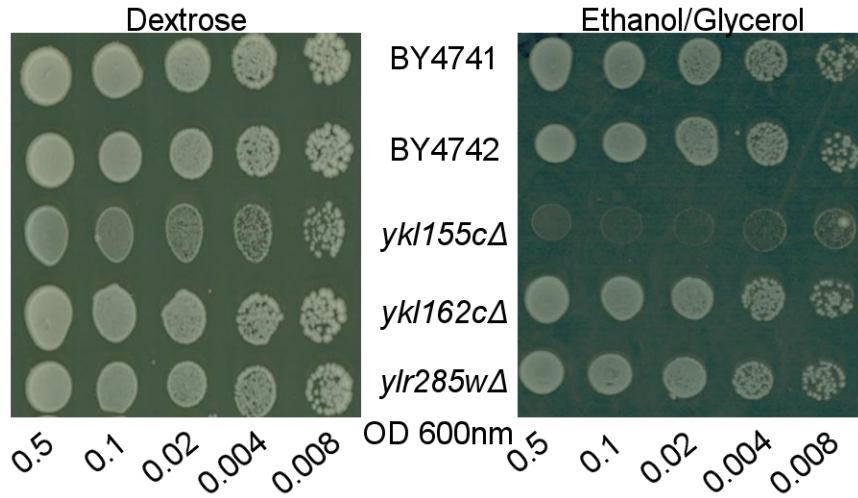


Figure 3-1. Example of a spot test assay for growth of *Saccharomyces cerevisiae*. Methyltransferase gene deletion strains were grown at 30 °C until an optical density at 600 nm of 0.4-0.8. Serial dilutions of each gene deletion strain (OD_{600nm} starting from 0.5 to 0.008) were plated on a YPD plate and on a YPEG (ethanol/glycerol) plate. After 3 days of growth at 30 °C, plates were photographed. Two mitochondrial putative methyltransferases (*ykl155cΔ* and *ykl162cΔ*) were tested in addition to the negative control (*ylr285wΔ*), lacking the eukaryotic elongation factor methyltransferase Efm7.

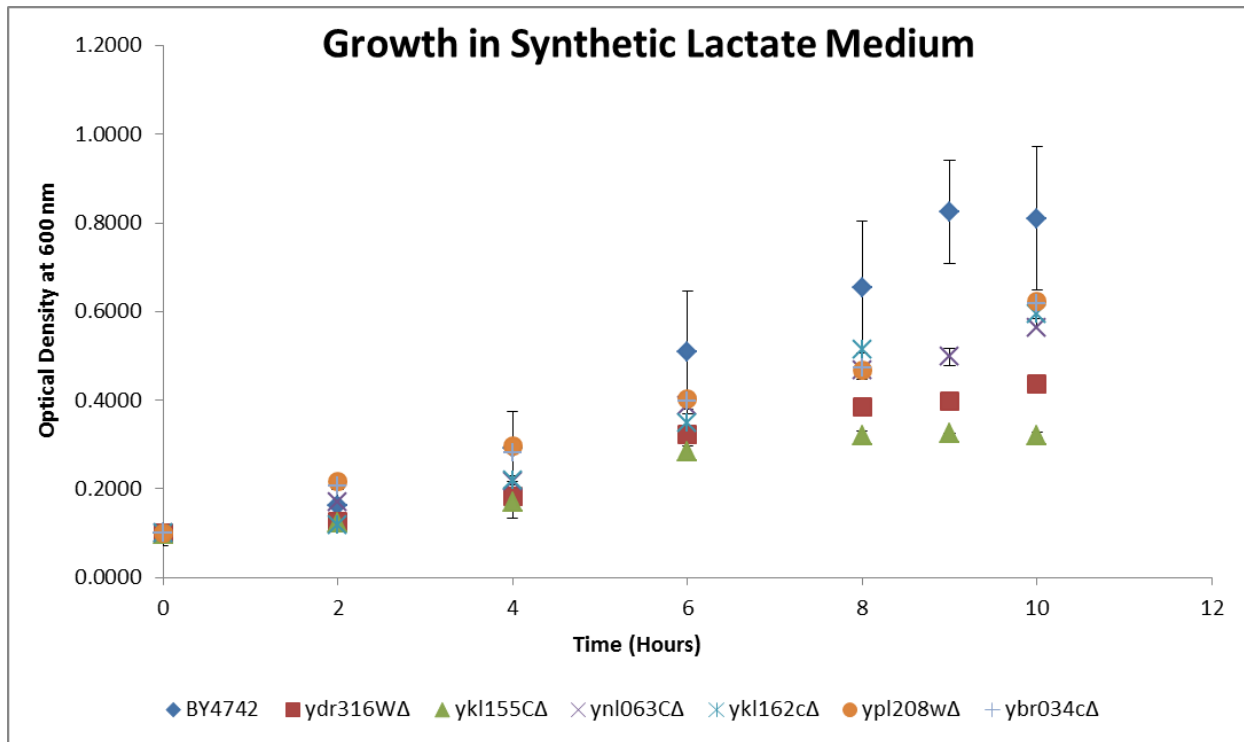


Figure 3-2. Growth of null mutants of putative mitochondrial methyltransferases in *Saccharomyces cerevisiae* in semisynthetic lactate liquid media. The optical density at 600 nm of cultures of yeast strains is plotted as a function of time in hours. BY4742 (wild type) is in blue, *ydr316wΔ* is in red, *ykl155cΔ* is in green, *ynl063cΔ* is in purple, *ykl162cΔ* is in light blue, *ypl208wΔ* is in orange, and *ybr034cΔ* is in gray. Error bars are standard deviation and n=1-3 (see Table 3-3).

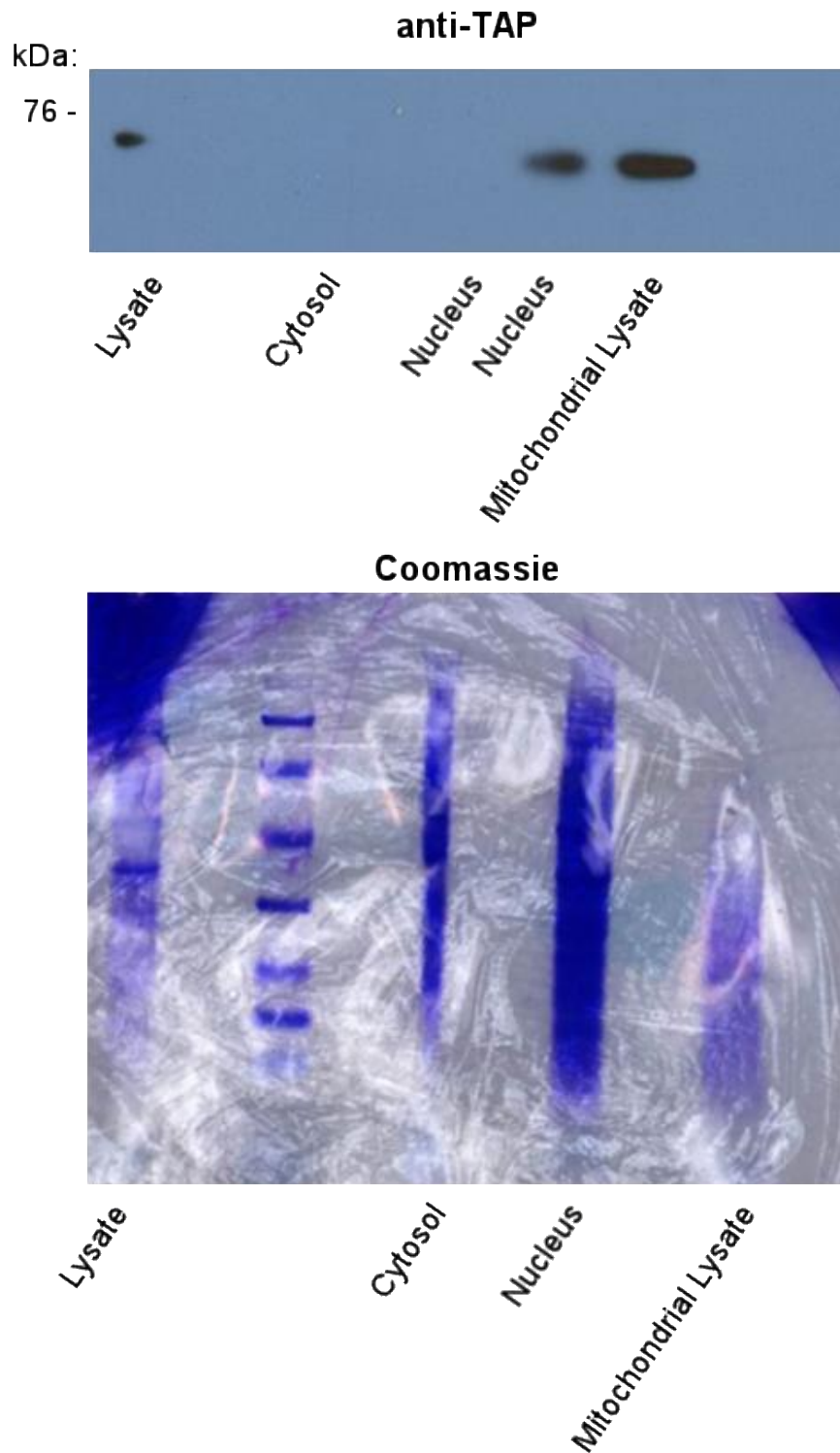


Figure 3-3. Mitochondrial localization of TAP-tagged Ykl162c in *Saccharomyces cerevisiae*.

20 µg of cell lysate, cytosol, nucleus, and mitochondrial lysate protein were prepared for SDS-

PAGE on a pre-cast 4-12% BisTris gel. Proteins from the gel were transferred to a PVDF membrane and the membrane was blocked in 5% non-fat dry milk in TBST overnight. Protein bands were visualized by incubation with an anti-TAP antibody (1:1000) for 1.5 hours and an anti-rabbit IgG antibody conjugated to HRP (1:6666) for 1.5 hours. Chemiluminescence was detected using ECL reagent. Film was exposed for 1 minute. Top panel is the anti-TAP immunoblot. Bottom panel is a representative Coomassie stained gel. Ykl162C is 46.5 kDa. With the TAP tag, the molecular weight of the protein should be around 67.5 kDa.

TABLES

Table 3-1. Protein BLAST of mitochondrially localized putative methyltransferases. Both protein-protein BLAST (blast-p)³³ and Domain Enhanced Lookup Time Accelerated BLAST (DELTA BLAST)²¹ were performed. BLAST searches were most recently done in February 2017.

BLAST type	Name	ORF	Human homolog	E value (yeast to human)	Percent Identity (yeast to human)	Mutual best hits	E value (human to yeast)
blast-p	Oms1	YDR316W	No significant similarity found				
DELTA	Oms1	YDR316W	METTL7B	0.004	27%	2 nd hit	0.001
blast-p	Rsm22	YKL155C	METTL11D	0.018	38%	Yes	0.008
DELTA	Rsm22	YKL155C	None with an E-Value better than threshold				
blast-p	Ykl162c	YKL162C	No significant similarity found				
DELTA	Ykl162c	YKL162C	No significant similarity found				
blast-p	Mtf1	YMR285W	No significant similarity found				
DELTA	Mtf1	YMR285W	TFB1M	0.36	27%	2 nd hit	0.012

Table 3-2. Known respiration and stress associated phenotypes of putative and known methyltransferases with mitochondrial substrates. Data from the Saccharomyces Genome Database, accessed January 2017.

Name	ORF	Strain	Experiment	Carbon source/ stressor	Phenotype	Respiration deficient
Oms1	YDR316W	null, S288C	classical genetics ³⁴	glycerol	petite	yes
Oms1	YDR316W	null, S288C, homozygous diploid	competitive growth ³⁵	glycerol	decreased growth	
Oms1	YDR316W	null, S288C, homozygous diploid	competitive growth ³⁵	ethanol	decreased growth	
Oms1	YDR316W	null, S288C, homozygous diploid	competitive growth ³⁵	lactate	decreased growth	
Ctm1	YHR109W	null, S288C, homozygous diploid	competitive growth ³⁶	3 mM hydrogen peroxide	decreased growth	
Rsm22	YKL155C	null, S288C, homozygous diploid	competitive growth ³⁵	glycerol	decreased growth	
Rsm22	YKL155C	null, S288C, homozygous diploid	competitive growth ³⁵	ethanol	decreased growth	
Rsm22	YKL155C	null, S288C, homozygous diploid	competitive growth ³⁵	lactate	decreased growth	
Rsm22	YKL155C	null, S288C, homozygous diploid	competitive growth ³⁶	glycerol	decreased growth	
Rsm22	YKL155C	null, W303-1A or MGD353-13D	classical genetics ³ (data for the reported phenotype was not shown in this report)	glycerol	no growth	yes
Rsm22	YKL155C	null, S288C	systematic mutation set ³⁷	glycerol	no growth	yes

Rsm22	YKL155C	null, S288C	systematic mutation set ³⁸	glycerol	no growth	yes
Ykl162c	YKL162C					
Mtf1	YMR228W	null, S288C	systematic mutation set ³⁷	glycerol	no growth	yes
Mtf1	YMR228W	null, S288C	systematic mutation set ³⁸	glycerol	no growth	yes
Mtf1	YMR228W	null, S288C, homozygous diploid	systematic mutation set ³⁹	glycerol	no growth	yes
Mtq1	YNL063W	null, B-8114, haploid <i>MATa</i>	classical genetics ¹	ethanol	slight decrease in growth	

Table 3-3. Experimentally determined growth phenotypes of null mutants of known and putative methyltransferases. Mutants obtained from GE Dharmacon in the strain background of BY4741 or BY4742. Mutants are ordered alphabetically by ORF.

Name	ORF	Experiment	Carbon source	Growth (compared to wild type)	Repli-cates
Rkm3	YBR030W	spot plate	dextrose	no effect	1
Rkm3	YBR030W	spot plate	glycerol	no effect	1
Rmt1	YBR034C	spot plate	dextrose	no effect	2
Rmt1	YBR034C	liquid culture	galactose	no effect	1
Rmt1	YBR034C	spot plate	glycerol	no effect	2
Rmt1	YBR034C	liquid culture	lactate	slight decrease	1
Hsl7	YBR133C	spot plate	dextrose	no effect	1
Hsl7	YBR133C	spot plate	glycerol	no effect	1
Rkm4	YDR257C	spot plate	dextrose	no effect	1
Rkm4	YDR257C	spot plate	glycerol	no effect	1
Oms1	YDR316W	liquid culture	dextrose	no effect	3
Oms1	YDR316W	spot plate	dextrose	no effect	2
Oms1	YDR316W	liquid culture	galactose	no effect	1
Oms1	YDR316W	spot plate	glycerol	no effect/ decrease	2
Oms1	YDR316W	liquid culture	glycerol	decrease	1
Oms1	YDR316W	liquid culture	lactate	decrease	3
Oms1	YDR316W	liquid culture	raffinose	decrease	1
Rmt2	YDR465C	liquid culture	galactose	no effect	1
Rmt2	YDR465C	spot plate	glycerol	no effect	1
Set5	YHR207C	spot plate	dextrose	no effect	3
Set5	YHR207C	spot plate	glycerol	no effect	3
Bmt5	YIL096C	spot plate	dextrose	no effect	1
Bmt5	YIL096C	liquid culture	galactose	no effect	1
Bmt5	YIL096C	spot plate	glycerol	no effect	1
Bmt5	YIL096C	liquid culture	raffinose	no effect	1
Hpm1	YIL110W	spot plate	dextrose	no effect	1
Hpm1	YIL110W	spot plate	glycerol	no effect	1
Set4	YJL105W	spot plate	dextrose	no effect	1
Set4	YJL105W	spot plate	glycerol	no effect	1
Efm3	YJR129C	spot plate	dextrose	no effect	1
Efm3	YJR129C	spot plate	glycerol	no effect	1
Rsm22	YKL155C	spot plate	dextrose	slight decrease	4
Rsm22	YKL155C	liquid culture	dextrose	slight decrease	3
Rsm22	YKL155C	spot plate	ethanol	decrease	1

Rsm22	YKL155C	spot plate	ethanol/glycerol	no growth	1
Rsm22	YKL155C	liquid culture	galactose	decrease	1
Rsm22	YKL155C	spot plate	glycerol	no growth	3
Rsm22	YKL155C	liquid culture	glycerol	decrease	1
Rsm22	YKL155C	liquid culture	lactate	decrease	3
Rsm22	YKL155C	spot plate	lactate	no growth	1
Ykl162c	YKL162C	spot plate	dextrose	no effect	3
Ykl162c	YKL162C	liquid culture	dextrose	no effect	1
Ykl162c	YKL162C	spot plate	ethanol	no effect	1
Ykl162c	YKL162C	spot plate	ethanol/glycerol	no effect	1
Ykl162c	YKL162C	liquid culture	galactose	no effect	1
Ykl162c	YKL162C	spot plate	glycerol	no effect	2
Ykl162c	YKL162C	liquid culture	glycerol	no effect	1
Ykl162c	YKL162C	spot plate	lactate	no effect	1
Ykl162c	YKL162C	liquid culture	lactate	no effect	1
Set3	YKR029C	spot plate	dextrose	no effect	1
Set3	YKR029C	spot plate	glycerol	no effect	1
Efm7	YLR285W	spot plate	dextrose	no effect	1
Efm7	YLR285W	spot plate	ethanol	no effect	1
Efm7	YLR285W	spot plate	ethanol/glycerol	no effect	1
Efm7	YLR285W	spot plate	lactate	no effect	1
Ymr209c	YMR209C	spot plate	dextrose	no effect	2
Ymr209c	YMR209C	liquid culture	galactose	no effect	1
Ymr209c	YMR209C	spot plate	glycerol	no effect	2
Ymr209c	YMR209C	liquid culture	raffinose	no effect	1
Mtf1	YMR228W	spot plate	dextrose	no effect	2
Mtf1	YMR228W	liquid culture	galactose	no effect	1
Mtf1	YMR228W	spot plate	glycerol	no growth	2
Mtf1	YMR228W	liquid culture	raffinose	no effect	1
Efm6	YNL024C	spot plate	dextrose	no effect	1
Efm6	YNL024C	spot plate	ethanol	no effect	1
Efm6	YNL024C	spot plate	ethanol/glycerol	no effect	1
Efm6	YNL024C	spot plate	lactate	no effect	1
Mtq1	YNL063W	spot plate	dextrose	no effect	1
Mtq1	YNL063W	liquid culture	dextrose	no effect	1
Mtq1	YNL063W	spot plate	glycerol	no effect	1
Mtq1	YNL063W	liquid culture	lactate	slight decrease	2
Sfm1	YOR021C	spot plate	dextrose	no effect	1
Sfm1	YOR021C	spot plate	glycerol	no effect	1
Rkm1	YPL208W	liquid culture	lactate	slight decrease	1

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CHAPTER FOUR

Determining the Mitochondrial Methyl Proteome in
Saccharomyces cerevisiae using Heavy Methyl SILAC

Introduction

After I determined the limitations of the biochemical and immunological characterization of yeast mitochondrial methylation in Chapters Two and Three, I decided to take a different approach using mass spectrometry. First, I attempted to use intact mass spectrometry to determine whether mitochondrial ribosomal proteins (MRPs) were methylated. This strategy, comparing the experimental mass with the theoretical mass to identify differences of 14 Da, has been employed to identify a number of methylation sites on the cytoplasmic ribosomal proteins¹⁻³. However, due to the presence of any cytoplasmic ribosomal proteins in the MRP preparation and potential variance in N-terminal presequences (Appendix 1), changing the theoretical masses of the MRPs, this strategy was not successful.

I thus shifted to a more proteomics based approach in collaboration with Dr. James Wohlschlegel (UCLA) and his laboratory. First, we compared methylation sites identified on MRPs isolated from wild type and methyltransferase gene deletion yeast strains. This ultimately gave a long list of putative methylation sites (in Supplemental Table 2, available on ProQuest.com), but we had no way of validating the sites determined. This led us to use heavy methyl stable isotope labeling with amino acids in cell culture (SILAC). Of the sites previously identified in the previous attempts to characterize the methylation sites of mitochondrial proteins, one was validated in this study, the monomethylation of K186 on Mrpl40. Interestingly, Mrpl40 has a molecular weight of 33.8 kDa (Appendix 1). It is quite possible this MRP is responsible for the methyllysine signal at 33 kDa in the immunoblotting experiments in Chapter Two. In addition to Mrpl40, we were able to identify, with confidence, 11 methylation sites on mitochondrial proteins using heavy methyl SILAC.

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Determining the Mitochondrial Methyl Proteome in *Saccharomyces cerevisiae* using Heavy Methyl SILAC

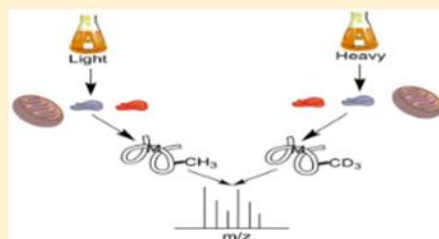
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Supporting Information

ABSTRACT: Methylation is a common and abundant post-translational modification. High-throughput proteomic investigations have reported many methylation sites from complex mixtures of proteins. The lack of consistency between parallel studies, resulting from both false positives and missed identifications, suggests problems with both over-reporting and under-reporting methylation sites. However, isotope labeling can be used effectively to address the issue of false-positives, and fractionation of proteins can increase the probability of identifying methylation sites in lower abundance. Here we have adapted heavy methyl SILAC to analyze fractions of the budding yeast *Saccharomyces cerevisiae* under respiratory conditions to allow for the production of mitochondria, an organelle whose proteins are often overlooked in larger methyl proteome studies. We have found 12 methylation sites on 11 mitochondrial proteins as well as an additional 14 methylation sites on 9 proteins that are nonmitochondrial. Of these methylation sites, 20 sites have not been previously reported. This study represents the first characterization of the yeast mitochondrial methyl proteome and the second proteomic investigation of global mitochondrial methylation to date in any organism.

KEYWORDS: heavy methyl SILAC, methylation, methyl proteome, mitochondria, MudPIT, yeast, protein lysine methylation, protein arginine methylation



INTRODUCTION

Often associated with the histone code, protein methylation is gaining recognition as a significant post-translational modification that can lead to enhanced function and regulation.¹ Much effort has been placed into identifying nonhistone protein substrates of methyltransferases,^{2–5} especially in the baker's yeast, *Saccharomyces cerevisiae*, which has one of the most fully characterized methyltransferasomes.^{6–9} A third of *S. cerevisiae* genes are orthologous to human genes, and half of the essential orthologous human genes exhibit functional complementarity in yeast.¹⁰ Global methylation in yeast has been investigated through the use of high-throughput proteomics.^{11–15} Because these studies have been performed with yeast grown in D-glucose, conditions where production of mitochondrial proteins is suppressed, mitochondrial methylation remains largely uncharacterized. Much of what is known about human mitochondrial defects has come from yeast studies.¹⁶ Therefore, understanding the methylation of yeast mitochondrial proteins may increase our understanding for other eukaryotes.

The majority of protein methylation occurs on lysine and arginine residues. Known substrates for protein arginine and lysine methyltransferases are generally localized in the nucleus

and cytoplasm^{17–19} with significant methylation present in the cytoplasmic translational apparatus,²⁰ which contains some of the more abundant proteins in the cell. Hence, the majority of methylation sites being identified in large-scale protein modification studies via proteomics would be from these more abundant cellular compartments. Mitochondrial proteins may be in much lower abundance than their nuclear and cytoplasmic counterparts,²¹ presenting a potential problem for proteomic studies of mitochondrial methylation.

Furthermore, less focus has been placed on the proteomic study of mitochondrial methylation. To date, there has only been one proteomic study of protein arginine methylation in the mitochondria, performed in *Trypanosoma brucei*.²² Only two methylated mitochondrial proteins have been well-characterized in *S. cerevisiae*. Cytochrome *c* is trimethylated on a lysine residue by Ctm1 prior to import,²³ a modification that has been implicated in import, stability, protein–protein interactions, and apoptosis.^{24–26} The mitochondrial translation release factor (Mrf1) is methylated by Mtq1 on a conserved glutamine residue, which is substoichiometric yet required for

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translation.²⁹ However, there are four putative protein methyltransferases^{6–8} that are localized to the mitochondria but have unknown substrates: Rsm22,³⁰ Mtf1,³¹ Oms1,³² and Ykl162c.³³ This suggests that more mitochondrial protein substrates for methylation have yet to be identified.

A bottom-up approach to identify global mitochondrial protein methylation is desirable due to postimport processing of mitochondrial proteins³⁴ that complicates top-down methods. This approach is not without its drawbacks, especially when identifying methylation sites. There are two major issues with large-scale studies to detect methylation sites in proteins: a high false discovery rate and a low abundance of many methylated proteins.³⁵ Some of the artifactual methylation seen in large-scale studies that use $\Delta 14$ Da mass shift based identification to detect methyl groups is due to sample preparation or processing for mass spectrometry, especially for samples prepared in methanol and ethanol.^{36–38} Many amino acid substitutions can result in changes of 14, 28, and 42 Da, so relying on mass shift alone could also incur misidentification of methylation on peptides.³⁹ Search algorithms can contribute to false-positives due to the misidentification of modifications as well.⁴⁰ Additionally, methylation may be substoichiometric or on low abundance proteins, making the sites difficult to detect in the background of more abundant species.⁴¹ To make matters worse, low precursor ion abundances can lead to poor fragmentation.³⁵ As a result, there is often little to no overlap between the results of various yeast proteomics studies.^{12,14,15} Thus, some previously reported methyl identifications may be artifactual, and many physiological methylation sites have likely escaped detection.

To overcome both of these problems, isotopic labeling coupled to analysis of purified subcellular fractions can be very useful. Heavy methyl stable isotope labeling by amino acids in cell culture (SILAC) can allow for unambiguous assignment of methylation to peptides.³⁹ This has been used with much success in human proteomic studies of methylation,^{42–46} with improvements being made to the method,⁴⁷ but is less utilized in yeast.^{15,48} Recently, using heavy methyl SILAC in *S. cerevisiae*, Hart-Smith et al.⁴⁹ found that large-scale mass spectrometry identifications of methylation sites typically have high false discovery rates and emphasized the need for this type of methylpeptide validation. We have adapted heavy methyl SILAC for use with *S. cerevisiae* under respiratory conditions to allow for a full complement of mitochondria. Here we report the identification of 26 methylation sites, likely to be on lysine and arginine residues, in 20 proteins across three protein fractions, including 20 novel sites. This study presents the first characterization of the mitochondrial methyl proteome in yeast. Coupled to the results from previous proteomic studies, our work suggests that we may have only identified a fraction of biologically relevant modifications.

■ MATERIALS AND METHODS

Yeast Strains

Yeast wild-type BY4741 (*MATa his3 Δ 1, leu2 Δ 0, met15 Δ 0, ura3 Δ 0*) and *met6 Δ* (*MATa his3 Δ 1, leu2 Δ 0, met15 Δ 0, ura3 Δ 0, met6::kanMX*) strains were obtained from Open Biosystems (now GE Dharmacon).

Heavy Methyl SILAC

Cells were grown in 2 L of synthetic lactate media⁵⁰ modified for heavy methyl SILAC. Instead of yeast extract, 1.75 g/L yeast nitrogen base without amino acids or ammonium sulfate and 5

g/L ammonium sulfate was used. For light cultures, 0.79 g/L complete supplement mixture powder (CSM, MP Biomedicals) was added. For heavy cultures, CSM was omitted and the medium was supplemented with 0.75 g/L methionine dropout amino acid mix (CSM-Met, MP Biomedicals) and 20 mg/L L-methionine (*methyl-D₃*, 98%) (Cambridge Isotope Laboratories). Cells were grown in a shaker incubator at 30 °C at 250 rpm to an optical density at 600 nm of ~ 3 and harvested by centrifugation at 5000g for 5 min.

Protein Fractionation

Crude mitochondria were obtained as previously described⁵⁰ from each of the 2 L heavy and light cultures described above. Mitochondria were lysed using 1.3% Triton X-100 in 350 mM ammonium chloride, 20 mM magnesium acetate, 1 mM EDTA, 2 mM β -mercaptoethanol, 20 mM Tris-HCl, pH 7.5. The lysates (~ 5 mL) were cleared by centrifugation at 30 000g for 10 min at 4 °C,⁵¹ and the protein concentration was determined by Lowry assay⁵² after 10% trichloroacetic acid precipitation. For preparation of samples of crude mitochondria for mass spectrometry, equal amounts of protein from the light and heavy lysates were combined (500 μ g each for a total of 1 mg of protein) and subjected to detergent removal and desalting using Pierce polyacrylamide spin desalting columns (7K MWCO) (Life Technologies). The desalted preparation was then precipitated with trichloroacetic acid as described.⁵³ Combined heavy and light mitochondrial lysates were stored at -20 °C until preparation for mass spectrometry analysis.

A fraction of mitochondrial ribosomal proteins was prepared from the crude mitochondrial lysates described above. Lysates (4 mL) were centrifuged on top of a sucrose cushion (1 M sucrose, 0.1 M potassium chloride, 10 mM magnesium chloride, 0.1 mM EDTA, 1 mM dithiothreitol, 2 mM TES, pH 7.6) at 180 000g for 3 h⁵⁴ at 4 °C with a Ti65 rotor (Beckman). The mitochondrial ribosomal proteins were acetic acid extracted, as previously described,⁵⁵ and protein concentrations were determined. Equal amounts of protein (5–10 μ g each for a total of 10–20 μ g of protein) from the light and heavy fractions were combined. Extracted proteins were dried using vacuum centrifugation and stored at -20 °C until preparation for mass spectrometry analysis.

A third fraction was prepared from the supernatant of the initial mitochondrial pelleting described above. This largely cytosolic fraction was centrifuged at 159 000g for 2 h at 4 °C with a Ti65 rotor (Beckman) to pellet ribosomes. The ribosomal proteins were acetic acid extracted,⁵⁵ and their protein concentration was determined. Equal amounts of heavy and light protein (100 μ g each for a total of 200 μ g of protein) were combined. Extracted proteins were dried in a vacuum centrifuge and stored at -20 °C until preparation for mass spectrometry analysis.

Heavy methyl SILAC was performed in biological triplicate for cytoplasmic ribosomal proteins and mitochondria and biological duplicate for mitochondrial ribosomal proteins. Each biological replicate came from independently grown cultures.

Protease Digestion

Light- and heavy-labeled protein samples derived from purified cytoplasmic ribosome, mitochondria, or purified mitochondrial ribosomes were mixed in a 1:1 ratio. Purified samples were precipitated using trichloroacetic acid and then resuspended in digestion buffer (100 mM Tris, pH 8.5, 8 M urea). Reduction and alkylation were performed sequentially by incubating the samples in 5 mM TCEP-HCl for 20 min at room temperature,

followed by 10 mM iodoacetamide for 20 min at room temperature in the dark. The samples were then digested with Lys-C and trypsin proteases. First, Lys-C was added at an enzyme to substrate ratio of 1:100 and incubated for 4 h at 37 °C. Following Lys-C digestion, the samples were diluted to 2 M urea using 100 mM Tris, pH 8.5, CaCl₂ was added to a final concentration of 1 mM, and trypsin was added at an enzyme to substrate ratio of 1:50 for 12 h at 37 °C. The trypsin digestion was stopped by the addition of formic acid at a final concentration of 5%.^{56,57} Samples were desalted using C18 tips according to the manufacturer's instructions, resuspended in 10 μ L of 5% formic acid, and then analyzed by LC-MS/MS as described below.

Mass Spectrometry Analysis

The digested peptide mixture was desalted and concentrated using C18-packed pipet tips (Thermo Fisher) and fractionated online using a 75 μ m inner diameter fritted fused silica capillary column with a 5 μ m pulled electrospray tip and packed in-house with 15 cm of Luna C18(2) 3 μ m reversed-phase particles. An EASY-nLC 1000 ultra-high-pressure liquid chromatography (UHPLC) system (Thermo Fisher) was used to deliver the gradient, and MS/MS spectra were collected on a Q Exactive mass spectrometer (Thermo Fisher) as described.^{58,59} Data analysis was carried out using the ProLuCID and DTASelect2 implemented in the Integrated Proteomics Pipeline - IP2 (Integrated Proteomics Applications, San Diego, CA).^{60–62} To identify methylated peptides, a differential modification search was employed that considered variable mass shifts of 14.0157 (monomethylation), 28.0314 (dimethylation), and 42.0471 (trimethylation) on lysines and arginines to identify unlabeled peptide. The heavy search considered a static mass shift of 3.0189 Da from L-[methyl-D₃]-methionine and variable mass shift of 17.0346 (monomethylation), 34.0692 (dimethylation), and 51.1038 Da (trimethylation) on lysine or arginine. Database searches were conducted without enzyme specificity. Peptide identifications were filtered using DTASelect and required a unique fully tryptic peptide with a spectrum-level false-positive rate of 5%, as estimated by a decoy database strategy.⁶³ The Skyline software package was used to manually evaluate the presence of heavy-light peptide doublet from extracted ion chromatograms generated for each peptide.⁶⁴

For additional analysis to identify lysine, arginine, aspartate, or glutamate containing methyl peptides, a differential modification search was employed that considered variable mass shifts of 14.0157 (monomethylation), 28.0314 (dimethylation), and 42.0471 (trimethylation) on lysines, arginines, aspartates, or glutamates to identify unlabeled peptide. The heavy search considered a static mass shift of 3.0189 Da on methionine corresponding to L-[methyl-D₃]-methionine and variable mass shift of 17.0346 (monomethylation), 34.0692 (dimethylation), and 51.1038 Da (trimethylation) on lysine, arginine, aspartate, or glutamate. Database searches were conducted without enzyme specificity using the MSGP+ search algorithm.⁶⁵ The search results were analyzed using Percolator and filtered at a spectrum-level *q* value of 0.01.⁶⁶ Methyl site localization and site probability was determined using the PTMRS algorithm.⁶⁷ The Skyline software package was used to manually evaluate the presence of heavy-light peptide doublet from extracted ion chromatograms generated for each peptide.⁶⁴

Data Visualization and Structural Modeling

Sequence logos were generated using WebLogo.⁶⁸ Sequences of 10 amino acids N-terminal and C-terminal of the methylated residue were inputted. The Venn diagram comparing candidates in the three protein fractions was prepared using a Venn diagram webtool (Bioinformatics & Evolutionary Genomics, Ghent University). Structures were modeled using Protein Homology/analogy Recognition Engine V 2.0 (Phyre²).⁶⁹ Structural figures of the Phyre² models were made using PyMol (Schrödinger).

Comparison using UniProt Database

Data obtained from this study and others like it were compared using proportional Venn diagrams. Proportional Venn diagrams were made from these methylated protein lists from cited studies using BioVenn.⁷⁰ The curated list from the Universal Protein Resource (UniProt) Database⁷¹ used for these comparisons was made as follows. The list was generated by first going to "keywords" in UniProt. From here, the category of Post-Translational Modification was selected, and methylation specifically was chosen from the keywords navigation. Only "Reviewed" proteins were mapped to the keyword. The reviewed methylation keyword results were then filtered by organism (*Saccharomyces cerevisiae* (strain ATCC 204508/S288c) (Baker's yeast)). This data set, once downloaded, comprised the "UniProt 2015" protein list. These comparisons are based on the open reading frame and not on the methylation site.

RESULTS

Labeling of Yeast Strains with L-[methyl-D₃]-methionine

To detect methylated yeast mitochondrial proteins, we first attempted a large-scale study of mitochondrial fractions from wild type and methyltransferase knockout strains using MudPIT⁷² without any isotopic labeling. This approach yielded many methylated peptide hits, but we were concerned that we did not consistently observe such peptides in various strains, leading us to question whether some or many of the hits could be false-positives. We then optimized a heavy methyl SILAC protocol for labeling methyl groups in yeast proteins with methyl-deuterium-labeled methionine. This method has been used with much success for unambiguously determining methylated peptides in a variety of human cell lines.^{39,42–46} Yeast take up exogenous methionine rapidly⁷³ and convert it into *S*-adenosyl-L-methionine (AdoMet),⁷⁴ which is then used as the primary methyl donor by methyltransferases in the cell. Other methyl donors include O⁶-methylguanine of DNA (used by Mgt1),⁷⁵ 5,10-methylenetetrahydrofolate (used by Cdc21),⁷⁶ and N⁵-methyltetrahydrofolate (used by Met6).⁷⁷

To optimize such labeling, it is important to shut down endogenous methionine biosynthesis that can dilute the label. The yeast wild-type BY4741 strain is already a methionine auxotroph with a deficiency in the *MET15* gene (also known as *MET17*, *MET25*), encoding the enzyme forming the homocysteine precursor of methionine.⁷⁸ We first utilized this strain for heavy labeling with L-[methyl-D₃]-methionine. With heavy labeling alone, we found incomplete and inconsistent levels of incorporation in different peptides (Figure 1), resulting in variable levels of light methylation being present in the heavy-labeled culture. Because of the importance of having a 1:1 ratio of heavy and light proteins for the analysis of the peptides, the dilution of the label was problematic. If only

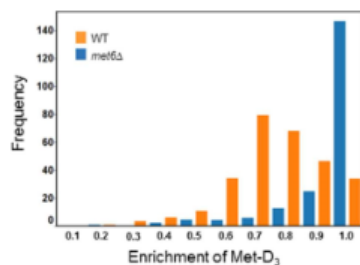


Figure 1. Improved enrichment of the deuterated methionine label into proteins of *S. cerevisiae* strains with a deletion in the *MET6* gene encoding the cobalamin-independent methionine synthase. As described in the **Materials and Methods**, wild type (WT) and *met6Δ* strains were heavy-labeled, mitochondrial ribosomal proteins were isolated, digested with trypsin/Lys-C and analyzed by LC-MS/MS. For each peptide identified, the ratio of the areas of the extracted ion chromatograms for each labeled and unlabeled peptide pair was calculated to obtain a fractional enrichment of *L*-[methyl-D₃]-methionine (Met-D₃).

the light form of the methylated peptide is present in the heavy culture (e.g., no label incorporation), then it will not be identified as methylated as there will be no heavy-light doublet in the MS1 spectra, only a light peak for that peptide from both the heavy and light cultures. It is likely here that unlabeled methyl groups can be introduced in the *MET15* deletion strain via the Met6 methionine synthase from *N*⁵-methyltetrahydrofolate.^{77,79} The *MET6* gene deletion strain has a mild phenotype aside from methionine auxotrophy.⁸⁰ We have observed that *met6Δ* cells grew similarly to BY4741 cells in synthetic nonfermentable medium, allowing for mitochondrial fractionation. We thus utilized a strain containing a *MET6* deletion mutation that prevents the methylation of homocysteine to methionine.⁷⁷ The use of this strain resulted in increased efficiency in labeling (Figure 1).

The *met6Δ* cells were thus grown in both light and heavy media under respiratory conditions and harvested; then, extracts were separated into three protein fractions: mitochondria, mitochondrial ribosomal proteins, and cytoplasmic ribosomal proteins, as described in the **Materials and Methods**. The latter fraction was prepared as a positive control because the methylation status of these proteins has been well established.⁹ The resulting heavy and light protein fractions were combined in a 1:1 ratio for mass spectrometric analysis. A total of three independent cell-labeling experiments were done; three independently prepared fractions of the mitochondria and of the cytoplasmic ribosomes and two independently prepared fractions of mitochondrial ribosomal proteins were digested with a trypsin/Lys-C protease mixture and analyzed by mass spectrometry as described in the **Materials and Methods**.

Identification of Peptides Containing Methylated Residues

To analyze the collected peptide mass spectral data for methylated peptides, two ProLuCID searches were set up for each protein fraction replicate in the IP2 platform, as described in the **Materials and Methods**. Both were closed searches for methylation of lysine (mono-, di-, and tri-) and arginine (mono-, and di-) specifically. One differential modification search considered only "light" peptides: peptides with a variable mass shift of 14.0157 (monomethylation), 28.0314 (dimethy-

lation), and 42.0471 (trimethylation) Da. The other differential modification search considered "heavy" peptides: peptides with a static mass shift of 3.0189 Da (from *L*-[methyl-D₃]-methionine) and variable mass shifts of 17.0346 (monomethylation), 34.0692 (dimethylation), and 51.1038 Da (trimethylation). We also performed additional searches to include the detection of methylated aspartate and glutamate residues, as described in the **Materials and Methods**. The peptide lists from these searches were then exported into Skyline libraries for evaluation of the presence of the expected heavy-light doublet in the extracted precursor ion chromatograms generated from each identified light/heavy methylated peptide pair. An example of this is seen in Figure 2A. We confirmed the heavy-light doublet by matching the observed isotope distribution of each precursor ion with the theoretical distribution (Figure 2B). Novel methylated peptides were further evaluated based on their presence in multiple replicates and the quality of their MS/MS spectra generated by HCD fragmentation.

From the data in all eight mass spectrometric analyses (two or three replicates from each of the three protein fractions), 112 peptides were identified using IP2 and Skyline as having a mass shift consistent with methylation but did not show the expected light/heavy doublet (an example is shown in Figure 2C,D; peptides are listed in Table S-1, tab 2). However, we did identify 36 peptides from 20 proteins in these experiments that displayed the expected light/heavy doublet (Table S-1, tab 3). Thus only 25% of peptides identified as methylated by the ProLuCID search algorithm were validated using heavy methyl SILAC. In rare cases, AdoMet is not used as a methyl donor.^{75,76} However, it appears that many of these identifications may be false-positives. Our results suggest that studies that do not validate methylated peptide identifications using heavy methyl SILAC or other quantifiable methods may overestimate the number of methylation sites occurring in the cell, potentially reporting methylation sites that are not present.

Here we report 26 methylation sites on 20 proteins across the three protein fractions. There was little overlap between methylated candidates in the three protein fractions, as seen in Figure 3. Of the proteins that overlapped, two were highly abundant, Ssa2 and Ssa4, which are part of the Hsp70 family of proteins, and two that are known to be methylated as well as abundant, Tef1/2 (eEF1A) and Rpl42ab. These results indicate that discrete pools of methylated proteins exist in the cytoplasm and the mitochondria.

Validation of Approach by Analysis of the Cytoplasmic Ribosomal Fraction

The cytoplasmic ribosomal protein fraction was used as a reference set for the heavy methyl SILAC method (Table 1). We validated four sites on three ribosomal proteins: dimethyl K106 and K110 on Rpl23ab,⁸¹ monomethyl K55 on Rpl42ab,⁸² and monomethyl R67 on Rpl12ab,⁸³ as seen in Table 1. The following lysine and arginine methylations on ribosomal proteins were not found in our analysis: trimethyl K3 on Rpl42ab,⁸² monomethyl K46 on Rpl1,⁸⁴ monomethyl K40 on Rpl42ab,⁸² mono- or dimethyl R11 on Rps2,⁸⁵ and monomethyl R145 on Rps3.⁸⁵ It is unclear if these sites were missed due to incomplete sampling of precursor ions during our data-dependent acquisition method or due to the absence of the modification under respiratory rather than fermentative growth conditions. It would be intriguing if these sites were required for function under fermentation but not under

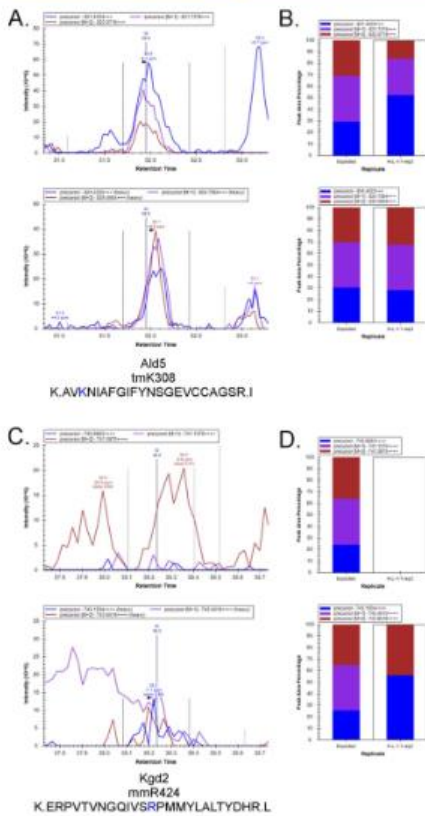


Figure 2. Examples of good and poor methylated peptide identifications in MS1 ion chromatograms. (A) Extracted precursor ion chromatograms for light (top) and heavy (bottom) peptides containing a candidate trimethyl K308 on Ald5. The zero, +1, and +2 isotopes are shown in blue, magenta, and red, respectively. The methylated residue is highlighted in blue in the peptide sequence. (B) Corresponding peak areas of both experimental and theoretical precursor ions for the heavy and light peptide containing this modification. (C,D) Extracted precursor ion chromatograms for light and heavy peptides containing an identified, but unlikely monomethyl R424 on Kgd2, analyzed as in panels A and B. These chromatograms are from the mitochondrial fraction.

respiratory conditions. It is also possible that the trimethyl K3 site on Rpl12ab may have been missed because it would be found in a peptide with a modified dimethylproline residue at position 1,⁸⁶ which was not considered during the database search. Arginine methylation at position 11 of Rps2 is substoichiometric,⁸⁷ which may be why this modification was missed. We also identified previously unseen methylation variants of Rpl23ab, including trimethylation and monomethylation at K106 and K110. These forms are distinct from the well-characterized dimethyl K106/dimethyl K110 species

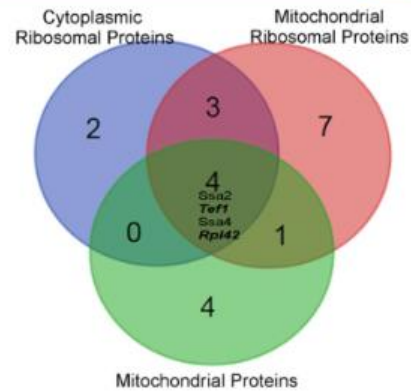


Figure 3. Overlap of methylated proteins between cytoplasmic ribosomal proteins (blue), mitochondrial ribosomal proteins (pink), and mitochondrial protein fractions (green). The four methylated proteins found in all three protein fractions are listed. Known methylated proteins are bolded and italicized.

(Table 1, Figure S-1, Figure S-2). However, such methylation site assignments were based primarily on the presence of a single site-determining ion and may represent sites that are mislocalized by the search algorithm.

We also found a number of ribosome-associated methylated proteins in this fraction. Eukaryotic elongation factor 1A (eEF1A), encoded by *TEF1/TEF2*, is known to be highly methylated on five lysine residues: monomethyl K30, trimethyl K79, dimethyl K316, monomethyl K390, and a C-terminal lysine methyl ester.^{88,89} Our approach here identified three out of the five sites, as seen in Figure 4: monomethyl K30, trimethyl K79, and monomethyl K390, the latter of which may be substoichiometric and at lower occupancy than the other methylated lysines on eEF1A.⁹⁰ We also identified a peptide containing the potentially novel monomethylation of K30 and K35. On the basis of the fragmentation pattern for this peptide (Figure S-1), however, this methylation site assignment could be incorrect as the site-determining ions support dimethylation of K30 as well. The other elongation factor methylation sites, trimethyl K509 and dimethyl K613 on EF2 and trimethyl K187, K196, and K789 on EF3,⁹¹ were not identified; again, it is unclear whether these sites are not methylated under respiratory conditions or were simply missed in the analysis. We also detected methylation on the Ded1 RNA helicase required for translation initiation for yeast mRNA.⁹² Here we confirm the dimethyl R51 site previously found.⁹³

Finally, we were able to detect methylation sites on several other proteins. We were able to confirm the dimethylation of abundant histone H3 protein (Hht1) at K37⁹⁴ that may have contaminated the cytoplasmic ribosome preparation. We additionally find a potentially novel monomethyl K38 on histone H3 (Figure S-1). K38 methylation is supported by two diagnostic site-determining ions, so further investigation is needed. Importantly, novel methylation sites are identified as follows: monomethyl K422 on Ssa4, monomethyl K421 on Ssa2, and dimethyl R89 on Hsp26. Ssa4, Ssa2, and Hsp26 are all cytoplasmic heat-shock-induced chaperones^{95,96} that poten-

Table 1. Validating Methods for Cytoplasmic Ribosomal Fraction

novel methylation sites found						
protein	site ^a	sequence ^b	Percolator q value	methyl site probability ^c	localization ^d	
Rpl23ab	tmK106, mmK110	R.DGVFLYFEDNAGVIANPKGEMKGSAITGPVGKE				cytoplasm
Rpl23ab	dmK110, dmK120	R.DGVFLYFEDNAGVIANPKGEMKGSAITGPVGKE				cytoplasm
Histone H3	mmK38	K.SAPSTGGVKKPHRY				nucleus
eEF1A	mmK30, mmK35	K.STTTGHLIYKCGGIDKR				cytoplasm
Ssa2/4	mmK421/422	K.KSEVFSTYADNPQVLIQVFEGERT				cytoplasm, mitochondria, nucleus, vacuole
Hsp26	dmR89	K.EVARPNNYAGALYDPRDETLDDWFQNDLSLFPSPGFGFPK	0	0		cytoplasm, nucleus
known ribosomal protein methylation sites found						
protein	site	sequence	Percolator q value	methyl site probability	localization	
Rpl12ab	mmR67	K.IQNRAQAAASVPSASSLVITALK.E	0	100		cytoplasm
		K.IQNRAQAAASVPSASSLVITALK.EPPRD	0	100		
Rpl23ab	dmK106, dmK110	R.DGVFLYFEDNAGVIANPKGEMKGSAITGPVGKE	0.00134386	K106:50.11 K110:99.77		cytoplasm
Rpl42ab	mmK55	R.KQSGFGGQTKPVFHK.K	0	100		cytoplasm
		K.QSGFGGQTKPVFHK.K	0	100		
		K.QSGFGGQTKPVFHK.K.A	5.34 × 10 ⁻⁵	100		
known ribosome-associated methylation sites found						
protein	site	sequence	Percolator q value	methyl site probability	localization	
eEF1A	mmK30	K.STTTGHLIYKCGGIDK.R	0	100		cytoplasm
		K.STTTGHLIYKCGGIDK.R.T	0	100		
eEF1A	tmK79	R.GITIDIALWKFETPK.Y	0	100		cytoplasm
		R.ERGITIDIALWKFETPK.Y				
eEF1A	mmK390	K.KLEDHPKFLK.S				cytoplasm
Ded1	dmR51	R.NNNSNYNNNGYNGGRGGGFFSNNR.R	0	100		cytoplasm, nucleus
histone H3	dmK37	K.SAPSTGGVKKPHRY	0	99.71		nucleus
known ribosomal protein methylation sites missed						
protein	site	potential reason	localization			
Rpl12ab	tmK3	presence of dimethyl proline in position 1				cytoplasm
Rpl42ab	mmK40	unclear				cytoplasm
Rpl1	mmK46	unclear				cytoplasm
Rps2	mm and dmR11	substoichiometric				cytoplasm
Rps3	mmR145	unclear				cytoplasm

^aMethylated residues are abbreviated as follows: mm, monomethyl; dm, dimethyl; tm, trimethyl. ^bMethylated residue is bolded and underlined. On the basis of residue level probabilities, also underlined are other potentially methylated residues. ^cMethyl site probabilities for bolded and underlined residue. ^dLocalization determined from *Saccharomyces* Genome Database.⁸⁰

tially copurified with cytoplasmic ribosomal proteins through interaction with nascent polypeptide chains.

As described above and in the **Materials and Methods**, known methylation sites were validated with additional analyses for lysine, arginine, glutamate, and aspartate methylation, with the exception of Rpl23ab, where the first dimethylation event has an even probability of being on K106 or E108 (Table 1). Additionally, although the majority of peptides gave unambiguous assignment of the trimethyl to K79 via site probability, a portion of the peptides did have equal site probability between K79 and E81. As this methylation is well characterized,⁸⁸ we can determine that the trimethylation occurs on K79.

Many of the novel methylated peptides were not identified in the additional analysis, including Rpl23ab, histone H3, eEF1a, and Ssa2/4. Hsp26, containing a C-terminal dimethyl arginine on R89, has a region of likely dimethylation, spanning from D64 to D77. On the basis of a spectrum to spectrum comparison between the two searches, the likely residues containing the methylation due to probability are D74 and D76, as they share an equal probability. Because dimethyl

aspartate is highly unlikely, the methylation possibly occurs on R66.

Analysis of the Mitochondrial Ribosomal Fraction

In the mitochondrial ribosomal protein fraction, three mitochondrial ribosomal proteins were identified as methylated: Mnp1, Mrpl4, and Mrpl40 (Table 2, Figure 5, Figure S-2). Like their cytoplasmic counterparts, mitochondrial ribosomal proteins are also subject to this modification. Mnp1, Mrpl40, and Mrpl4 are all large subunit components.⁹⁷ Methylation of mitochondrial ribosomal protein L11 has been reported in *Arabidopsis thaliana*;⁹⁸ we find no evidence of the methylation of the corresponding yeast protein in our study. A number of methylation sites of mitochondrial ribosomal proteins were also reported in *Trypanosoma brucei*,²² but confirmatory isotope labeling was not used in that study. The *T. brucei* homologue of Mnp1, Tb927.7.4550, was found to be methylated on an arginine residue in that study.

The novel methylation sites found in the mitochondrial ribosomal protein fraction are monomethyl R55 on Pdb1, monomethyl K144 on Pst2, monomethyl K558 on Ecm10,

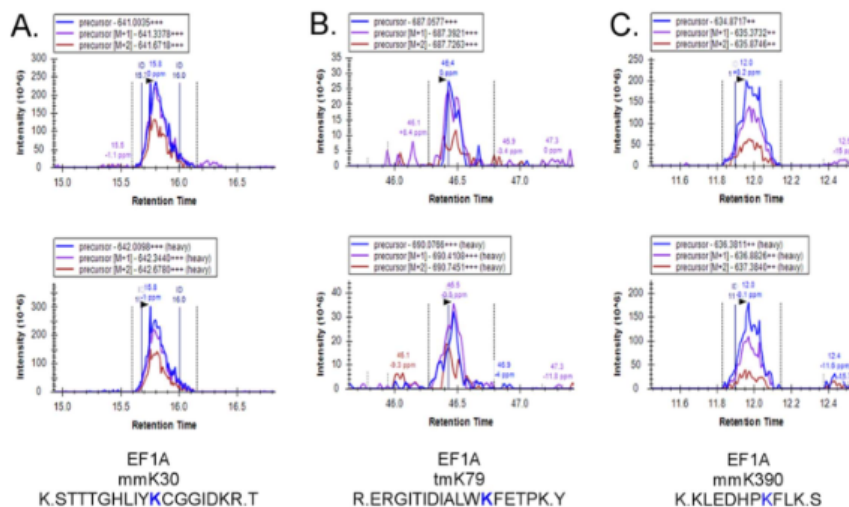


Figure 4. Confirmation of known methylation sites on EF1A. Extracted precursor ion chromatograms for light (top) and heavy (bottom) peptides containing (A) monomethyl K30, (B) trimethyl K79, and (C) monomethyl K390. The zero, +1, and +2 isotopes are shown in blue, magenta, and red, respectively. The methylated residue is highlighted in blue in the peptide sequence. Chromatograms are representative of three replicates from the cytoplasmic ribosomal protein fraction.

monomethyl K422 on Ssa4, dimethyl K56 on Mnp1, monomethyl K49 on Rps2, monomethyl K421 on Ssa2, monomethyl R204 on Mrpl4, monomethyl R260 on Lat 1, dimethyl K340 on Lat1, and monomethyl K186 on Mrpl40. The presence of methylated histone H3 is a possible sign of nuclear contamination. There was expected cytoplasmic ribosomal protein contamination in the mitochondrial ribosomal protein fraction, as demonstrated by the presence of methylated peptides from Rpl23ab, eEF1A, Rpl12ab, Rpl42ab, and a novel methylation on Rps2 (Table 2).

Analyzing the data for aspartate and glutamate methylation in addition to lysine and arginine methylation yielded additional confirmation of known sites in this fraction, with the exception of methyl peptides from eEF1a and Rpl23ab. Although alternative and novel methylation states are identified with opening up the search for D/E methylation (Table 2), the known methyl peptide containing dimethyl K106 and dimethyl K110 on Rpl23ab was no longer identified. The peptide identified corresponded better to the novel trimethyl K106 and monomethyl K110 peptide, although it was found in the scan number for the dimethyl K106 and K110 peptide. The methyl peptide containing monomethyl K30 had equal probability for the monomethylation to occur on a nearby aspartate (D35). However, as mentioned previously, given the well-studied nature of eEF1a lysine modifications, it is more likely that the methylated residue truly is K30.⁸⁸ Additionally, the novel methylation of histone H3 on K38 appears to be more likely on the known residue of K37.

While the methyl peptides were not identified for any of the three MRPs, a shortened methyl peptide that did not contain K56 had a high probability of methylation on E68 on Mnp1. Similarly, the methyl peptide containing R260 on Lat1 was not

identified, while the peptide containing K340 had equal probability of the dimethyl on K340 or D343. The methylation of Pdb1 cannot be clearly assigned to R55 in this fraction but spans a region from E51 to D58. For the corresponding peptides from the previous search, it appears to be an equal probability of methylation on either E51 or E52. Neither of the exact matching novel methyl peptides of trimethyl/monomethyl K106 and monomethyl/trimethyl K110 on Rpl23ab were identified. Similar peptides yielded methyl site probabilities that indicated possibility of E108 methylation. There is stronger basis for trimethyl K106 and monomethyl K110 based on probability. However, within this search, all methylations were initially misassigned to E96 and D97.

Clear site assignments occurred for the methyl peptides of Ssa2/4, Pst2, Ecm10, and Rps2. While the original site assignment for Ssa2/4 was K421/422, the additional analysis gave this methyl peptide a high probability for methylation on E423/E424. Similarly, Pst2 monomethylation has a high probability on E121 instead of K144. Ecm10 monomethylation likely occurs on K558 having been identified in the search for just K/R and in K/R/D/E with a high probability for that residue. The same is true for monomethylation of Rps2 at K49, which is a new modification for the ribosome under respiratory conditions.

Analysis of Methylated Proteins in the Mitochondrial Fraction

The majority of candidate methyl peptides identified were mitochondrial in origin, as seen in Table 3 (Figure S-3). The newly identified sites in this protein fraction were as follows: monomethyl R55 on Pdb1, trimethyl K308 on Ald5, monomethyl K422 on Ssa4, monomethyl K421 on Ssa2,

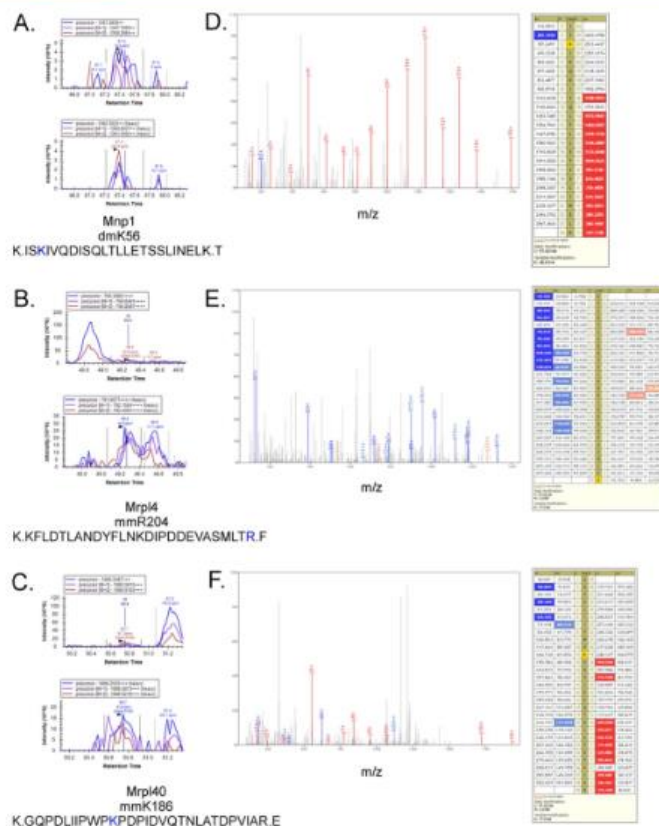


Figure 5. Novel methylated peptides found in mitochondrial ribosomal protein fraction. Extracted precursor ion chromatograms for light (top) and heavy (bottom) peptides containing (A) dimethyl K56 on Mnp1, (B) monomethyl R204 on Mrp14, and (C) monomethyl K186 on Mrp140. The zero, +1, and +2 isotopes are shown in blue, magenta, and red, respectively. The methylated residue is highlighted in blue in the peptide sequence. Fragmentation patterns of product ions for (D) Mnp1, (E) Mrp14, and (F) Mrp140 methyl peptides. Methylated residue is highlighted in yellow in the peptide sequence.

trimethyl K490 on Hsp60, trimethyl K345 on Ald4, and dimethyl K360 on Aco1. None of these have been previously identified. Methylated peptides from both Rpl42ab and eEF1A were also identified, likely due to the abundance of these proteins.

In additional database searches for methyl lysine, arginine, glutamate, and aspartate, these methylpeptides were confirmed, albeit putatively on alternative sites (Table 3). It is apparent from the fragmentation pattern of the methyl peptide containing the monomethyl arginine on Pdb1 as well as the methyl site probabilities that the methylation cannot be localized. Here we are confident given results from the MRP fraction that the peptide contains a methylation mark; however, it cannot be assigned to R55 but is confined to the methylatable residues from E51 to D58 as in the MRP fraction. On the contrary, the trimethylation of K308 on the methylpeptide from

Ald5 appears to be correctly assigned based on methyl site probabilities. This occurred for K490 on Hsp60 as well. The potential methylations of Ssa2/4 and Aco1 present an interesting divergence from the previous search algorithm. The monomethylation on Ssa2/4 was assigned to E423/4 with high probability, as opposed to K421/422. The dimethyl on Aco1 was not able to be localized based on probability. Although the exact corresponding peptide from the original search was not identified, similar peptides contained equal probability on the methylatable residues from D323 to E345. It is clear that two methyl groups are present on this peptide, whether as one dimethyl lysine or two monomethylations as dimethyl D or E is not biologically possible.

Clear assignments were seen for known sites of monomethyl K55 on Rpl42ab and monomethyl K30 on eEF1a with high methyl site probabilities. This is to be expected as (1) these

Table 2. Methylated Sites Found in the Mitochondrial Ribosomal Fraction

novel mitochondrial ribosomal methylation sites found						
protein	site ^a	sequence ^b	Percolator <i>q</i> value	methyl site probability ^c	localization ^d	
Mnp1	dmK56	KISKIVQDISQLTLLETSSLINELK.T			mitochondria	
Mrpl4	mmR204	K.KFLD TL LANDYFLNKDIPDDEVASMLTRF			mitochondria	
Mrpl40	mmK186	K.GQPD LI PWPKPDPIDVQJNLATDPVIARE			mitochondria	
novel mitochondrial ribosome-associated methylation sites found						
protein	site	sequence	Percolator <i>q</i> value	methyl site probability	localization	
Lat1	mmR260	K.QSSQTS G AAAAATPAAATSSTTAGSAPSPSSTASYEDVPIS TM RS			mitochondria	
Lat1	dmK340	K.FKNVQVSVAVATPTGLLTPIVK.N	0	50	mitochondria	
Pdb1	mmR55	R.EALNSAMAEEL DR DDVFLIGEEVAQYNGAYKV	0	0	mitochondria	
Ecm10	mmK558	R.NAIE TAN KADQLANDTENSIFEFGK.L	0	99.99	mitochondria	
novel methylation sites found						
protein	site	sequence	Percolator <i>q</i> value	methyl site probability	localization	
Pst2	mmK144	K.VAGCFVSTGTGGGNEATIMNSLSTLAHHGIIFVPLGYK.N	0	0	cytoplasm, mitochondria	
Ssa2/4	mmK421/422	K.KSEVFSTYADNQPGLIQVFEGEGER.T	0	0.36	cytoplasm, mitochondria, nucleus, vacuole	
Rps2	mmK49	K.AGK IT TIEIFLHSLPVK.E	0	100	cytoplasm	
Rpl23ab	tmK106, mmK110	R.DGVFLYFEDNAGVIANPKGEMKGSAITGPVKGK.E			cytoplasm	
Rpl23ab	mmK106, tmK110	R.DGVFLYFEDNAGVIANPKGEMKGSAITGPVKGK.E			cytoplasm	
histone H3	mmK38	K.SAPSTGGVKKPHRY	0	0.34	nucleus	
known methylation sites found						
protein	site	sequence	Percolator <i>q</i> value	methyl site probability	localization	
Rpl23ab	dmK106, dmK110	R.RRDGVFLYFEDNAGVIANPKGEMKGSAITGPVKGK.E	0.001962164		cytoplasm	
Rpl42ab	mmK55	K.QSGFGGQTKPVFHK.K	0	100	cytoplasm	
		R.KQSGFGGQTKPVFHK.K	0	100		
Rpl12ab	mmR67	K.IQNRQAAASVPSASSLVTTALKEPPR.D	0	100	cytoplasm	
		K.IQNRQAAASVPSASSLVTTALKEPPRDR.K	0	100		
eEF1A	mmK30	K.STTTGHLIYKCGGIDK.R.T	0	50	cytoplasm	
histone H3	dmK37	K.SAPSTGGVKKPHRY	0	99.64	nucleus	

^aMethylated residues are abbreviated as follows: mm, monomethyl; dm, dimethyl; tm, trimethyl. ^bMethylated residue is bolded and underlined. Based on residue level probabilities, also underlined are other potentially methylated residues. ^cMethyl site probabilities for bolded and underlined residue. ^dLocalization determined from *Saccharomyces* Genome Database.⁸⁰

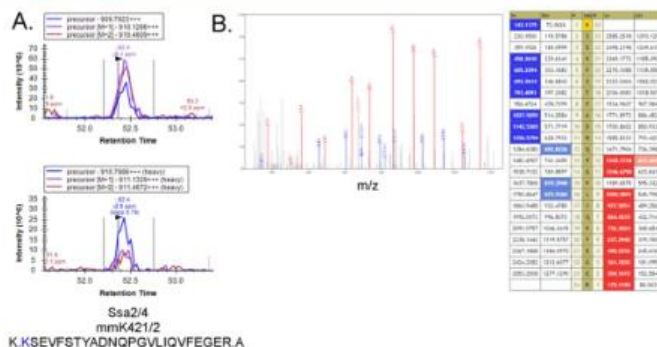
Table 3. Methylated Peptides Found in the Mitochondrial Fraction

novel mitochondrial methylation sites found						
protein	site ^a	sequence ^b	Percolator <i>q</i> value	methyl site probability ^c	localization ^d	
Pdb1	mmR55	R.EALNSAMAEEL DR DDVFLIGEEVAQYNGAYKV	0	0.2	mitochondria	
Ald5	tmK308	K.AVKNIAFGIFYNSGEVCCAGSR.I	0	100	mitochondria	
Ssa2/4	mmK421/422	K.KSEVFSTYADNQPGLIQVFEGEGER.T	0	0.37	cytoplasm, mitochondria, nucleus, vacuole	
Hsp60	tmK490	K.QJIENAGEEGSVIIGKLIIDEYGD DF AK.G	0	100	mitochondria	
Ald4	tmK345	R.VYVEESIYDKFIEEFKAASESIK.V	0	33.33	mitochondria	
Aco1	dmK360	K.DLLSADKDAEYDEVVEIDLNTLEPYINGPPTDLATPVSK.M			mitochondria	
known methylation sites found						
protein	site	sequence	Percolator <i>q</i> value	methyl site probability	localization	
Rpl42ab	mmK55	K.QSGFGGQTKPVFHK.K	0	100	cytoplasm	
eEF1A	mmK30	K.STTTGHLIYK.C	0	100	cytoplasm	
		K.STTTGHLIYKCGGIDK.R	0	100		
eEF1A	tmK79	R.GITIDIALWKFETPK.Y	0	100	cytoplasm	

^aMethylated residues are abbreviated as follows: mm, monomethyl; dm, dimethyl; tm, trimethyl. ^bMethylated residue is bolded and underlined. Based on residue level probabilities, also underlined are other potentially methylated residues. ^cMethyl site probabilities for bolded and underlined residue. ^dLocalization determined from *Saccharomyces* Genome Database.⁸⁰

methylation events are unambiguously on lysine residues and (2) these methyl peptides contain few, if any, acidic residues for assignment. The assignment of trimethyl 79 on eEF1a in the methyl peptide was less clear. While the exact matching peptide

from the original search carried unambiguous probability for trimethylation solely on K79, in most of the methyl peptides identified in the K/R/D/E search, the methyl site probability split for localization to either K79 or E81. However,



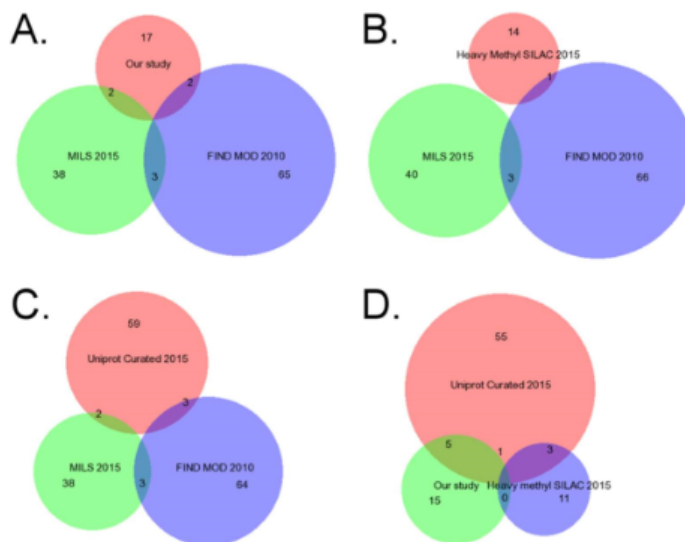


Figure 8. Overlap of methylated proteins identified from the literature and curated from Uniprot. (A) Comparison between "Our study" (red), "MILS 2015" (green), and "FIND MOD 2010" (blue). (B) Comparison between known studies "Heavy methyl SILAC 2015" (red), "MILS 2015" (green), and "FIND MOD 2010" (blue). (C) Comparison with "Uniprot Curated 2015", "MILS 2015" (green), and "FIND MOD 2010" (blue). (D) Comparison to "Uniprot Curated 2015", "Our study" (green), and "Heavy methyl SILAC 2015" (blue). "Our study" was candidate proteins identified in this work. "FIND MOD 2010" proteins are from Pang et al., 2010, "MILS 2015" proteins are from Wang et al., 2015, "Heavy methyl SILAC 2015" proteins are from Plank et al., 2015, and "Uniprot Curated 2015" proteins were manually curated from Uniprot in March 2015.

and Hsl7) in yeast are localized in the cytoplasm and nucleus.¹⁰⁴ Additionally, there does not appear to be any sequence specificity for lysine (Figure 7A,B) or arginine (Figure 7C) methylated residues determined in this study. Again, the common arginine methylation motif (RGG), which Rmt1 is known to methylate¹⁰⁵ is not seen, except in the known Rmt1 substrate, Ded1, presumably because no other methylated arginine site is an Rmt1 target. Thus structural elements appear to be required for the modification at these sites in place of or in addition to the amino acid sequence adjacent to the methylatable residue. On the basis of structural models, most of the newly identified methylation sites are surface-exposed in structurally accessible regions of the proteins (Table S-1, Table 1, Figure S-4). Surface-exposed residues are more likely to be accessible as substrates for a methyltransferase.

DISCUSSION

Approach

Our study presents the first look at global protein methylation in the mitochondria of *Saccharomyces cerevisiae* through the use of heavy methyl SILAC. This work identifies potential substrates for the mitochondrially localized candidate protein methyltransferases. Our finding that mitochondrial ribosomal proteins specifically are methylated is consistent not only with reports of methylated mitochondrial ribosomal proteins from trypanosomes and *Arabidopsis* but also as a hallmark modification for ribosomal proteins as a whole.²⁰ Identification of these methylation reactions may offer insight into the

functional and regulatory detail of mitochondrial translation, metabolism, control, and dynamics.

Many other proteomic studies have attempted to characterize the methyl proteome of *S. cerevisiae*. Even with validation techniques, such as isotopic labeling, there is little to no overlap between methylated proteins determined from established methyl proteome studies and those curated from Uniprot (Figure 8, Table S-2). It thus appears that the methylation sites identified in any given study represent only a fraction of the methylation that occurs on the cellular level. The lack of overlap with the study presented here is likely also due to our focus on analyzing mitochondrial fractions rather than the entire proteome.

Opening searches up to methyl E and D resulted in more E and D assignments than K/R. Many may not be biologically relevant, for example, trimethyl E81, as opposed to trimethyl K79 on eEF1 α , which is a known and well-characterized modification. However, many monomethylation events on glutamate or aspartate appear to be possible as on Pdb1 or Ssa2/4. There are few examples of acidic residue methylation in *S. cerevisiae*. Pab1 in yeast and other organisms has been reported to be methylated on various glutamate residues needed for function.¹⁰⁶ There have been whole cell investigations of glutamate and aspartate methylation in yeast. However, these experiments performed were dependent on in-gel digests, and it is unclear if methanol was avoided in sample preparation.¹⁰³ Additionally, the only well-characterized example of an acidic residue methyltransferase is CheR in the prokaryote chemotaxis signaling system.¹⁰⁷ This does not have

a homolog in yeast. Although there are many putative methyltransferase with substrates that have yet to be identified,⁹ there currently is no identified glutamate or aspartate methyltransferase in yeast. While possible, it seems unlikely that newly identified sites are indeed localized on D/E residues, unless acidic residue methyl esterification may be more common in the mitochondria. Until all of the methyltransferases in yeast have been characterized, this remains a possibility.

Mitochondrial Methylation

Methylated mitochondrial protein candidates serve a variety of functions. Mitochondrial fraction candidates are involved in metabolism or mitochondrial genome maintenance in some capacity. Both Ald4 and Ald5 are mitochondrial aldehyde dehydrogenases. Constitutively expressed Ald5 is the minor isoform, while Ald4 is the major isoform.¹⁰⁸ Pdb1 is the E1 beta subunit of pyruvate dehydrogenase.¹⁰⁹ Ald4 and Pdb1, along with candidates Hsp60 and Aco1, are mitochondrial nucleoid proteins, suggesting a role of methylation in the maintenance of the mitochondrial genome.^{110,111} Interestingly, of these, the aconitase Aco1¹¹² is a multifunctional protein that acts not only in the TCA cycle but also in mtDNA maintenance in an enzyme activity-independent manner. Previous studies suggest that Aco1 couples metabolism to mitochondrial genome maintenance.^{110,113} Although Aco1 is known to be phosphorylated,^{114–116} it is unclear how this modulates Aco1 function. Coupled now to Aco1 methylation, further work is needed to understand how its many post-translational modifications affect its functions.

Methylation may certainly affect translation, similar to the cytoplasmic translational apparatus. Mnp1 and Mrp4 have essential functions in mitochondrial translation.^{97,117,118} Additionally, Mnp1, the L7/12 homologue¹¹⁹ on the yeast mitochondrial ribosome, is vital not only for mitochondrial translation but also for cell cycle progression as well.¹²⁰ Gene deletion of Mnp1 not only affects respiratory growth but also is lethal under fermentative conditions. Methylation of these proteins could represent a point of regulation of mitochondrial translation or be important for assembly or function, as is the case for cytoplasmic ribosomal proteins.^{101,121}

Although not a mitochondrial ribosomal protein, Lat1, the E2 component of the pyruvate dehydrogenase complex,¹²² was recently found to associate with mitochondrial ribosomes.¹²³ Our finding of methylated Lat1 peptides in the MRP fraction suggests that Lat1 does indeed interact with the mitochondrial ribosomes. Other potentially mitochondrial ribosome associated proteins were Pdb1, also the E1 beta subunit of pyruvate dehydrogenase,¹⁰⁹ Ecm10, one of the mitochondrial Hsp70s,¹²⁴ and Pst2, a protein of unknown function found in mitochondrial proteome studies.¹²⁵ Like Lat1, Ecm10 and Pdb1 may also interact with mitochondrial ribosomes, which may explain their presence in this fraction. Both Ecm10 and Pdb1 localize to mitochondrial nucleoids.^{101,126} It has been recently discovered that mitochondrial ribosomes form complexes at mitochondrial nucleoids, termed MIOREX complexes.¹²³ The presence of Ecm10, Pdb1, and Lat1 in the MRP fraction may be indicative of these MIOREX complexes.

Chaperone Methylation and the “Chaperone Code”

Many molecular chaperone proteins were identified as methylated in this study, including Hsp60 and Ssa2/4. Hsp60 is the major chaperone in the mitochondria. In humans, HSPD1, the Hsp60 homologue, interacts with both ETFBKMT

and METTL21B.¹²⁷ ETFBKMT and METTL21B are lysine methyltransferases; this interaction could facilitate a methylation reaction with HSPD1 as a substrate. There are no known interactions of Hsp60 with methyltransferases in yeast.

Methylation of Ssa2/4 is unexpected as the methylation did not occur on the conserved methylation site that was previously reported for Hsp70 proteins in human cells.^{127–130} Methylation of Hsp70 proteins in humans has been implicated as part of a “chaperone code”,¹³¹ and enhanced methylation is often seen in cancer.¹³⁰ However, this site, K556 in Ssa2 and K559 in Ssa4, has been reported as unmethylated in yeast.⁹⁰ We also did not find evidence of lysine 556/559 methylation under respiratory conditions. Monomethylation of K421 or K422 under respiratory conditions presents a unique departure from their human counterparts, particularly if this site is truly on E423 or E424. Monomethylation on lysine 421/422 or E423/424 is novel and presents a divergence from the known methylation state in human Hsps. As the conserved methylation has been implicated in human malignancies,¹³⁰ a further study is required to determine whether this novel methylation site affects Hsp70 function as well.

CONCLUSIONS

Importantly, we were able to adapt heavy methyl SILAC for use in commercially available *met6Δ* strain of yeast. While we have found that methyl proteome studies that do not use isotope labeling have the propensity to overestimate biologically relevant methylations, current heavy methyl SILAC studies using the *MET6+* BY4741 strain^{15,48} likely underestimate the methylations reported. This is due to the fact that *Met6* can catalyze the formation of light methionine using *N*⁵-methyltetrahydrofolate. Gene deletion of *MET6* ensures that heavy cultures will contain only heavy methylated proteins. It is our hope that with this knowledge heavy methyl SILAC can be more widely used in the yeast methyl proteome community.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.6b00521.

Supporting Information table of contents and supplementary table and figure legends. (PDF)

Supplementary Figure S-1. Extracted precursor ion chromatograms and fragmentation patterns of candidates from cytoplasmic ribosomal protein fraction. (PDF)

Supplementary Figure S-2. Extracted precursor ion chromatograms and fragmentation patterns of candidates from mitochondrial ribosomal protein fraction. (PDF)

Supplementary Figure S-3. Extracted precursor ion chromatograms and fragmentation patterns of candidates from mitochondrial fraction. (PDF)

Supplementary Figure S-4. Structural models of newly identified methylation sites. (PDF)

Supplementary Table S-1. Tables of candidate methylated proteins, candidate peptides found in each fraction, and all peptides found across the three subcellular fractions. (XLSX)

Supplementary Table S-2. Tables of overlapping methylated proteins from studies used in Figure 7. (XLSX)

Supplementary Table S-3. Raw data tables from ProLuCID searches through the IP2 pipeline. (XLSX)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest. Raw mass spectrometry files are available for upload at MASSIVE repository (accession number MSV000080107).

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ABBREVIATIONS

Cyto RP, cytoplasmic ribosomal proteins; Mito, mitochondrial; MRP, mitochondrial ribosomal proteins; MudPIT, multidimensional protein identification technology; SILAC, stable isotope labeling by amino acids in cell culture

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CHAPTER FIVE

Physiological Implications of Mitochondrial Protein Methylation

Introduction

Following the characterization of the mitochondrial methyl proteome, described in Chapter Four, I set out to further validate these new methyltransferase substrates using methylation-specific antibodies via immunoblotting. Detection of methyl groups by immunoblotting using such antibodies is currently a standard analytical tool and for many labs it is the only approach used to determine new substrates for methyltransferases. This method is not without its pitfalls, however, and concerns have been raised regarding the specificity of commercial and laboratory-produced antibodies¹.

An additional problem arises when tagged proteins are analyzed by immunoblotting. As discussed in Chapter Two, I found that mitochondrial lysates alone do not have an informative methyl lysine signal due to contaminating cytosolic proteins that react with the antibody and have increased signal over other proteins. It is difficult to detect MRP methylation above the signal of the highly methylated cytosolic ribosomal proteins and elongation factors. Purification of tagged individual proteins can be used to avoid these problems. However, as the targets of validation are mitochondrial, the proteins must be C-terminally tagged as the N-terminal sequence is often important to importing proteins² and N-terminal tags may be cleaved by the proteolytic import machinery². An N-terminal tag may also affect import into mitochondria, which would affect function or potential methylation.

There are few choices for commercially available C-terminally tagged yeast ORFs. One of which are the tandem affinity purification (TAP) tagged ORF yeast strains. The TAP tag has been used in proteomics extensively for the identification of protein complexes³. Additionally, libraries of the yeast TAP-tagged ORF strains have led to characterization of global yeast protein complexes^{4,5} and protein expression⁶. One downside to the TAP system is that the ORF is still

under the endogenous promoter, which may not be ideal for a mitochondrial protein of lower abundance. Other overexpression systems may overcome this, but overexpressing a substrate may affect methylation levels.

I also sought out to understand the physiological implications of the mitochondrial protein methylation using bioinformatics tools. After the work presented in Chapter Four had been published, I found two critical publications that were relevant to the physiological aspects of the proteins being methylated. First, a complete structure of the *Saccharomyces cerevisiae* mitochondrial ribosome (mitoribosome) has been determined⁷. This confirmed the transient association of Rsm22 with the mitoribosome (discussed in more detail in Chapter Three), suggesting its appearance on the mitochondrial ribosome is as a result of its methyltransferase activity. However, Rsm22 is a small subunit MRP, or at least associates with the small subunit⁸. The methylated mitochondrial ribosomal proteins that have been identified so far are components of the large subunit⁹. Second, Larsen *et al.* determined the monomethyl arginine profile of human cells¹⁰.

To further understand the mitochondrial protein methylation, I turned to the human system. This second study is notable as over 370 of the protein targets suggested to contain monomethyl arginine residues by mass spectrometry were mitochondrial¹⁰. By looking into these new mitochondrial targets, more clues into the conservation of mitochondrial methylation from yeast to humans can be obtained.

Methods

Immunoblotting with methyl specific antibodies

Yeast TAP tagged open reading frame strains were obtained from Dr. Michael Carey (UCLA). Aco1, Mnp1, Ald5, and Rpl42a TAP positive strains were selected for by growth on selectable media (SC-His) and purified via standard methods¹¹⁻¹⁴ with the exception that cells were grown in semisynthetic lactate media (described in detail in Chapter Two), rather than YPD, in a shaker incubator at 30 °C to an optical density at 600 nm greater than 1. Cells harvested by centrifugation at 5,000 x g for 5 minutes and washed twice with water. Approximately 100 µL of major steps in the purification were aliquoted and stored at -20 °C.

Protein fractions and lysates (30 µL) were added to an equal volume of 2X SDS running buffer and heated for 3 minutes at 100 °C. Samples and full-range Amersham Rainbow Molecular Weight Markers (GE Healthcare Life Sciences, RPN800E) were loaded on 4-12% gradient BisTris PAGE gel (ExpressPlus, GenScript, M41212). Gels were run at 140 V using a Tris-MOPS-SDS running buffer system (GenScript powder, M00138). Proteins from the gel were transferred to a PVDF membrane (Amersham Hybond, GE Healthcare Life Sciences, Cat 10600023) for 1 hour at 30 V in a Tris-glycine buffer with 10% methanol in an XCell II Blot Module (ThermoFisher Scientific EI9051). Depending on the antibody, membranes were blocked in 5% non-fat dry milk in either phosphate or Tris buffered saline with 0.1% (v/v) Tween 20 (PBST/TBST) or in 5% bovine serum albumin in Tris buffered saline with 0.1% (v/v) Tween 20 (TBST).

The following antibodies were used at the given dilutions and incubation times: anti-TAP Tag antibody; 1:1000 in 1% non-fat dry milk in TBST; 1 hour (ThermoFisher Scientific, CAB1001), anti-methylated (ϵ -N) lysine; 1:1000 in 1% bovine serum albumin in TBST;

overnight at 4 °C (Immunechem, ICP0501), anti-trimethyllysine, conjugated to HRP; 1:5000 in 1% non-fat dry milk in PBST; 1.5 hours (Immunechem, ICP0602, a gift from Dr. Joanna Goldberg (Emory University School of Medicine)¹⁵), and anti-rabbit IgG, conjugated to HRP; 1:6666 in 1% non-fat dry milk or bovine serum albumin in TBST; 1 hour (Cell Signaling, 7074). Protein bands were visualized by chemiluminescence using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, RPN2232) and exposed to Hybond CL Autoradiography Film (Danville Scientific, 1001364). The amount of proteins transferred to the membrane was determined qualitatively by staining with 0.5% (w/v) Ponceau S in 1% acetic acid.

Gene Ontology term enrichment for protein fractions

Gene Ontology (GO)¹⁶ term enrichment analysis was performed directly through the GO Consortium webtool¹⁷. Data were obtained by inputting the gene lists for each protein fraction methyl candidate list from Caslavka Zempel *et al.*⁹ and searching for molecular functions, cellular compartments, and biological processes (respectively) in *Saccharomyces cerevisiae*. GO annotations used were from the GO Ontology database (released 2016-11-30) and the analysis tool used was the PANTHER classification system (released 2016-7-15). Results were corrected for multiple comparisons using the Bonferroni correction. GO annotations were accessed in December 2016.

Structural location of mitochondrial ribosomal protein methylation sites

The recent structure of the mitochondrial ribosome⁷ (PDB: 5MRC) was downloaded. Using PyMol (Schrödinger, Inc.), the methylated MRPs were selected and colored. The

methylated amino acid was selected, displayed as “sticks”, and colored. Measurements were taken atom to atom between I126 of Mrpl27 (mL41, chain 3) and R204 of Mrpl4 (uL29, chain T). Mrpl40 (uL24, chain Q) was also selected.

Comparison of yeast mitochondrial protein methylation to human protein mitochondrial methylation

The data from Larsen *et al.*¹⁰ were downloaded and manually curated for mitochondrial proteins. Keyword “mitochondrial” was used to sort the identified methylated proteins. Other proteins were eliminated as were duplicate proteins. Using Basic Local Alignment Search Tool (BLAST, NCBI)¹⁸, FASTA files of human mitochondrial proteins identified were searched against non-redundant protein sequences (nr) in *Saccharomyces cerevisiae* (taxid: 4932) in a protein-protein BLAST (blastp). Hits in *S. cerevisiae* were then searched against non-redundant protein sequences (nr) in Humans (taxid: 9606) in a protein-protein BLAST (blastp) to determine if the proteins were mutual best hits. FASTA sequences of Human proteins were aligned with FASTA sequences of *S. cerevisiae* homologs using Clustal Omega¹⁹ (EMBL-EBI).

Mitochondrial methyl proteins in Larsen *et al.*¹⁰ were compared to full list of candidate methyl proteins in Caslavka Zempel *et al.*⁹ using a Venn diagram webtool (Bioinformatics & Evolutionary Genomics, Ghent University).

Results

Immunoblotting with methyl specific antibodies

Three unconfirmed mitochondrial targets for methylation (Aco1, Mnp1, and Ald5) and one known methylated protein (Rpl42a) were purified using tandem affinity purification. After

tandem affinity purification, I found that cleavage with tobacco etch virus (TEV) protease was not successful in two cases (Figure 5-1). Ald5-TAP showed a band shifted due to the loss of the Protein A portion of the tag after cleavage. For Aco1-TAP, it is difficult to determine if TEV cleavage was successful given cross-reactivity of the fractions with either the anti-TAP or secondary antibody (Figure 5-1). However, subsequent immunoblotting showed a gel shift in the Aco1 band after TEV cleavage (Figure 5-2). There was no signal in the purified protein lanes for any of the proteins, except for Aco1, as seen in Figure 5-1.

With 2 L cultures as the starting material, there appeared to be very little protein in the fractions (Figure 5-1, Figure 5-2, C). Nonetheless, I continued to probe for methylation of the tagged proteins. Both of the full-length and cleaved variants of Aco1-TAP give a strong signal when immunoblotting for mono- and dimethyllysine, as seen in Figure 5-2, panel A. However, each TAP tagged protein was detected as methylated in the cleared lysates, indicated by large, dark bands (Figure 5-2, A). This pattern was seen again when immunoblotting for trimethyllysine (Figure 5-2, B), even though the majority of the TAP tagged proteins are not trimethylated⁹. Ald5 was identified as trimethylated on K308⁹. However, no signal for trimethyllysine was observed in the lane containing Ald5-TAP after cleavage (Figure 5-2, B). Unlike the lysate bands for each of the TAP-tagged proteins, Aco1-TAP after cleavage did not have signal for trimethylated lysine, suggesting that the band in Figure 5-2, panel A, is specific for mono- and dimethyllysine. This suggests that the identification of Aco1 as dimethylated on a lysine residue is correct.

Gene Ontology term enrichment for protein fractions

GO terms were analyzed for enrichment within the methylated candidates for each protein fraction (described in detail in Chapter Four). This was not very informative. The GO terms for both cellular compartments and molecular function corresponded primarily to the specific protein fraction each methyl candidate was in (Figure 5-3). For cellular compartments, cytoplasmic ribosomal fraction was enriched for elongation factor complexes and translation machinery, the mitochondrial fraction mitochondrial matrix and nucleoid, and the mitochondrial ribosomal fraction in translation (Figure 5-3). Molecular function enrichment was similar in that the enrichment corresponded to the function of the fraction as a whole.

Structural location of mitochondrial ribosomal protein methylation sites

The current structure of the mitoribosome was downloaded to investigate positioning the methylated proteins and methylated residues in the context of the two subunits as a whole. Both Mrpl4 and Mrpl40 are positioned near the surface of the large subunit, maintaining more protein-protein interactions as opposed to core proteins making protein-RNA interactions (Figure 5-4, A). It is interesting to note that Mrpl4 and Mrpl40 are in close proximity to each other, interacting in the structure of the mitoribosome. The methylated residue of Mrpl40 (K186) is surface exposed in a flexible loop (Figure 5-4, B) while the methylated residue of Mrpl4 is on an alpha helix interacting with another protein, Mrpl27, potentially through interactions with the backbone of I162 (Figure 5-4, C). In the mitoribosome structure, it appears as though methylated residues are maintaining protein-protein interactions as opposed to the protein-RNA stabilizing effect the methylated residues on many cytoplasmic ribosomal protein provide buried deep in the ribosome²⁰.

Comparison of yeast mitochondrial protein methylation to human protein mitochondrial methylation

Using the data from Larsen *et al.*¹⁰, methylated mitochondrial proteins were BLASTed to determine their yeast homolog. Not every mitochondrial protein that was identified as methylated had a yeast homolog and this resulted in a list of 98 homologous yeast proteins. These proteins were then compared to the entire list of methylated proteins, not just those that were validated by heavy methyl SILAC, in Caslavka Zempel *et al.*⁹. Thirteen proteins overlapped between the putative methylated proteins in yeast and the yeast homologs of methylated human proteins (Figure 5-5). Of these, seven had been confirmed by heavy methyl SILAC in yeast: Ald5, Ald4, Lat1, Ecm10, Aco1, Ssa4, and Pdb1 (Table 5-1). Similar mitochondrial proteins appear to be methylated in both yeast and human mitochondria.

Notably, Pdb1 and the human PDHB, the beta subunit of E1 of pyruvate dehydrogenase, have 63% identity in the BLAST search. When aligned, the majority of the sequence appears to be conserved (Figure 5-6). Larsen *et al.* identified a monomethyl arginine on R49 of PDHB¹⁰. This is in the same position and a similar sequence to the Pdb1 monomethyl arginine on R55⁹. Both methylated arginines are surrounded by acidic residues (Figure 5-6), indicating a similar sequence motif for the methylation reaction and perhaps related methyltransferases. The presence of this methyl mark in both human and yeast proteomic analyses suggests that it is conserved.

Discussion

Through immunoblotting with methylation specific antibodies, the dimethylated lysine residue of Aco1 appears to be confirmed. As for the other TAP tagged proteins, it remains

unclear if the signal observed is due to methylation or cross-reactivity with the antibody. The TAP-tagged proteins contain a Protein A tag. Protein A, from *Staphylococcus aureus*, binds immunoglobulins very well²¹. This allows for affinity purification. This may be what is causing the artefactually large signal of the TAP tagged proteins in the lysate. If the immunoglobins used for immunoblotting can recognize the Protein A epitope even after linearization by SDS and transfer to a membrane, there will be a signal for the TAP-tagged protein regardless of the antibodies used. As the Aco1-TAP signal after TEV cleavage is presumably on a protein with the Protein A portion of the tag cleaved, this appears to be a real result.

This confirmation of the methylation of Aco1 is interesting for many reasons. The human ACO2 and other TCA cycle components have been implicated in cancer pathogenesis²². Additionally, also in human cells, citrate synthase, another TCA cycle enzyme, has been identified as methylated²³. Citrate synthase was identified as methylated on the homologous lysine residue in the data from Chapter Four, though this did not pass the heavy-light doublet test⁹. Methylation may then be a mechanism for regulation of the TCA cycle or for association of the TCA cycle enzymes with each other. Recent evidence suggests that TCA cycle enzymes form super complexes, termed the metabolon²⁴. Additional methyl groups or retaining the positive charge on lysine residues could facilitate this interaction as, at least in human systems, the loss of the methyl groups on citrate synthase do not affect protein amount or enzymatic activity²³.

In the structure of the mitoribosome, Mrpl40 and Mrpl4 interact with each other on the large subunit. The surface exposure of K186 on Mrpl40 and the contacts Mrpl4 R204 makes with Mrpl27 suggest an involvement of the methylated residues on protein-protein interactions and thus, mitoribosome structure. Mrpl4 is indispensable not only for mitochondrial translation, but

for growth on fermentable carbon sources as well²⁵. The portion of Mrpl40 where K186 is located is in a mitochondrial specific C-terminal extension (from residues 86 to 297)²⁶. It has been shown that Mrpl40 is in close proximity to Oxa1, potentially through interaction with this C-terminal domain. This could be an interface between the mitoribosome and Oxa1²⁶. Additionally, the absence of the C-terminal region causes defects in oxidative phosphorylation, through the loss of mitochondrially translated products²⁶. Mrpl40's positioning in the exit tunnel and close to the membrane may allow for facilitation of co-translational insertion into the membrane via this association to Oxa1^{26,27}. Methylation may regulate or facilitate this. This allows points toward the methylation of mitochondrial proteins as an important mechanism for facilitating protein-protein interactions^{23,28}.

Comparisons between proteomic data from yeast and humans indicate that similar proteins are methylated in the mitochondria in both organisms. Direct evidence of the same site being modified on both yeast and human pyruvate dehydrogenase (Pdb1 and PDHB) seems to demonstrate that some of these sites are conserved from lower to higher eukaryotes. The function of these methyl marks and the role of methylation for the structure and function of the proteins may affect mitochondrial processes. This further opens the door to continuing investigations into the methylation state of mitochondrial proteins, not only in yeast, but in the human homologs as well.

FIGURES

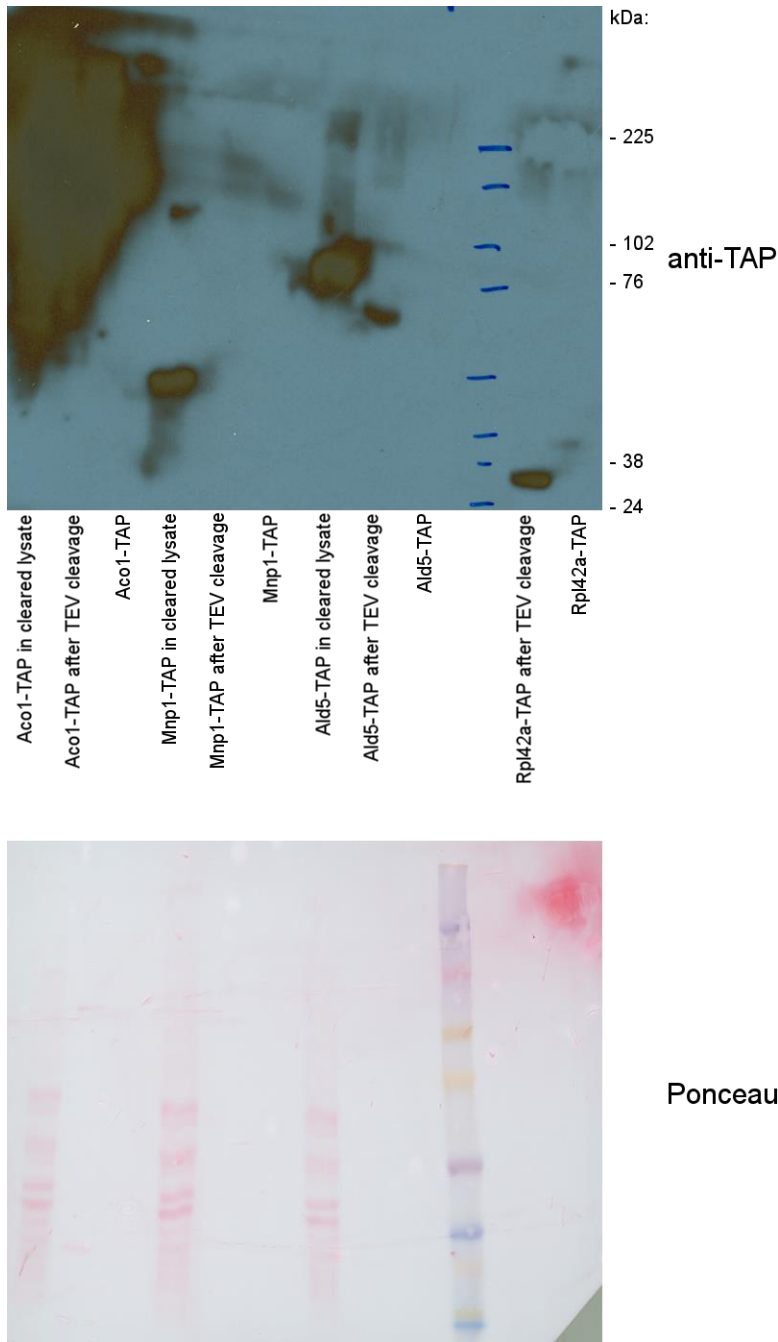


Figure 5-1. Determination of successful tandem affinity purification of methylated protein candidates. Tagged proteins under the endogenous promoter were purified using tandem affinity purification. Cleared lysate, the fraction after tobacco etch virus (TEV) protease cleavage, and the final elution containing purified protein were loaded on a pre-cast 4-12% BisTris gel.

Molecular weights are as follows (+tag, -Protein A portion of TAP tag): Rpl42a- 33.2 kDa, 18.2 kDa; Aco1- 104.6 kDa (-N-terminal MTS), 89.6 kDa; Ald5- 75 kDa (-N-terminal MTS), 60 kDa; Mnp1- 37.9 kDa (-N-terminal MTS), 22.9 kDa. Top panel: protein bands were visualized using an anti-TAP tag antibody (1:1000) and an anti-rabbit IgG antibody conjugated to HRP (1:6666) with chemiluminescence detected using ECL reagent. The image is of a 5 second exposure of the membrane to autoradiography film. Bottom panel: membrane stained with Ponceau S.

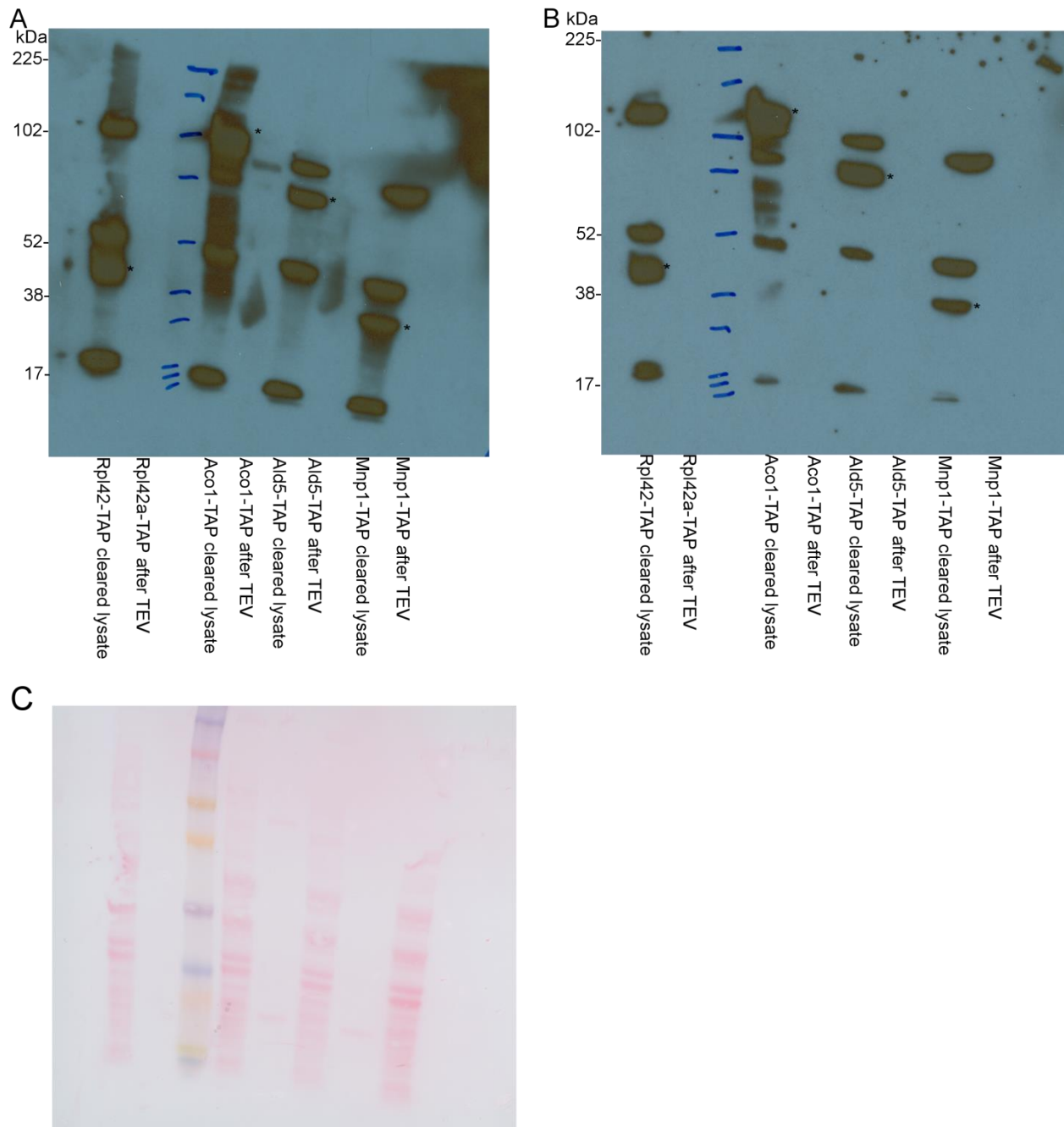


Figure 5-2. Immunoblotting of tandem affinity purification fractions for methyl lysine.

Tagged proteins under the endogenous promoter were purified using tandem affinity purification. Cleared lysate and the fraction after tobacco etch virus (TEV) protease cleavage were loaded on a pre-cast 4-12% BisTris gel. Asterisks indicate the band associated with the molecular weight of the tagged-protein construct present in the lysate. Molecular weights are as follows (+tag, -

Protein A portion of TAP tag): Rpl42a- 33.2 kDa, 18.2 kDa; Aco1- 104.6 kDa (-N-terminal MTS), 89.6 kDa; Ald5- 75 kDa (-N-terminal MTS), 60 kDa; Mnp1- 37.9 kDa (-N-terminal MTS), 22.9 kDa. A.) Protein bands were visualized using an anti-methyl lysine antibody (specific for monomethyl and dimethyllysine, 1:1000) and an anti-rabbit IgG antibody conjugated to HRP (1:6666) with chemiluminescence detected using ECL reagent. The image is of a 1 minute exposure of the membrane to autoradiography film. B.) Protein bands were visualized using an anti-trimethyllysine antibody conjugated to HRP (1:5000) with chemiluminescence detected using ECL reagent. The image is of a 5 minute exposure of the membrane to autoradiography film. C.) Membrane stained with Ponceau S.

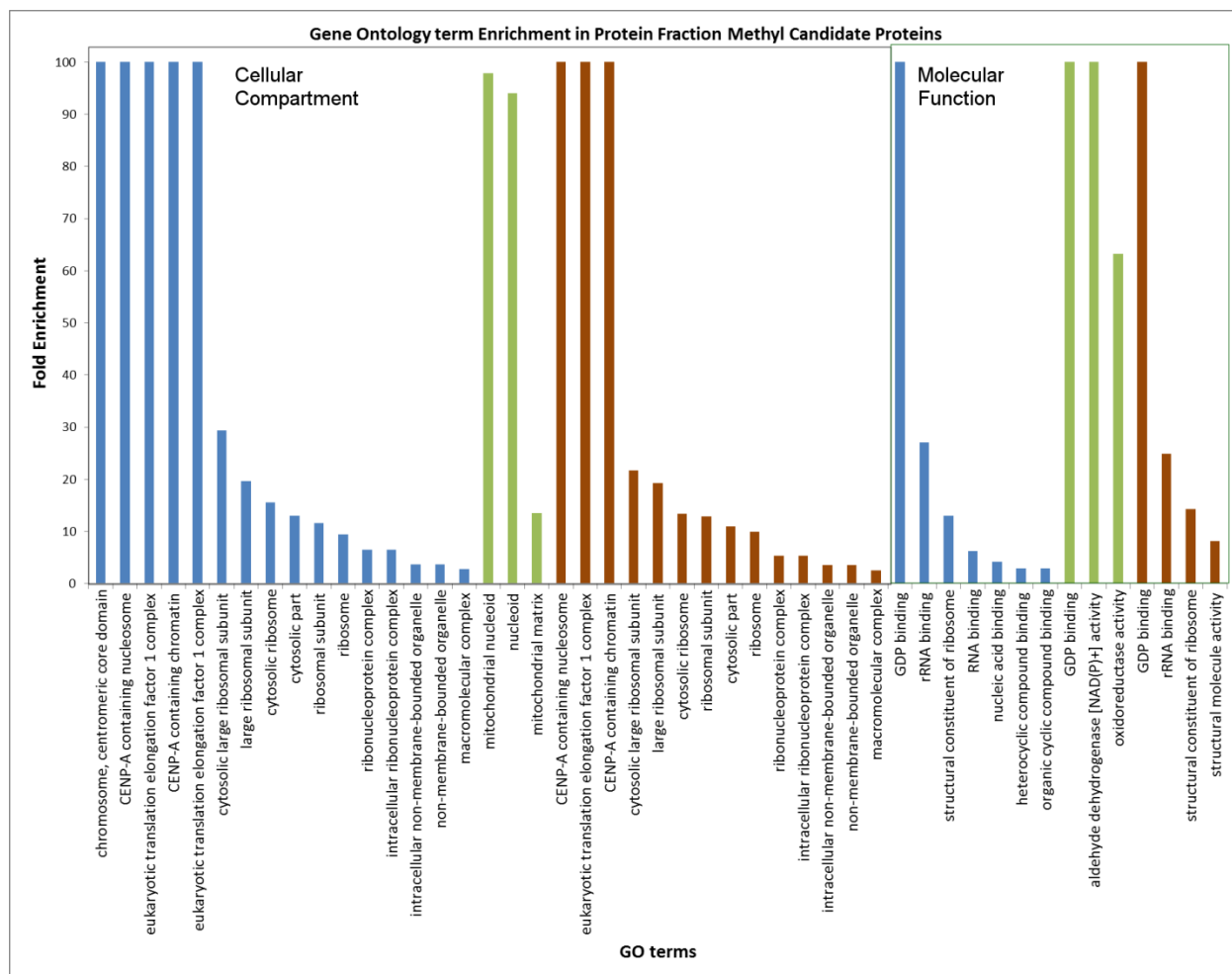


Figure 5-3. Gene Ontology term fold enrichment. GO term enrichment analysis was performed on methyl candidate proteins from each of the protein fractions. Enrichment by cellular compartment (gray box) and molecular function (green box) was investigated. Fold enrichment at 100 is indicative of >100 fold enrichment in the analysis. Cytoplasmic ribosomal protein fraction methyl candidates are in blue, mitochondrial fraction methyl candidates are in green, and mitochondrial ribosomal protein fraction methyl candidates are in orange.

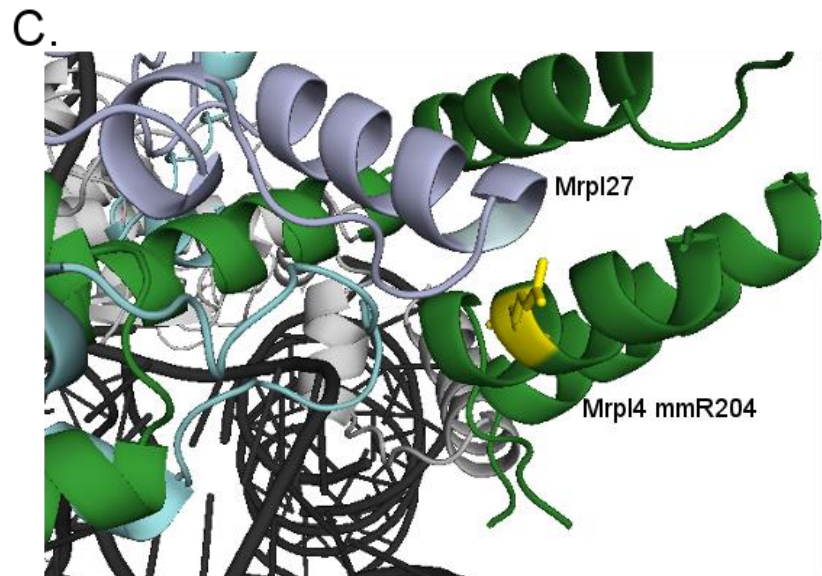
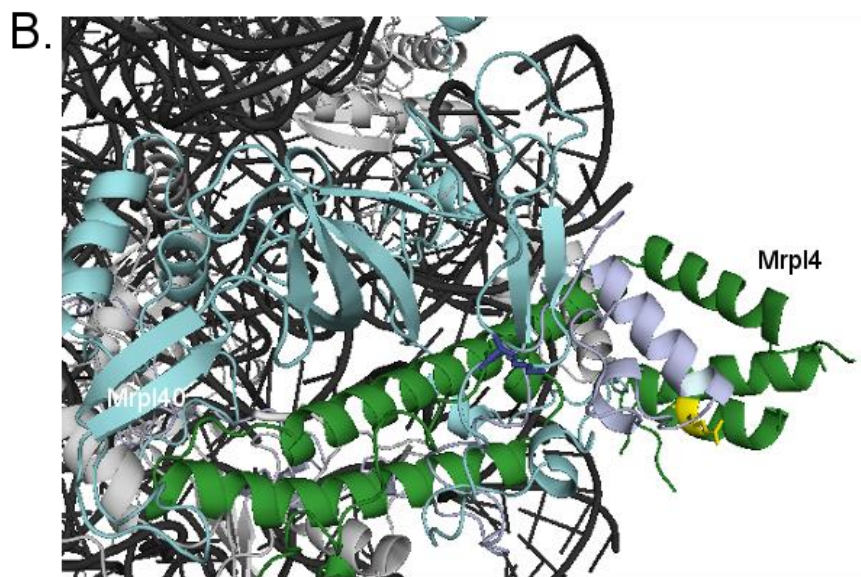
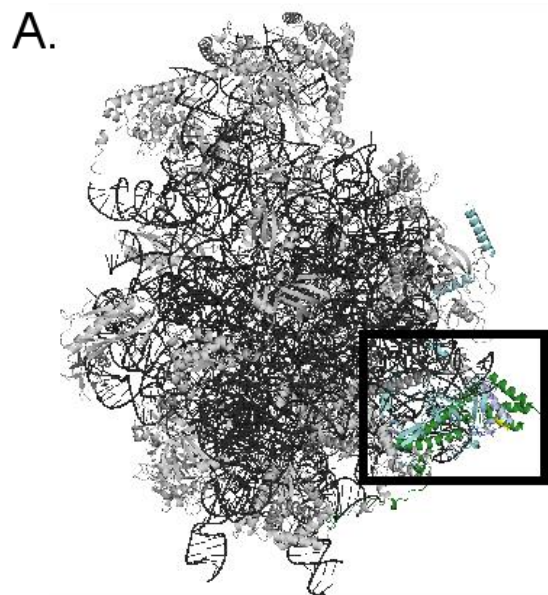


Figure 5-4. Structural positioning of methyl groups within the context of the mitoribosome.

A.) Structure of the complete mitoribosome in *S. cerevisiae*. Methylated proteins are highlighted in cyan and green (Mrpl40 and Mrpl4 respectively, Mnp1 is not present in the crystal structure). RNA is in black with other mitochondrial proteins shown in shades of gray. B.) Zoom-in of the boxed portion of the mitoribosome in panel A. Mrpl40 is highlighted in cyan with K186 in blue (potential site of monomethylation). Mrpl4 is in green with R204 in yellow (also the potential site of monomethylation). C.) Mrpl4 R204 interacts with Mrpl24 (in blue gray). (PDB: 5MRC)

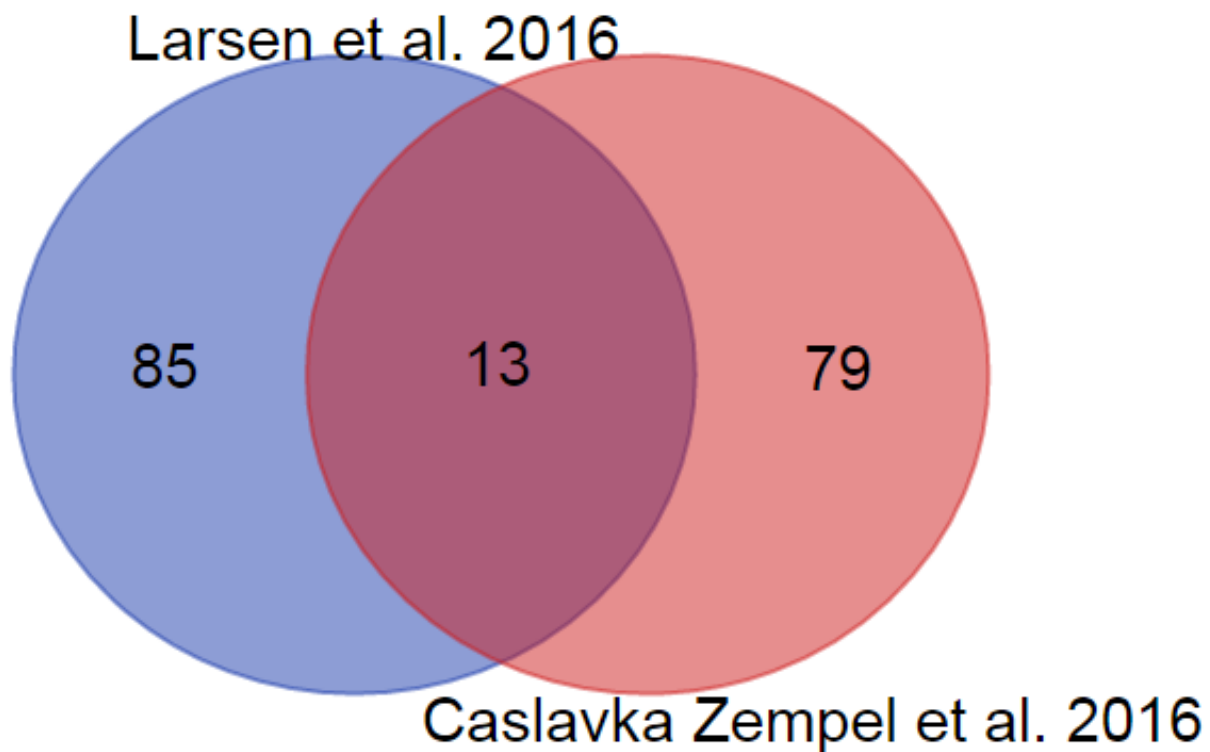


Figure 5-5. Overlap of yeast mitochondrially localized methylated proteins with human mitochondrially-localized methylated proteins. Human mitochondrial proteins identified to be methylated in Larsen *et al.* 2016¹⁰ were BLASTed against yeast proteins. The resulting list of yeast homologs were directly compared to the putatively methylated proteins from Caslavka Zempel *et al.* 2016⁹.

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hPDHB -MAAV-SGLVRRPLREVSG-LLK--RRFHWTAPAAALQVTVRDAINQGMDEELERDEKVF 54
scPDB1 MFSRLPTSLARNVARRAPTSFVRPSAAAAALRFSSTKTMTVREALNSAMAEELDRDDDFV 60
      :: : .*.* *..   ::   :: :***:*:*.* ***:***:**

hPDHB LLGEEVAQYDGAYKVS RGLWKKYGDKRIIDTPISEMGFAGIAVGAAMAGLRPICFMTFN 114
scPDB1 LIGEEVAQYNGAYKVS KGLLDRFGE RRVDTPITEYGF TGLAVGAALKGLKPIVEFMSFN 120
      *:*****:*****:**   ::*::::*****:* **:*:*****: **.* **.***

hPDHB FSMQAIDQVINSAAKTYMSSGGLQPVPVIVFRGPNGASAGVAAQHSQCFAAWYGHCPGLKV 174
scPDB1 FSMQAIDHVNSAAKTHYMSGGTQKQMVFRGPNGAAVGVGAQHSQDFSPWYGSIPGLKV 180
      *****:*:*****:***** *   :*****:.* **.* **.* **.*

hPDHB VSPWNSEDAKGLIKSAIRDNNPVVWLENELMYGVPEFPPEAQSKDFLIPIGKAKIERQG 234
scPDB1 LVPYSAEDARGLLKA AIRDPNPVVFLENELLYGESFEISEEALSPEFTLPY-KAKIEREG 239
      : *::**:*:*:*:**** ***,*****:** **:* ** * :* :* *****:**

hPDHB THITVVSHSRPVGHCLEAAAVLS-KEGVECEVINMRTIRPMDMETIEASVMKTNHLVTVE 293
scPDB1 TDISIVTYTRNVQFSL EAAEILQKKYGVSAEVINLR SIRPLDTEAIIKTVKKNHLITVE 299
      *.*:~::~:* * .*.**** :* * **..****:*:***.* *:* .* *****:**

hPDHB GGWPQFGVGAEICARIMEGPAFNFLDAPAVRVTGADVPMYAKILEDNSIPQVKDIIFAI 353
scPDB1 STFPSFGVGAEIQAQVMESEAFDYLDAPIQRVTGADVPTPYAKELEDFAFPDTPYIVKAV 359
      . :*.***** *::**.* **:*:**** ***** **.* **.* **.* **.*

hPDHB KKT LNI-          359
scPDB1 KEVLSIE         366
      *:.*. *

```

Figure 5-6. Alignment of hPDHB to scPDB1. The amino acid sequences of human and yeast pyruvate dehydrogenase E1, subunit beta were aligned in CLUSTAL Omega. The top amino acid sequence is the human protein (hPDHB) and the bottom amino acid sequence is the yeast protein (scPDB1). The blue box contains R49 (hPDHB) and R55 (scPDB1), a residue that was shown to be methylated in both organisms.

TABLES

Table 5-1. Common methylated mitochondrial proteins in humans and yeast. UniProt accession numbers of methylated human proteins¹⁰ are listed with the corresponding yeast homolog⁹ and E values from the BLAST search using the human protein as a query. BLAST accessed in December 2016 and January 2017.

Human protein UniProt accession number	Yeast homolog(s)	Percent amino acid identity	E value
A2A274	Aco1	67%	0
A5YVE9	Pda1	56%	2.00E-122
O00330	Lat1	34%	1.00E-68
P05091	Ald4	62%	0.00E+00
P11177	Pdb1	63%	1.00E-152
P25705	Atp1	74%	0
P30837	Ald4	50%	0
P31040	Sdh1	66%	0
P38646	Ssc1, Ecm10, Ssq1 ^a , Ssa3,Ssa4	66-54%	0
P47985	Rip1	58%	9.00E-73
P48735	Idh2	64%	0
P50213	Idh2	56%	1.00E-122
Q02252	Ald4	29%	1.00E-65
Q3SY69	Ald5	45%	2.00E-145

^aDue to the high sequence similarity of Hsp70 proteins, Ssq1 was identified as a homolog. Ssq1 was not determined to be methylated in yeast.

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CHAPTER SIX

Comparing Methylated Proteins in *Saccharomyces cerevisiae* under Fermentative and Respiratory Growth Conditions

Introduction

In Chapter Four, I reported on the presence of methylated proteins in cytoplasmic ribosomal, mitochondrial ribosomal, and whole mitochondrial fractions of *Saccharomyces cerevisiae* grown under respiratory conditions. I was surprised to find that not all of the cytoplasmic ribosomal proteins previously demonstrated to be methylated were identified as such with our method. I suggested various reasons as to why that might have been¹, including the possibility that not all protein methylation reactions that occur in yeast grown under fermentative conditions would also occur under respiratory conditions.

It has already been demonstrated that ribosomal proteins can have different methylation states given different growth conditions. In bacteria, the methylation of lysine residues on rpL7 and L12 decreases with increasing temperature². In budding yeast, there is a decrease of methylation of Rps1b and a 10-fold increase in methylation of Rps2 in stationary phase (after 48 hours of growth) compared to exponential growth³. In the fission yeast *Schizosaccharomyces pombe*, methylation levels of Rpl42 affects growth in stationary phase⁴. The growth phase that cells are in while harvested affects methylation levels of known methylated proteins. In addition to growth under respiratory conditions, light and heavy labeled cells in Caslavka Zempel *et al.* were harvested after about 3 days of growth¹, which could also affect methylation levels.

Growth medium and carbon source can affect protein abundance, which can in turn affect identification of methylation sites. As yeast are facultative anaerobes, they must metabolically adapt based on the carbon source available⁵. In an investigation comparing mitochondrial proteins identified under fermentative and respiratory conditions, there was significant variation in protein abundance, particularly a decrease in abundance of certain proteins under fermentation⁵. It is possible this change in protein content of the mitochondria can occur on a

global level as well given specific carbon sources. More importantly, changes in phosphorylation of mitochondrial proteins were also carbon source specific, varying with the medium used for growth⁵. Therefore, other posttranslational modifications, such as methylation, may not only be affected by protein abundance under different growth conditions, but may change levels based on the metabolic needs of the cell.

Cytoplasmic ribosomal proteins may be one such target for changes in modifications with varying carbon sources. Given the evidence from log phase compared to stationary phase, methylation levels are likely to change for cytoplasmic ribosomal proteins under other cellular conditions, including carbon source in the growth medium. Our laboratory has some evidence for varying methylation levels with growth on non-fermentable carbon sources. This was first observed on Rps2, which is substoichiometrically modified on an arginine residue that varies in methylation level depending on the carbon source⁶. This was further supported later while investigating the methylation levels of cytoplasmic ribosomal proteins after cell growth on non-fermentable carbon sources⁷. In this chapter, I sought to further determine the changes in methylation level of cytoplasmic proteins under fermentation and respiration using the heavy methyl SILAC approach described in Chapter Four. Specifically, I aimed to determine if the missed cytoplasmic ribosomal proteins were not identified as methylated due to the method of identification or due to the growth conditions.

Methods

Yeast strain used

The methionine auxotroph strain *met6Δ* (*MATa* his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, met6::kanMX) was used in this study. It was obtained from GE Dharmacon (YSC6273-201937518).

Heavy labeling of cell cultures- respiration

Cells were grown under respiratory conditions in 100 mL modified semisynthetic lactate medium (LM, described in Chapter Two). In both heavy and light cultures, 1.75 g/L yeast nitrogen base without amino acids or ammonium sulfate and 5 g/L ammonium sulfate was used in place of yeast extract. For light cultures, 0.79 g/L complete supplement mixture powder (CSM, MP Biomedicals) was added as an amino acid source. For heavy cultures, the medium was supplemented with 0.75 g/L methionine dropout amino acid mix (CSM-Met, MP Biomedicals) and 20 mg/L L-methionine (*methyl-D₃*, 98%) (Cambridge Isotope Laboratories). As with other non-mitochondrial heavy methyl SILAC proteomic investigations⁸, cells were grown in a shaker incubator at 30 °C at 250 rpm to an optical density at 600 nm of 0.7-1.0 and harvested by centrifugation at 5,000 x g for 5 minutes at 4 °C. Two biological replicates were performed for each condition (heavy and light).

Heavy labeling of cell cultures- fermentation

Cells were grown under fermentative conditions in 100 mL synthetic complete (SC) medium, either with light amino acids or supplemented with heavy methionine. Light cultures were grown in SC with 1.7 g/L yeast nitrogen base, 5 g/L ammonium sulfate, 2% dextrose, and

0.79 g/L complete supplement mixture powder (CSM, MP Biomedicals). Heavy cultures were grown in SC+ D₃-methionine: 1.7 g/L yeast nitrogen base, 5 g/L ammonium sulfate, 2% dextrose, 0.75 g/L methionine dropout amino acid mix (CSM-Met, MP Biomedicals) and 20 mg/L L-methionine (*methyl-D*₃, 98%) (Cambridge Isotope Laboratories). Cells were grown in a shaker incubator at 30 °C at 250 rpm to an optical density at 600 nm of 0.7-1.0 and harvested by centrifugation at 5,000 x g for 5 minutes at 4 °C. Two biological replicates were performed for each condition (heavy and light).

Cytosolic isolation

Cytosolic isolation of both heavy and light cultures under both conditions was performed similarly to mitochondrial isolation⁹. Cell pellets were thawed on ice and resuspended with 1 mL 0.1 M Tris-SO₄, pH 9.4 with 10 mM dithiothreitol. This suspension was incubated at 30 °C with shaking at 250 rpm for 15 minutes. Cells were pelleted by centrifugation for 5 minutes at 2,000 x g and at 4 °C. The pellets were resuspended in 1.6 mL 1.4 M sorbitol, 20 mM phosphate, pH 7.4. Cells were pelleted by centrifugation for 5 minutes at 2,000 x g and at 4 °C. Cells were converted to spheroplasts by incubation with 2 mL of 1.25 mg/mL zymolyase 20T for 30 minutes at 4 °C with shaking at 250 rpm. Spheroplasts were centrifuged at 4000 x g for 5 minutes at 4 °C. Spheroplasts were washed twice in 1.6 mL 1.4 M sorbitol, 20 mM phosphate, pH 7.4. Washed spheroplasts were resuspended in 2 mL 0.6 M sorbitol, 20 mM HEPES, pH 7.4 with 0.5 mM phenylmethylsulfonyl fluoride. Spheroplasts were dounce homogenized with 15 strokes using a type B pestle. Homogenized spheroplasts were brought to a volume of 5 mL with 0.6 M sorbitol, 20 mM HEPES, pH 7.4 with 0.5 mM phenylmethylsulfonyl fluoride. The lysate was cleared by centrifugation at 1,500 x g for 5 minutes. Supernatant was reserved and the homogenization step

was repeated using the pellet. Combined supernatants were centrifuged for 10 minutes at 12,000 x g. Mitochondrial pellets were discarded. The supernatant (cytosol) was transferred to a fresh 15 mL conical tube. Two 1 mL aliquots were reserved in microcentrifuge tubes. Cytosols were stored at -20 °C.

Preparation of samples for mass spectrometry

After quantification of the amount of protein in each cytosol, heavy and light cytosols of each replicate in each condition were combined in a 1:1 ratio based on protein amount. 75 µg of heavy cytosol protein and 75 µg of light cytosol protein combined to give a total of 150 µg of protein. Combined heavy:light cytosols were de-salted and precipitated with trichloroacetic acid as previously described¹. Protein pellets were stored at -20 °C until analysis by mass spectrometry.

Mass spectrometry analysis

Sample preparation, protease digestion, and mass spectrometry analysis were performed as previously described¹. To identify methylated peptides, a differential modification search was employed which considered variable mass shifts of 14.0157 (monomethylation), 28.0314 (dimethylation) and 42.0471 (trimethylation) on lysine or arginine residues. The heavy search considered a static mass shift of 3.0189 Da on methionine corresponding to L-[*methyl-D*₃]-methionine and variable mass shifts of 17.0346 (monomethylation), 34.0692 (dimethylation) and 51.1038 Da (trimethylation) on lysine or arginine. Database searches were conducted considering only fully tryptic peptides using the MS-GF+ search algorithm¹⁰. The search results were analyzed using Percolator and filtered at a spectrum-level q-value of 0.01¹¹ and a precursor

mass error of less than 2 ppm. Methyl site localization was performed using the PTMRS algorithm and only peptides with a greater than a localization probability of greater than 0.05 were considered further¹². The Skyline software package was used to manually evaluate the presence of heavy-light peptide doublet from extracted ion chromatograms generated for each peptide¹³. Raw data can be found in Supplemental Table 3, available on ProQuest.com.

Skyline evaluation of heavy and light doublets

Peptides were selected as passing the heavy-light doublet test if 1) both heavy and light peptides were present and 2) they were present in a reasonable ratio. Presence of heavy or light peptides was confirmed through comparing the isotope distribution of both the heavy and light peptides to the expected isotope distribution. Peptides were not included if one peptide (either heavy or light) had an isotope distribution that was not as expected. Additionally, the peak areas of heavy and light peptides were compared to confirm the expected 1:1 ratio. More heavy or light peak area was allowed as long as the peak area was not significantly more or less than the peak area of the corresponding heavy or light peptide.

Peptides meeting these criteria were further split into two categories: those that were found in both biological replicates or those that were found in just one biological replicate. The resulting methyl peptide lists were then further narrowed down in confidence by the fragmentation patterns in the MS2 spectra for the peptides. Peptides that may have passed the heavy-light doublet test were not included in the final methylated peptide list if there were not informative ions for that peptide, as visualized in the Skyline software program¹³. However, peptides with lower quality spectra were included in the list if the methylation site on the peptide was previously identified. Fermentation and respiration lists were curated separately to avoid

bias in the peptides determined to pass the heavy-light doublet test. Fragmentation patterns, isotope distributions, and peak areas for the peptides included as methylated are included in Appendix 2. Where applicable, ions of the fragmented methyl peptide were generated using MS-Product in ProteinProspector 5.19.1 Basic (UCSF).

Comparison of methyl peptides identified in fermentation and respiration

The resulting manually curated methyl peptide lists were compared for presence of similar peptides between the two conditions. First, methyl peptides were compared for presence in the list of passing methyl peptides for either condition. Second, “true” methyl peptides from one condition were searched for in the entire dataset of the other condition to observe mere presence of that peptide in the other condition. Peptides that were unique to either fermentation or respiration were noted. Peptides were also classified as known or unknown in terms of methylation state based on UniProt¹⁴, arginine methylation¹⁵, and mitochondrial methylation¹ datasets. Additionally, proteins containing methyl peptides identified in either fermentation or respiration were compared using a Venn diagram webtool (Bioinformatics & Evolutionary Genomics, Ghent University).

Results

Fermentation

The majority of peptides determined to be methylated from the two biological replicate cytosols isolated from fermenting yeast cells were on known methylated proteins (Table 6-1). Those that were new sites were either a different methylation state at a known methylation site (e.g. Rpl23a) or completely novel (e.g. Ssa1 and Srp40) (Table 6-1). A little over half of the

methylated peptides found in only one replicate were novel (Table 6-2). Known methylation sites on Rpl42b, eEF1A (Tef2), Nsr1, and Ded1 were found in only one replicate. Additionally, the lysine methylation on Ssa2/4, identified under respiratory conditions in Chapter Four, is also identified under fermentation.

The fermentation dataset allowed for the identification of the known lysine methylations of Rpl23a, arginine methylation of Rpl12b, both lysine methylation sites on Rpl42b, and all four methylation sites on eEF1A (save for the fifth methylation site, which is the methyl esterification of its C-terminus) (Table 6-1, 6-2). Not all the cytoplasmic ribosomal proteins were identified as methylated on lysine and arginine under either condition; Rpl1, Rps2, and Rps3. This is similar to the missing cytoplasmic ribosomal proteins that were not identified in Chapter Four. As this appears to be specific for the method, the four methylated translation associated proteins identified as methylated on known sites can serve as a benchmark for the method.

Additional methylation states were observed on eEF1A and Rpl23a. Rpl23a appears to be trimethylated on K106, with no methylation on K110 (Table 6-2) and monomethylated on K110 in addition to the dimethylation of K106 (Table 6-1). The trimethylation of K106 on Rpl23a is supported by two informative ions in the fragmentation of the peptide: y_{12} and y_{16}^{+2} (Figure 6-1). The possibility of another modification state, a dimethylation and monomethylation on K106 and 110, respectively, cannot be discounted, although there is less support for this state of the peptide in the fragmentation pattern (Appendix 2). Lysine 79 is variably methylated on eEF1A, which has previously only been shown to be di- or trimethylated^{16,17}. In this experiment, I observed monomethylation and confirmed the dimethylation of K79 (Table 6-2).

Respiration

Similar to the fermentation dataset, the majority of the methylated peptides identified under respiratory conditions in both isolated cytosols were known methylation sites (Table 6-3). Of the new methylation sites identified, methylation sites were either new methylation states at a known methylation site (e.g. Rpl23a), novel sites on known methylated proteins (e.g. Ssa2) or completely novel (e.g. Srp40 and Pub1). Known and new methylated peptides were identified in only one of the biological replicates (Table 6-4). Here, there is an increase of methylated mitochondrial proteins, such as the known methylation on cytochrome *c* (Cyc1) and a new methylation site on Ald5 (which was identified as methylated on a different lysine residue in Chapter Four). This site is distinct from the trimethylation on K360 that was previously reported¹. However, no ions in the fragmentation of the peptide actually contain the modified residue (Appendix 2), so there is little spectral support for the new methylation reported here.

Like in the fermentation dataset, characteristic methylation sites on Rpl23a, Rpl12b, Rpl42b, and eEF1A are also seen in respiration.

Comparison between fermentation and respiration

Most of the methylated peptides identified under either condition were identified as methylated in the other condition (Table 6-1 to 6-4). If proteins identified as methylated are compared between the two conditions, only 2 proteins are unique in fermentation and 4 proteins are unique in respiration (Figure 6-2). By comparing methylation sites identified between the two conditions, some of the peptides identified as methylated in one condition have different methylation states in respiration, such as Ssa2 under respiration (Table 6-3) or Nsr1 under fermentation (Table 6-2).

Only two methylation sites unique to fermentation: dmK190 on Kdx1 and tmK106 on Rpl23a (a different methylation state than previously determined) (Table 6-5). Methylation sites found only when yeast cells are respiring are as follows: dmK467 on Ald5, tmK78 on Cyc1, mmR23 on Ssa2, dmK390 on eEF1A (novel methylation state than previously identified), and mmR45 and mmR60 on Yer152c (Table 6-6). Unlike fermentation, respiration allowed for identification of more mitochondrial targets of methylation (Ald5 and Cyc1).

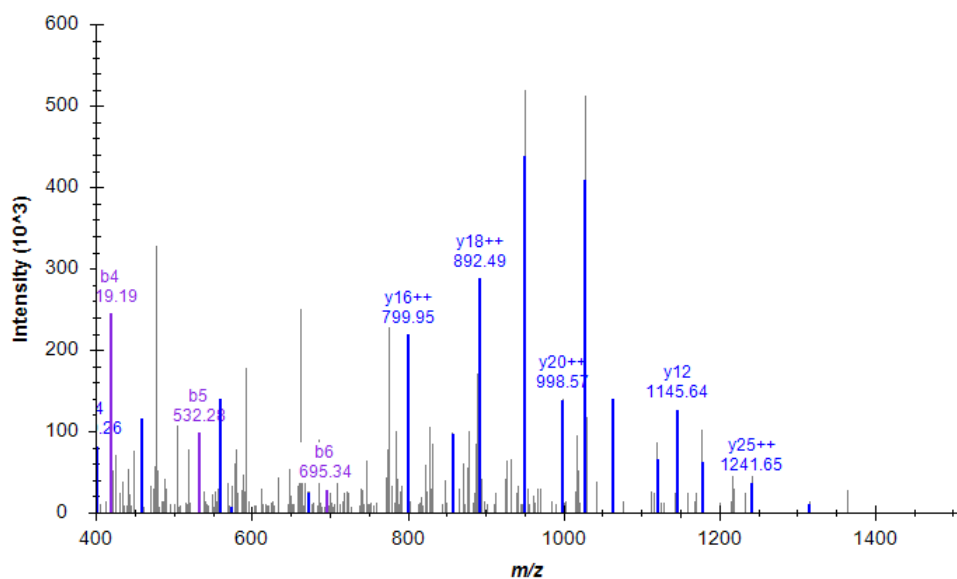
Novel methylation sites were shared in both conditions. Ssa1, Tsa2, Pub1, and Spr40 are all novel methylated proteins identified under both respiration and fermentation (Table 6-7). These methylations have not been identified previously in the literature. Interestingly, the site methylated in Ssa1 (mmR23) under both conditions is nearly identical to the site methylated on Ssa2 under respiration only (mmR23) with the only difference being a serine at position 21 as opposed to an alanine in Ssa1. Ssa1 and Ssa2 are 98% identical, but subtle differences in sequence have effects on their respective functions¹⁸.

Discussion

The majority of peptides identified as methylated were similar across both fermentation and respiration. Respiration specific methylation occurred on proteins related to mitochondria in their function either through localization or functional effects. This was expected as respiratory growth conditions would allow for the expression of more mitochondrial proteins. Novel proteins were methylated in both conditions that have yet to be identified: Ssa1, Tsa2, Pub1, and Spr40. In addition to the known and well-characterized proteins that have been identified as methylated, this investigation confirmed the lysine methylation on Ssa2/4, not only under respiration, but in fermentation as well. There do appear to be differences in methylation patterns

between respiring and fermenting yeast cells. However, these changes do not occur on highly methylated, well-characterized proteins. Future work is needed to thoroughly investigate the reason behind these differences.

FIGURES



Rpl23a
 DGVFLYFEDNAGVIANPK[+42]GEMKGSAITGPVVGK, charge 4

b	b+2		y	y+2	
---	---	1	D	32	---
173.0557	---	2	G	31	3208.6712
272.1241	---	3	V	30	3151.6498
419.1925	---	4	F	29	3052.5814
532.2766	---	5	L	28	2905.5130
695.3399	---	6	Y	27	2792.4289
842.4083	---	7	F	26	2629.3656
971.4509	---	8	E	25	2482.2971
1086.4779	---	9	D	24	2353.2546
1200.5208	---	10	N	23	2238.2276
1271.5579	---	11	A	22	2124.1847
1328.5794	---	12	G	21	2053.1476
1427.6478	---	13	V	20	1996.1261
1540.7318	---	14	I	19	1897.0577
1611.7690	---	15	A	18	1783.9736
1725.8119	---	16	N	17	1712.9365
1822.8646	---	17	P	16	1598.8936
1993.0067	997.0070	18	K(+42.0471)	15	1501.8408
2050.0282	1025.5177	19	G	14	1331.6988
2179.0708	1090.0390	20	E	13	1274.6773
2310.1112	1155.5593	21	M	12	1145.6347
2438.2062	1219.6067	22	K	11	1014.5942
2495.2277	1248.1175	23	G	10	886.4993
2582.2597	1291.6335	24	S	9	829.4778
2653.2968	1327.1520	25	A	8	742.4458
2766.3809	1383.6941	26	I	7	671.4087
2867.4286	1434.2179	27	T	6	558.3246
2924.4500	1462.7286	28	G	5	457.2769
3021.5028	1511.2550	29	P	4	400.2554
3120.5712	1560.7892	30	V	3	303.2027
3177.5927	1589.3000	31	G	2	204.1343
---	---	32	K	1	147.1128

Figure 6-1. Rpl23a trimethyllysine 106 fragmentation. MS2 spectrum of the peptide on Rpl23a identified as trimethylated. y-ions are labeled in blue and b-ions are labeled in purple on the spectrum. In the ion list for the peptide, doubly charged ions are grayed.

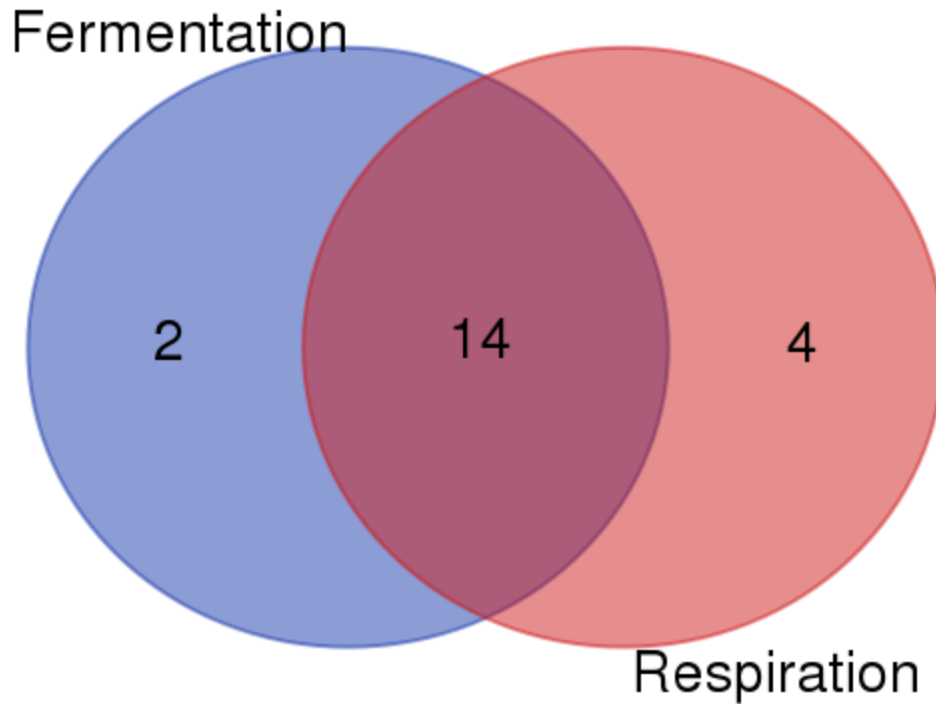


Figure 6-2. Comparison of identified methylated proteins identified under respiration and fermentation conditions. Methylated proteins (Table 6-3 and 6-4) identified in cytosols isolated from respiring yeast cells (red) were compared to methylated proteins (Table 6-1 and 6-2) identified in cytosols from fermenting yeast cells (blue).

TABLES

Table 6-1. Methylated peptides identified in cytosols isolated from fermenting yeast cells that were found in both biological replicates. Methylated residue is in bold; brackets indicate the number of methyl groups. Peptides are organized alphabetically by ORF. Whether a methylation site was known or unknown was determined through data available on UniProt¹⁴ (purple) and in Yagoub *et al.* 2015¹⁵ (light orange). Peptides that were also identified as methylated under respiratory conditions are denoted with an x and type of peptide are color coded as follows: green peptides were found in both replicates of the respiration condition, yellow were found in only one replicate under respiration, and red peptides were in the respiration dataset, but did not have a heavy-light doublet.

Protein Name	ORF	Peptide Modified Sequence	Known Methylation Site	Identified in Respiration data
Ssa1	YAL005C	AVGIDLGTTYSCVAHFANDR[+14]VDIILANDQGNR		x
Rp123a	YBL087C	DGVFLYFEDNAGVIANPK[+28]GEMK[+28]GSAITGPVVK	x	x
Rp123a	YBL087C	DGVFLYFEDNAGVIANPK[+28]GEMK[+14]GSAITGPVVK		x
Hht1	YBR010W	EIAQDFK[+14]TDLR	x	x
Hht1	YBR010W	EIAQDFK[+28]TDLR	x	x
Hht1	YBR010W	EIAQDFK[+42]TDLR	x	x
Tef2	YBR118W	STTTGHLIYK[+14]CGGIDK	x	x
Tef2	YBR118W	STTTGHLIYK[+14]CGGIDKR	x	x
Tef2	YBR118W	ERGITIDIALWK[+42]FETPK	x	x
Tef2	YBR118W	GITIDIALWK[+42]FETPK	x	x
Tef2	YBR118W	NVSVK[+14]EIR	x	x
Tef2	YBR118W	NVSVK[+28]EIR	x	x
Eft2	YDR385W	LVEGLK[+28]R	x	x
Rpl12b	YDR418W	IQNR[+14]QAAAASVVPSASSLVITALK	x	x
Npl3	YDR432W	GGYSR[+28]GGYGGPR	x	x
Npl3	YDR432W	GSYGGSR[+14]GGYDGPR	x	x
Rp42b	YHR141C	ASLFAQGK[+14]R	x	x
Rp42b	YHR141C	KQSGFGGQTK[+14]PVFHK	x	x
Rp42b	YHR141C	QSGFGGQTK[+14]PVFHKK	x	x
Srp40	YKR092C	INFEAWELTDNTYK[+14]GAAGTWGEK		x

Table 6-2. Methylated peptides identified in cytosols isolated from fermenting yeast cells that were found in only one biological replicate. Methylated residue is in bold; brackets indicate the number of methyl groups. Peptides are organized alphabetically by ORF. Whether a methylation site was known or unknown was determined through data available on UniProt¹⁴ (purple), in Yagoub *et al.* 2015¹⁵ (light orange), and in Caslavka Zempel *et al.* 2016¹ (blue). Peptides that were also identified as methylated under respiratory conditions are denoted with an x and type of peptide are color coded as follows: green peptides were found in both replicates of the respiration condition, yellow were found in only one replicate under respiration, and red peptides were in the respiration dataset, but did not have a heavy-light doublet.

Protein Name	ORF	Peptide Modified Sequence	Known Methylation Site	Identified in Respiration data
Rpl23a	YBL087C	DGVFLYFEDNAGVIANPK[+42]GEMKGSAITGPVGK		
Tef2	YBR118W	STTGHLYK[+28]CGGIDK		x
Tef2	YBR118W	GITIDIALWK[+14]FETPK		x
Tef2	YBR118W	GITIDIALWK[+28]FETPK	x	x
Tef2	YBR118W	KLEDHPK[+14]FLK	x	x
Tsa2	YDR453C	K[+14]FEDQGAQVLFASDSEYSLAWTNLPR		x
Ssa4	YER103W	K[+14]SEVFSTYADNQPGVLIQVFEGER	x	x
Nsr1	YGR159C	LDFSSPRPNDGGR[+28]GGSR	x	x
Nsr1	YGR159C	GGR[+28]GGFRPSGSGANTAPLGR	x	
Rpl42b	YHR141C	QSGFGGQTK[+14]PVFHK	x	x
Kdx1	YKL161C	VNDGFIK[+28]GYITSIWYK		
Ssa2	YLL024C	K[+14]SEVFSTYADNQPGVLIQVFEGER	x	x
Pub1	YNL016W	NYGNNNR[+14]GGFR		x
Ded1	YOR204W	R[+28]GGYNGGFFGGNNGSR	x	x

Table 6-3. Methylated peptides identified in cytosols isolated from respiring yeast cells that were found in both biological replicates. Methylated residue is in bold; brackets indicate the number of methyl groups. Peptides are organized alphabetically by ORF. Whether a methylation site was known or unknown was determined through data available on UniProt¹⁴ (purple). Peptides that were also identified as methylated under fermentative conditions are denoted with an x and type of peptide are color coded as follows: green peptides were found in both replicates of the fermentation condition and yellow were found in only one replicate under fermentation.

Protein Name	ORF	Peptide Modified Sequence	Known Methylation site	Identified in Fermentation data
Rp23a	YBL087C	DGVFLYFEDNAGVIANPK[+28]GEMK[+14]GSAITGPVGK		x
Rp23a	YBL087C	DGVFLYFEDNAGVIANPK[+28]GEMK[+28]GSAITGPVGK	x	x
Tef2	YBR118W	STTTGHLIYK[+14]CGGIDK	x	x
Tef2	YBR118W	ERGITIDIALWK[+42]FETPK	x	x
Tef2	YBR118W	KLEDHPK[+14]FLK	x	x
Rpl12b	YDR418W	IQNR[+14]QAAASVVPSASSLVITALK	x	x
Rp42b	YHR141C	ASLFAQGK[+14]R	x	x
Rp42b	YHR141C	KQSGFGGQTK[+14]PVFHK	x	x
Rp42b	YHR141C	QSGFGGQTK[+14]PVFHK	x	x
Srp40	YKR092C	INFEAWELTDNTYK[+14]GAAGTWGEK		x
Ssa2	YLL024C	AVGIDLGTTYSCVAHFSNDR[+14]VDIANDQGNR		
Pub1	YNL016W	NYGNNNR[+14]GGFR		x

Table 6-4. Methylated peptides identified in cytosols isolated from respiring yeast cells that were found in only one biological replicate. Methylated residue is in bold; brackets indicate the number of methyl groups. Peptides are organized alphabetically by ORF. Whether a methylation site was known or unknown was determined through data available on UniProt¹⁴ (purple), in Yagoub *et al.* 2015¹⁵ (light orange), and in Caslavka Zempel *et al.* 2016¹ (blue). Peptides that were also identified as methylated under fermentative conditions are denoted with an x and type of peptide are color coded as follows: green peptides were found in both replicates of the fermentation condition, yellow were found in only one replicate under fermentation, dark purple peptides are identified in the fermentation dataset, but under a different methylation state, and red peptides were in the fermentation dataset, but did not have a heavy-light doublet.

Protein Name	ORF	Peptide Modified Sequence	Known Methylation site	Identified in Fermentation data
Ssa1	YAL005C	AVGIDLGTTYSCVAHFANDR[+14]VDIANDQGNR		x
Hht1	YBR010W	EIAQDFK[+28]TDLR	x	x
Tef2	YBR118W	STTTGHLIYK[+14]CGGIDKR	x	x
Tef2	YBR118W	GITIDIALWK[+28]FETPK		x
Tef2	YBR118W	GITIDIALWK[+42]FETPK	x	x
Tef2	YBR118W	GITIDIALWK[+14]FETPK		x
Tef2	YBR118W	NVSVK[+28]EIR	x	x
Tef2	YBR118W	KLEDHPK[+28]FLK		
Np3	YDR432W	GGYSR[+28]GGYGGPR	x	x
Np3	YDR432W	NDYGPPR[+28]GSYGGSR[+14]GGYDGPR	x	x
Np3	YDR432W	GSYGGSR[+14]GGYDGPR	x	x
Tsa2	YDR453C	K[+14]FEDQGAQVLFASDSEYSLAWTNLPR		x
Ald5	YER073W	VK[+28]AGTVWINTYNNFHQNVFPGGFGQSGIGR		
Ssa4	YER103W	K[+14]SEVFSTYADNQPGVLIQVFEGER	x	x
YER152C	YER152C	EYDNDPYNR[+14]HPLTYGSDEGALWVR[+14]EQICTFLNDQLFK		
Nsr1	YGR159C	LDFFSPRPNDGGR[+28]GGSR	x	x
Rp42b	YHR141C	QSGFGGQTK[+14]PVFHKK	x	x
Cyc1	YJR048W	KNVLWDENNMSEYLTNPK[+42]K	x	
Cyc1	YJR048W	NVLWDENNMSEYLTNPK[+42]K	x	x
Ssa2	YLL024C	K[+14]SEVFSTYADNQPGVLIQVFEGER	x	x
Hef3	YNL014W	LK[+14]EFGFSDEMIEMPIASLSGGWK		x
Ded1	YOR204W	R[+28]GGYGNNGFFGGNNGGSR	x	x

Table 6-5. Proteins identified as methylated under fermentation and not respiration. Proteins identified as categorized as unique based on the absence of methylated peptides in the respiration dataset. Descriptions of protein function are from Saccharomyces Genome Database¹⁹.

Protein name	Methylated residue	Description
Kdx1	tmK190	Serine/threonine protein kinase
Rpl23a	tmK106	Large subunit ribosomal protein

Table 6-6. Proteins identified as methylated under respiration and not fermentation. Proteins identified as categorized as unique based on the absence of methylated peptides in the fermentation dataset. Descriptions of protein function are from Saccharomyces Genome Database¹⁹.

Protein name	Methylated residue	Description
Ald5	dmK467	Mitochondrial aldehyde dehydrogenase
Cyc1	tmK78	Cytochrome <i>c</i> , electron carrier
Ssa2	mmR23	Heat shock 70 protein; involved in protein folding and vacuolar import
Tef2	dmK390	Eukaryotic elongation factor 1a (eEF1A)
Yer152c	mmR45, mmR60	Unknown protein with transaminase activity

Table 6-7. Novel methylated proteins identified in cytosols isolated from both respiring and fermenting yeast cells. Descriptions of protein function are from Saccharomyces Genome Database¹⁹.

Protein name	Methylated residue	Description
Pub1	mmR264	Poly (A)+ RNA-binding protein
Srp40	mmK356	Nucleolar serine-rich protein; involved in preribosome assembly or transport
Ssa1	mmR23	Heat shock 70 protein; involved in protein folding and nuclear transport
Tsa2	mmK261	Stress inducible cytoplasmic thioredoxin peroxidase

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CHAPTER SEVEN

Future Goals and Perspectives

This dissertation is the first glimpse into global mitochondria methylation in the model organism *Saccharomyces cerevisiae* and opens the doors to a wide variety of further approaches to understanding the role of this type of modification. While I have fine-tuned methods for determining protein methylation in the yeast mitochondria, there is still much to learn about the methylated proteins and the corresponding methylation reactions.

Improvements on heavy methyl SILAC and use for mitochondrial protein methylation

The method described in Chapter Four for determining protein methylation has made it easier to not only identify mitochondrial methylated proteins, but to unambiguously assign mass shifts due to methylation on peptides. However, the results from Chapter Four demonstrate that modification site localization on methylated peptides remains to be a challenge, especially in peptides rich with both basic and acidic residues. While it is possible to confirm each site on each identified methyl protein by site-directed mutagenesis, this becomes a laborious undertaking given the large amount of data proteomics produces. Such a targeted approach works better if one protein identified as methylated were selected for further study, as is more commonly done for biochemical characterization of a substrate and its methyltransferase^{1,2}. For mass spectrometry analyses, further work to improve the enrichment of methylated proteins and peptides, the proteolytic digestion of protein samples, and the methods for analyzing data may be warranted³.

There are multiple strategies to help mitigate the issues of identifying specific methylation sites in proteins, each with their own limitations and benefits. In addition to the PTMRS tool used in Chapter Four, there are other methods for determining probability or confidence of site localization for covalent modifications³. Using two analytical tools for site

probability may add strength to the confidence of the proposed modified site as only methylation sites with high probabilities with both algorithms would be reported. A limitation here then could be that some genuine sites that might be excluded for not passing this type of “test”, particularly if one tool was better suited for methylation site localization over other types of modifications. Many of these analytical tools were not made for methylation site localization, but rather, like PTMRS, created for localization of more well-studied modifications like phosphorylation⁴.

Other ionization and fragmentation strategies may also aid in solving this problem³, but these will be dependent on what instruments are available for analysis. Digestion with different proteases may help in better characterizing methylation site localization as different peptides would be generated. The choice of protease can be very important in these types of investigations^{5,6}. The residues that can be methylated (K, R) are also targets for peptidyl cleavage by proteases⁶. This can affect the size and properties of methyl peptides generated.

Trypsin is the most widely used choice for digestion for many applications⁶. There is one caveat, however, in that trypsin cannot cleave after dimethylated or trimethylated lysine residues⁷. Additional miscleavages are observed with mono- and dimethylarginine residues⁸. Trypsin cannot cleave after KP or RP sequences; trypsin activity can also be affected by nearby acidic residues and basic residues that can result in missed cleavages at the desired K or R residues^{6,9}. These missed cleavages can be favorable, resulting in methylated peptides that are long enough to be suitable for mass spectrometry analysis. However, many of the identified methylated peptides detected in Chapter Four contain such sequences, which may result in their enrichment due to missed cleavages and perhaps an overestimation of their levels.

Methylated peptides containing both basic residues and acidic residues can confound methyl site localization due to the possibility of methylation at these residues. LysargiNase has

been shown to be an alternative to trypsin in methyl proteomics¹⁰. LysargiNase cleaves N-terminal to lysine and arginine residues, regardless of methylation state, allowing for better identification of methyllysine and methylarginine containing peptides¹⁰. However, both trypsin and lysargiNase may not be suitable for regions rich in arginine and lysine residues (often occurring in methylated proteins like histones¹¹) as the resulting peptides may be too short for mass spectrometry analysis. Ideally, large scale proteomic identification of methylation sites would be repeated multiple times using various proteases with different residue specificities in order to ensure no information is lost.

Enrichment for methylated peptides may also aid in the identification of methylated lysine and arginine residues specifically. Immunoprecipitation (IP) of methylated peptides using antibodies that are specific for either methylarginine or methyllysine have been used in methyl proteomics with some success¹². While methylarginine antibodies have been well-characterized and validated as specific for immunoprecipitation of pan methylarginine (recognizing all species) or more specific types of mono- and dimethylarginine species¹², antibodies against methyllysine residues are less commonly used¹³. Recently, more specific methyllysine and methylarginine antibodies have been developed to overcome this problem¹⁴. As these antibodies become more widely used, better identification of the myriad of lysine methylation sites will be possible. There are other methods for enrichment of methyllysine, such as utilizing the 3xMBT domain repeats of L3MBTL1^{15,16}. However, even though the laboratory that discovered it has touted its efficacy¹², our laboratory has not been able to replicate 3xMBT's specificity for methylated lysine residues (unpublished data). In any case, an attempt at enrichment may yield more or better validated methylated peptides.

It is also possible that true methylated sites may be missed by the method used here of manual evaluation of doublets in Skyline¹⁷. An automated pipeline for data analysis may increase methylated peptides that are validated with heavy-light doublets observed in Skyline. Certainly, automation would remove any bias present in manual evaluation.

Function characterization of mitochondrial protein methylation

The physiological importance of the identified methylation sites has yet to be explored. While I have speculated on accessibility and importance of the identified methylated mitochondrial proteins in both Chapter Four and Chapter Five, there is much to learn experimentally about these methylation sites. The accessibility of MRP methylation sites on the mitoribosome surface perhaps points away from importance in translation¹⁸, in facilitating contacts with the rRNA¹⁹, or in assembly²⁰. However, the proximity of the methylation site on Mrp4 may be needed for stability of the mitoribosome through protein-protein interactions. The methylation site on Mrpl40 may be needed for interaction with Oxa1²¹ or with the mitochondrial inner membrane. Whether either of these would affect mitochondrial translation can be easily determined experimentally. Site directed mutagenesis of the methylated site followed by *in organello* translation²² can be used to monitor mitochondrial translation by either radiolabeling or immunoblotting for the translation products to determine the effects of the methylation events on translation or specific translation products.

Many of the methylated proteins identified warrant individual characterization. Aco1, the yeast aconitase, is localized in both the cytosol and mitochondria and is already known to be heavily modified^{23,24}, in addition to the methylation site identified in Chapter Four. The additional identification of a modification of Aco1 by methylation may be important for its

localization or stability (as with cytochrome *c* methylation)^{25,26}, its regulation, or its protein-protein interactions²⁷, potentially within the metabolon of associated enzymes in the TCA cycle²⁸.

The conservation of mitochondrial methylation sites from yeast to humans is also intriguing. With both hPDHB and scPdb1 identified as methylated, this points to a conserved function of the methylation site and a conserved arginine methyltransferase (potentially a novel arginine methyltransferase in the mitochondria). What the function is and what methyltransferase is responsible in both yeast and humans would need to be determined.

The importance of the chaperone code²⁹ has expanded now to include the yeast Hsp70 proteins in both the cytosol and the mitochondria. How the methylation of Ecm10 and Ssa2/4 (identified in Chapter Four) contribute to this particular example of common methylated proteins would be an interesting question to investigate. Additionally, as Ssa2/4 are not methylated on the conserved residue from humans in the chaperone code^{30,31}, the divergence from yeast to humans warrants further work. In Chapter Six, three methylation sites on Hsp70 proteins were identified. It became evident that the previously identified Ssa2/4 methylation occurs under respiration and fermentation. Novel Ssa1 and Ssa2 arginine methylation add further to the yeast chaperone code.

Chapter Six demonstrated that mitochondrial proteins are more easily identified as methylated under respiration. Interestingly, there were some dissimilar proteins identified as methylated between fermentation and respiration. While the identified cytochrome *c* methylation has been well-characterized^{27,32,33}, the functional importance of the methylation on the remaining proteins identified solely under respiration represent another avenue of exploration.

Identifying and characterizing methyltransferases responsible for modifying mitochondrial substrates

I would like to end with the logical next step of the work presented in this dissertation. With the optimization of heavy methyl SILAC with the *met6*Δ strain and the identification of heavy methyl SILAC validated mitochondrial methyl peptides, the mitochondrial methyltransferases can now be characterized. Double mutants for *MET6* and candidate methyltransferase genes can be heavy labeled and then analyzed by mass spectrometry. Quantification using another labeled amino acid may be needed to fully determine the presence and absence of methylated and unmethylated peptides. In the original heavy methyl SILAC experiments, Ong *et al.* used deuterated lysine as an additional label in the heavy cultures³⁴. However, the method developed in this dissertation should allow for mitochondrial proteins to be observed as methylated or unmethylated in methyltransferase gene deletion strains, a result that I could not achieve with the other biochemical methods attempted (in Chapters 2, 3, and 5). Analyzing the deletions of the genes for the four putative methyltransferases (Rsm22, Mtf1, Oms1, and Ykl162c) would be the obvious starting point.

Once a possible methyltransferase-substrate pair is identified, the methylation reaction can be confirmed using *in vitro* methylation with purified proteins. Chemical crosslinking within the mitochondria coupled with affinity purification can confirm interactions of the substrate and methyltransferase³⁵. Such identifications have been a long-term goal of this project since I joined the laboratory. As standard biochemical methods for identifying possible substrates were not generally successful for me when working with mitochondria, the change of focus to simply identifying the methylated mitochondrial proteins slowed progress on characterizing the mitochondrial methyltransferases. Standard enzymology and biochemistry techniques can be

used to understand the function of the methyltransferase by investigating binding, kinetics, and regulation.

Recently, increasing numbers of mitochondrial protein methyltransferases have been identified in human cells³⁶⁻³⁸. This represents a renewed interest in mitochondrial protein modification. As more substrates are identified in both yeast and human mitochondria and methyltransferases are confirmed, our understanding of the role of protein methylation in the mitochondria will only increase.

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APPENDICES

Appendix 1. Yeast Mitochondrial Ribosomal Proteins (MRPs) and their N-terminal

Mitochondrial Presequences. The information in this appendix comes from a variety of sources in the scientific literature. Masses based on open reading frames are from Saccharomyces Genome Database in 2012-2014. Length of signal peptide and experimentally determined mass (empirical mass) is specific to each mitochondrial ribosomal protein (MRP): Graack *et al.* 1988¹, Matsushita *et al.* 1989², Kitakawa *et al.* 1990³, Graack *et al.* 1991⁴, Graack *et al.* 1992⁵, Grohmann *et al.* 1994⁶, Graack *et al.* 1995⁷, Pan and Mason 1995⁸, Kitakawa *et al.* 1997⁹, Graack and Wittmann-Liebold 1998¹⁰, Sato and Miyakawa 2004¹¹, Vogtle *et al.* 2009¹² (masses reported from this reference are the experimentally determined signal peptide mass subtracted from the theoretical mass). Parentheses indicate that an N-terminal import peptide has not been determined¹⁰. Also included is the mass reported by Woellhaf *et al.* 2014¹³. I have listed each MRP by name and by ORF, in increasing order of theoretical mass. Bacterial homologs are from Woellhaf *et al.* 2014¹³. MRPs with bacterial homologs are italicized and those that have methylated bacterial homologs (Polevoda and Sherman 2007¹⁴) are both italicized and bold. Type of methylation in bacteria is indicated by the following symbols: + = methylalanine, ' = methylglutamine, " = methyllysine, and # =methylmethionine. n.d. signifies “not determined” and dashes indicate absence in either report.

MRP	ORF	<i>E. coli</i> homolog	Mass Based on ORF (Da)	Length of signal peptide (number of residues)	Empirical Mass (Da)	Mass Reported by Woellhaf <i>et al.</i> 2014 ¹³ (Da)
MRPL39/ YML39	YML009C	-	7973	1 ¹⁰	7841 ¹⁰	-
<i>MRPL33</i>	<i>YMR286W</i>	<i>L30</i>	<i>9531</i>	<i>1</i> ¹⁰	<i>11012</i> ¹⁰	<i>9531</i>
<i>RSM19</i>	<i>YNR037C</i>	<i>S19</i>	<i>10275</i>	<i>24</i> ¹²	<i>7576</i>	<i>10275</i>
MRP10	YDL045W-A	-	10690	1 ¹⁰	10559 ⁹	10690

MRP	ORF	<i>E. coli</i> homolog	Mass (Da)	Length of signal peptide (number of residues)	Empirical Mass (Da)	Mass Reported by Woellhaf <i>et al.</i> 2014 ¹³ (Da)
<i>RTC6</i>	<i>YPL183W-A</i>	<i>L36</i>	10698	<i>n.d.</i>	(10698)	10698
YMR44/ MRPL44	YMR225C	-	11476	No signal peptide ¹⁰	11476 ²	11476
MRPL37	YBR268W	-	11949	24 ¹⁰	9185 ¹⁰	11949
<i>YDR115W</i>	<i>YDR115W</i>	<i>L34</i>	12030	<i>n.d.</i>	(12030)	12030
<i>RSM27</i>	<i>YGR215W</i>	<i>S33</i>	12393	No signal peptide ¹²	12393	12393
<i>YNL122C</i>	<i>YNL122C</i>	<i>L35</i>	13244	<i>n.d.</i>	(13244)	13244
<i>MRP2</i>	<i>YPR166C</i>	<i>S14</i>	13538	<i>n.d.</i> ; possibly 15 ¹⁰	(13538)	13538
<i>MRPS16</i>	<i>YPL013C</i>	<i>S16</i>	13639	<i>n.d.</i>	(13639)	13639
YMR31/ KGD4	YFR049W	S36	13689	8 ^{10,12}	12775 12792 ²	13689
MRPL38/ YML34	YKL170W	L14	14904	57 ¹⁰	8812 ⁹	14904
MRPL38/ YML38	YKL170W	L14	14904	No signal peptide ¹⁰	14904	14904
<i>MRP17</i>	<i>YKL003C</i>	<i>S6</i>	15021	71 ¹² ; No signal peptide ¹⁰	6738 17343 ⁹	15021
MRPL31	YKL138C	-	15520	12 ¹⁰	14246 ¹	15520
<i>RSM18</i>	<i>YER050C</i>	<i>S18</i>	15835	<i>n.d.</i>	(15835)	15835
MRP49	YKL167C	-	16021	1 ¹² ; possibly 19 ¹⁰	15872	16021
<i>SWS2</i>	<i>YNL081C</i>	<i>S13</i>	16089	<i>n.d.</i>	(16089)	16089
MRPL51	YPR100W	-	16124	<i>n.d.</i>	(16124)	16124
<i>MRPL50</i>	<i>YNR022C</i>	<i>L9</i>	16283	38 ¹² ; 9 ¹²	11842 15237	16283
IMG2	YCR071C	-	16380	80 ¹²	7540	16380
MRPL27	YBR282W	-	16492	54 ¹² ; 16 ¹⁰	10315 14798 ⁴	16492
<i>MRPL19</i>	<i>YNL185C</i>	<i>L11''</i>	16670	58¹²	10813 16669⁹	16670
<i>MRPS12</i>	<i>YNR036C</i>	<i>S12</i>	16917	<i>n.d.</i>	(16917)	16917
MRPL28	YDR462W	-	17342	44 ¹² ; 26 ¹⁰	12404 14387 ⁹	17342
<i>MRPS8</i>	<i>YMR158W</i>	<i>S8</i>	17470	<i>n.d.</i>	(17470)	17470
<i>MRPL49</i>	<i>YJL096W</i>	<i>L21</i>	18387	<i>n.d.</i>	(18387) (25402) ¹⁰	18387
<i>MRPL23</i>	<i>YOR150W</i>	<i>L13</i>	18463	<i>n.d.</i>	(18463)	18463

MRP	ORF	<i>E. coli</i> homolog	Mass (Da)	Length of signal peptide (number of residues)	Empirical Mass (Da)	Mass Reported by Woellhaf <i>et al.</i> 2014 ¹³ (Da)
MRPL25/ YMR26	YGR076C	-	18587	1 ¹⁰	18368 ¹⁰	18587
IMG1	YCR046C	L19	19394	15 ¹²	17546	19394
MRPL36	YBR122C	L31	20091	33 ¹⁰ ; 10 ¹²	18552 ¹⁰	20091
MRP21	YBL090W	S21	20394	24 ¹²	17558	20394
MRPL12/ MNP1	YGL068W	L7/L12''	20650	34 ¹¹	16891 ¹¹	20650
MRPL32	YCR003W	L32	21437	71 ^{10,12}	13362 13374 ¹⁰	21437
MRPL20	YKR085C	-	22392	18 ¹⁰	20626 ¹⁰	22392
RSM10	YDR041W	S10	23424	n.d.	(23424)	23424
MRPL6	YHR147C	L6	23889	16 ^{10,12}	21634 ⁹ 21881	23889
MRPS18	YNL306W	S11 ⁺	24563	59 ¹⁰	17734 ⁹	24563
MRPL16/ YML47	YBL038W	L16 [#]	26518	37 ¹² ; 13 ¹² ; possibly 41 ¹⁰	22433 24905 26517 ⁸	26518
MHR1	YDR296W	-	26895	n.d.	(26895)	-
MRPL8	YJL063C	L17	26945	1 ^{10,12}	26796 26822 ³	26945
MRPS17	YMR188C	S17	27635	n.d.	(27635)	27635
RSM7	YJR113C	S7	27816	n.d.	(27816)	27816
MRPL15	YLR312W-A	-	28167	28 ^{10,12}	24913 24929 ⁹	28167
MRPL11	YDL202W	L10	28515	31 ^{10,12}	24790 24804 ⁹	28515
MRPL9	YGR220C	L3'	29790	19 ¹⁰	27553 ⁵	29790
MRPL24/ MRPL14	YMR193W	L28	30049	35 ¹² ; 21 ¹⁰	25711 27532 ⁹	30049
RSM26	YJR101W	-	30223	73 ¹² ; 7 ¹²	22028 29374	30223
MRPL13	YKR006C	-	30271	86 ⁶	21619 ¹⁰	30271
RSM25	YIL093C	S23	30513	No signal peptide ¹²	30513	30513
MRP20	YDR405W	L23	30566	45 ¹⁰ ; 35 ¹⁰ ; 34 ¹² ; 33 ¹²	25711 ⁹ 26798 26911	30566

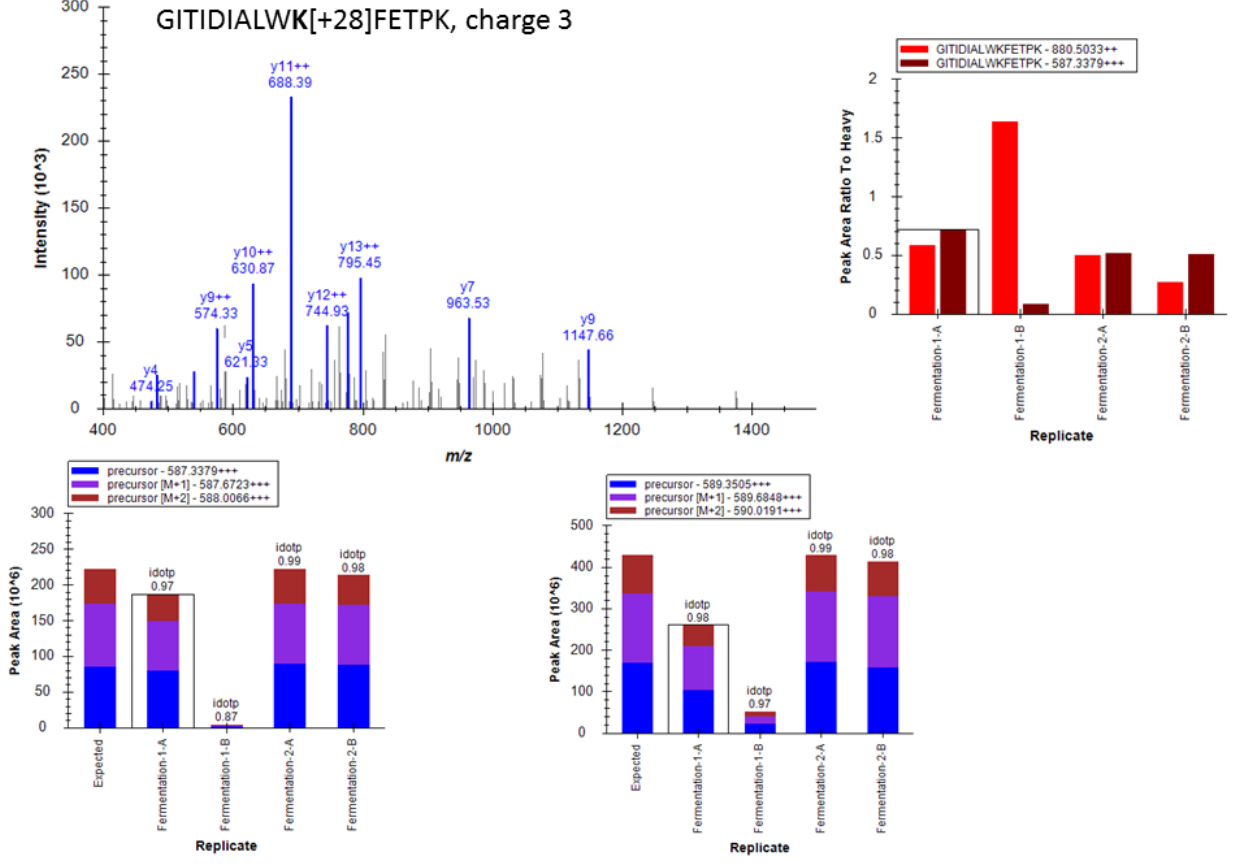
MRP	ORF	<i>E. coli</i> homolog	Mass (Da)	Length of signal peptide (number of residues)	Empirical Mass (Da)	Mass Reported by Woellhaf <i>et al.</i> 2014 ¹³ (Da)
MRPL1	YDR116C	L1	30996	28 ¹²	27847	30966
MRPS9	YBR146W	S9	31925	45 ¹² ; 24 ¹²	26551 29113	31925
YML6	YNL025C	L4	31969	26 ¹⁰ ; 16 ¹²	29040 ⁹ 30006	31969
MRPL17/ YML17	YNL252C	-	32212	19 ¹⁰	30253 ⁹	32212
MRPL17/ YML30	YNL252C	-	32212	19 ¹⁰	30926 ¹⁰	32212
MRPS28	YDR337W	S15	33057	33 ^{10,12}	29451 29467 ¹⁰	33057
MRPL7	YDR237W	L5	33099	19 ¹⁰	31754 ⁹	33099
MRPL40	YPL173W	L24	33751	1 ¹² ; No signal peptide ⁹	33602 33749 ⁹	33751
MRPS5	YBR251W	S5	34883	13 ¹²	33255	34883
MRPL22	YNL177C	L22	34897	34 ¹²	31066	34897
PET123	YOR158W	-	35998	1 ¹²	35849	35998
MRPL10	YNL284C	L15	36347	57 ^{10,12}	29844 29856 ⁹	36347
MRP1	YDR347W	-	36728	12 ¹²	35309	36728
MRPL4	YLR439W	L29	36965	14 ⁷	35295 ¹⁰	36965
RSM24	YDR175C	S35	37393	30 ¹² ; 28 ¹²	33892 34119	37393
MRP13	YGR084C	-	38988	67 ¹² ; 10 ¹⁰	31215 34676 ⁹	38988
MRP51	YPL118W	-	39445	1 ¹² ; No signal peptide ¹²	39296 39445	39445
MRPS35	YGR165W	-	39575	n.d.	(39575)	39575
RSM28	YDR494W	-	41216	14 ¹²	39486	41216
MRPL35	YDR322W	-	42825	n.d.	42825	42825
MRP7	YNL005C	L27	43226	31 ¹² ; 27 ^{10,12}	39620 40076 40092 ¹⁰	43226
RML2	YEL050C	L2	43786	n.d.	(43786)	43786
MRPL3	YMR024W	-	43999	59 ¹	37179 ¹⁰	43999
MRP4	YHL004W	S2	44151	n.d.	(44151)	44151
PPE1/ MRPS2	YHR075C	-	44887	35 ¹⁰	40770 ⁹	44887

MRP	ORF	<i>E. coli</i> homolog	Mass (Da)	Length of signal peptide (number of residues)	Empirical Mass (Da)	Mass Reported by Woellhaf <i>et al.</i> 2014¹³ (Da)
				<i>No signal peptide (encoded by mitochondrial DNA)</i>		
<i>VARI</i>	<i>Q0140</i>	<i>S3/S24</i>	<i>47123</i>		<i>46786</i> ¹⁰	<i>47123</i>
<i>RSM23</i>	<i>YGL129C</i>	<i>S29</i>	<i>50867</i>	<i>n.d.</i>	<i>(50867)</i>	<i>50867</i>
<i>NAM9</i>	<i>YNL137C</i>	<i>S4</i>	<i>56356</i>	<i>53</i> ¹² ; <i>possibly 34</i> ¹⁰	<i>50098</i>	<i>56356</i>
<i>MRPL45</i>	<i>YGL125W</i>	-	<i>68560</i>	<i>38</i> ¹⁰	<i>63936</i> ⁹	-
<i>RSM22</i>	<i>YKL155C</i>	-	<i>72190</i>	<i>24</i> ¹²	<i>69344</i> ¹²	<i>72190</i>

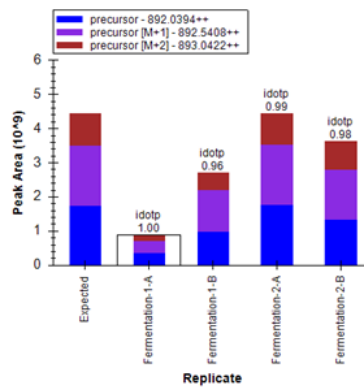
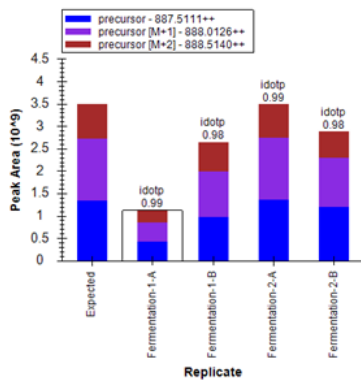
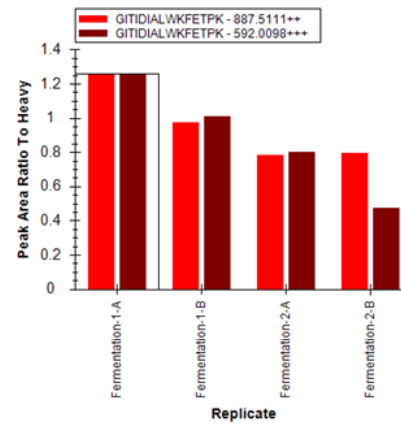
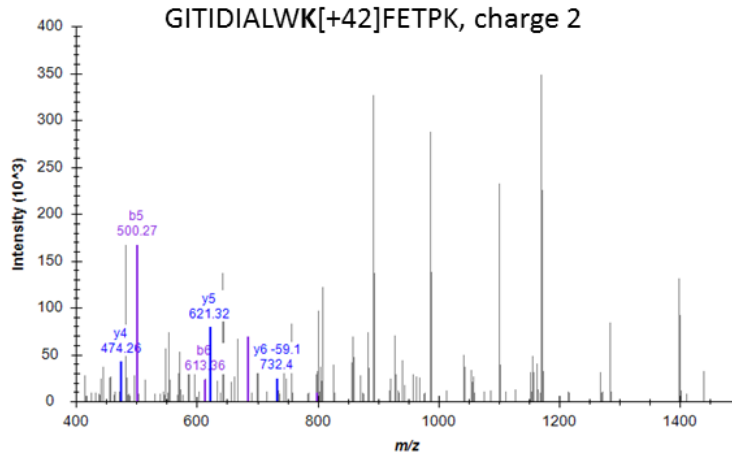
Appendix 2. Representative fragmentation patterns, isotope distribution, and peak areas for heavy and light peptides identified as methylated under fermentation and respiration.

Methods for how the data were obtained are explained in detail in Chapter Six. The Skyline software package¹⁵ was used for evaluation of the presence of heavy and light doublets and visualization of MS2 spectra. Isotope distributions are normalized to heavy peptide when applicable. Methyl peptides identified in fermentation are presented first followed by methyl peptides identified in respiration.

Fermentation: eEF1A Fragmentation and Heavy-Light Doublet Confirmation

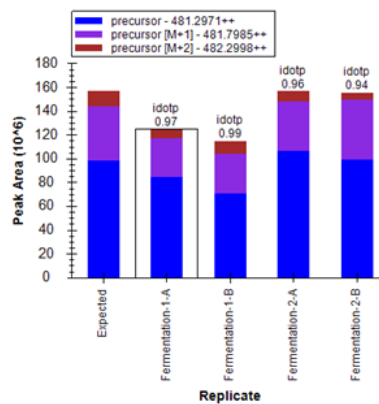
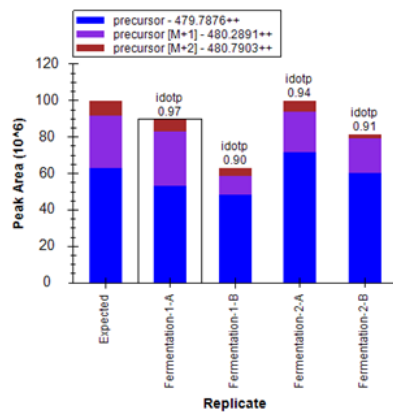
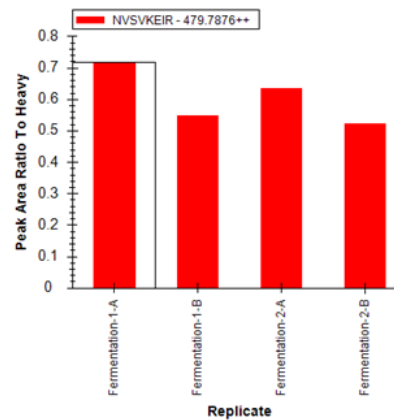
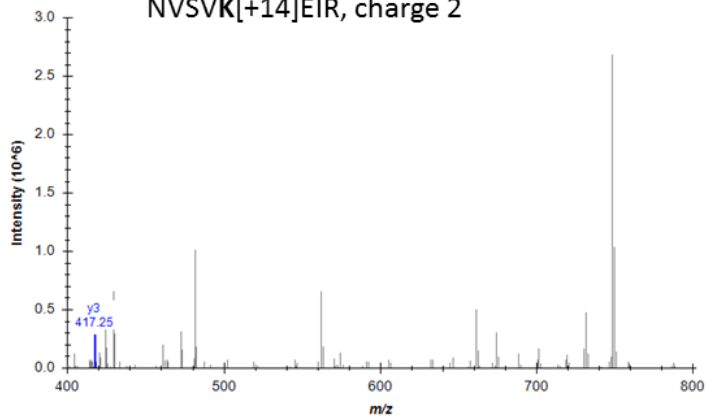


Fermentation: eEF1A Fragmentation and Heavy-Light Doublet Confirmation

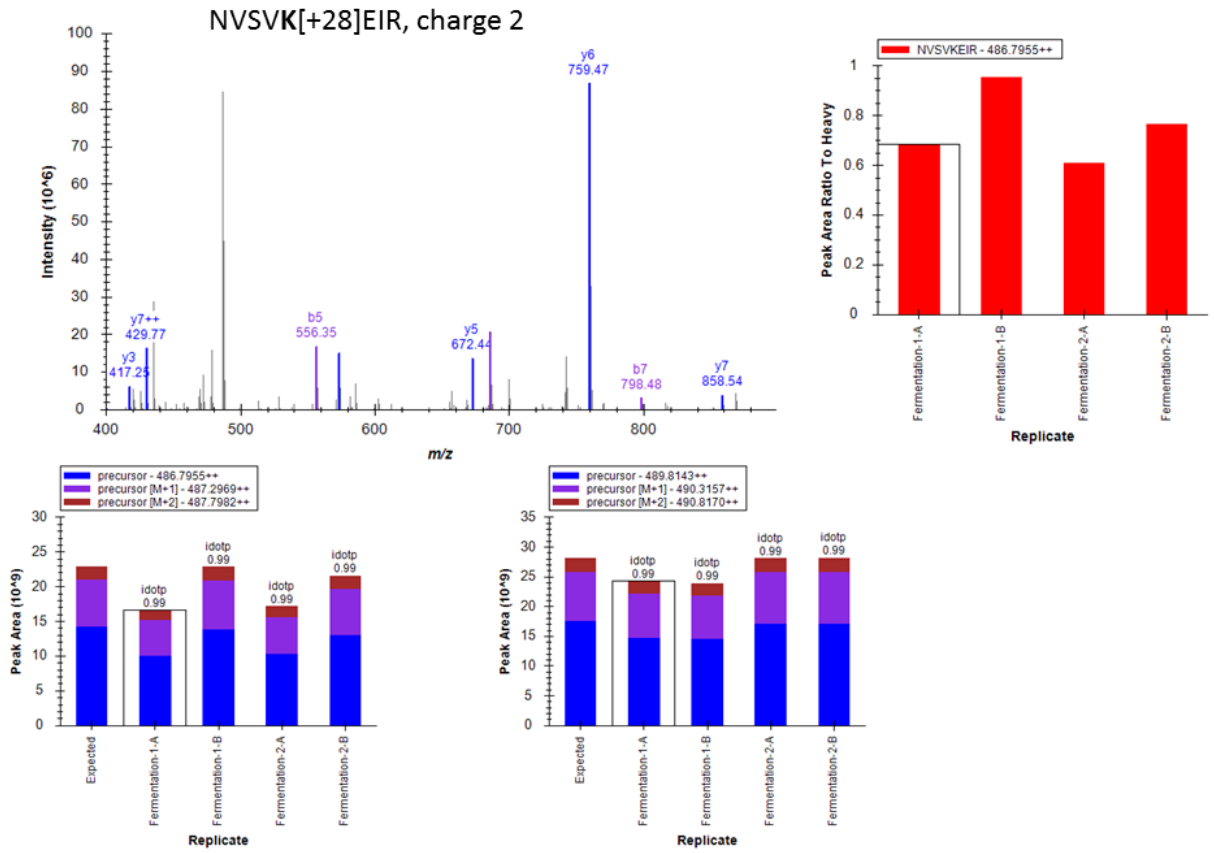


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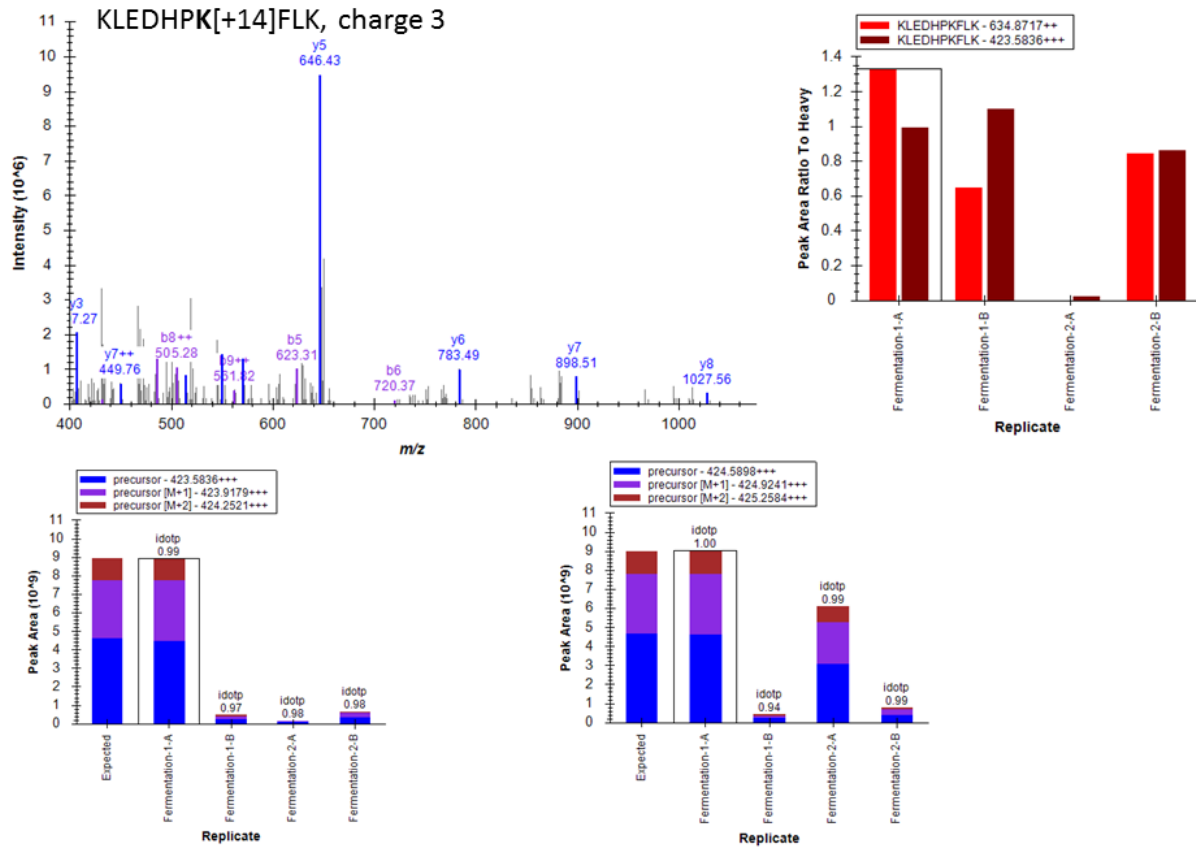
NVSVK[+14]EIR, charge 2



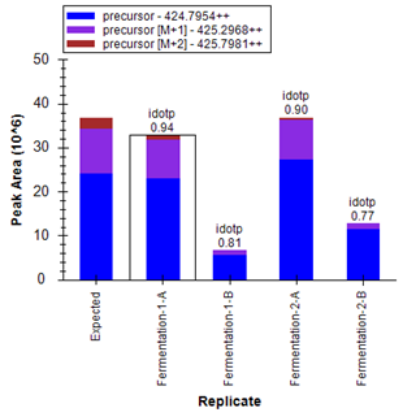
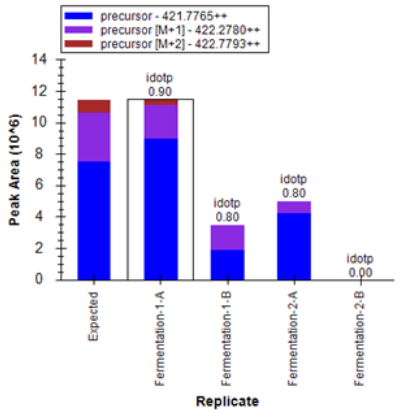
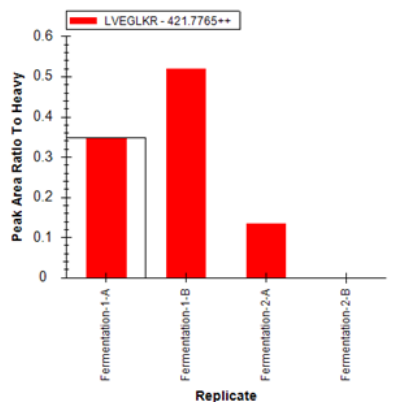
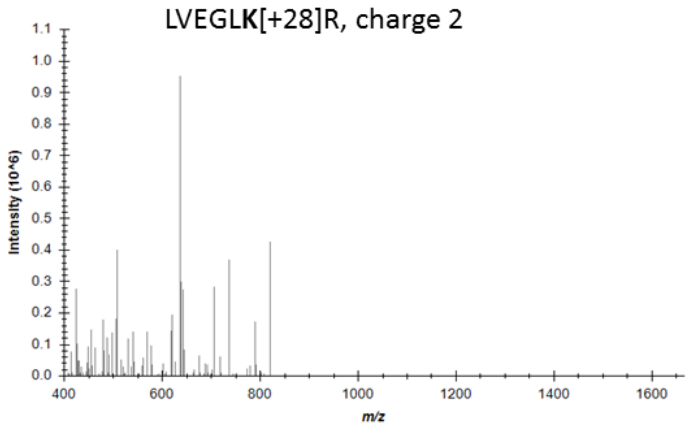
Fermentation: eEF1A Fragmentation and Heavy-Light Doublet Confirmation



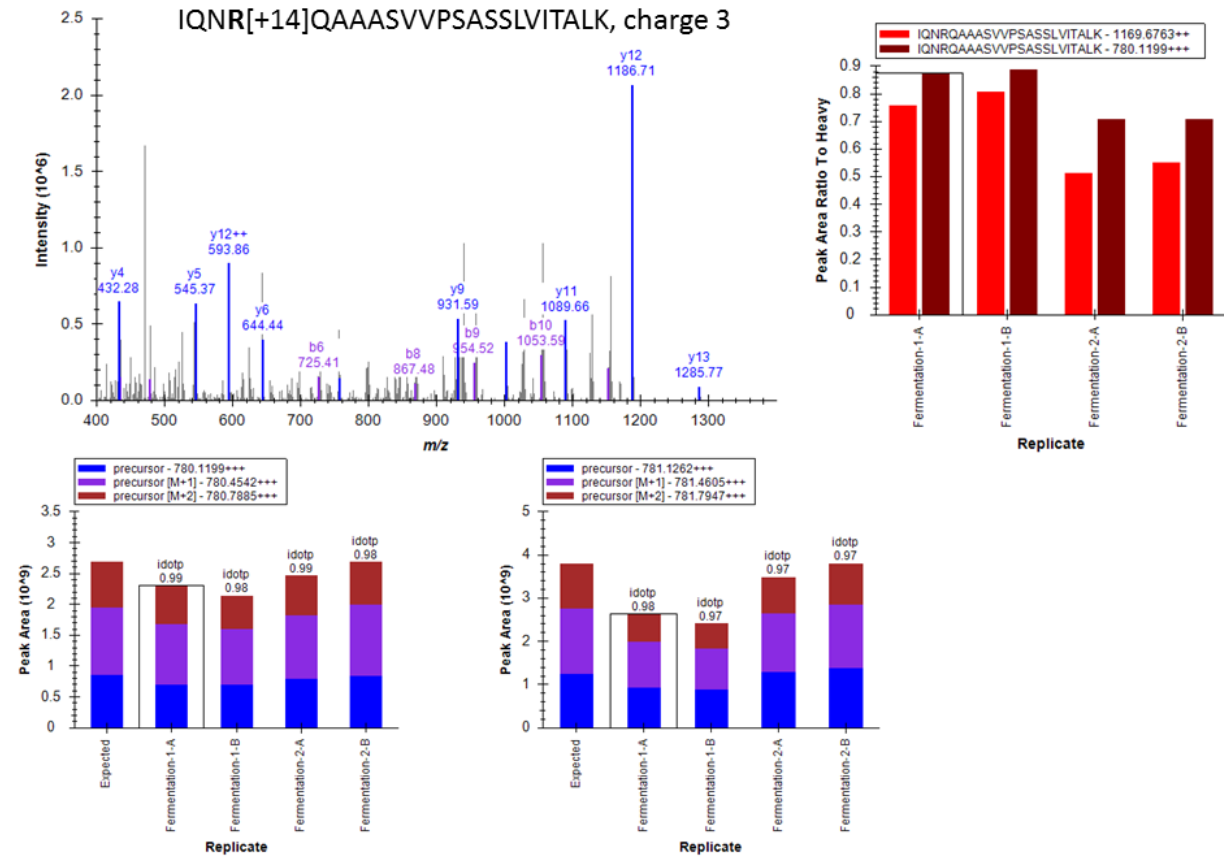
Fermentation: eEF1A Fragmentation and Heavy-Light Doublet Confirmation



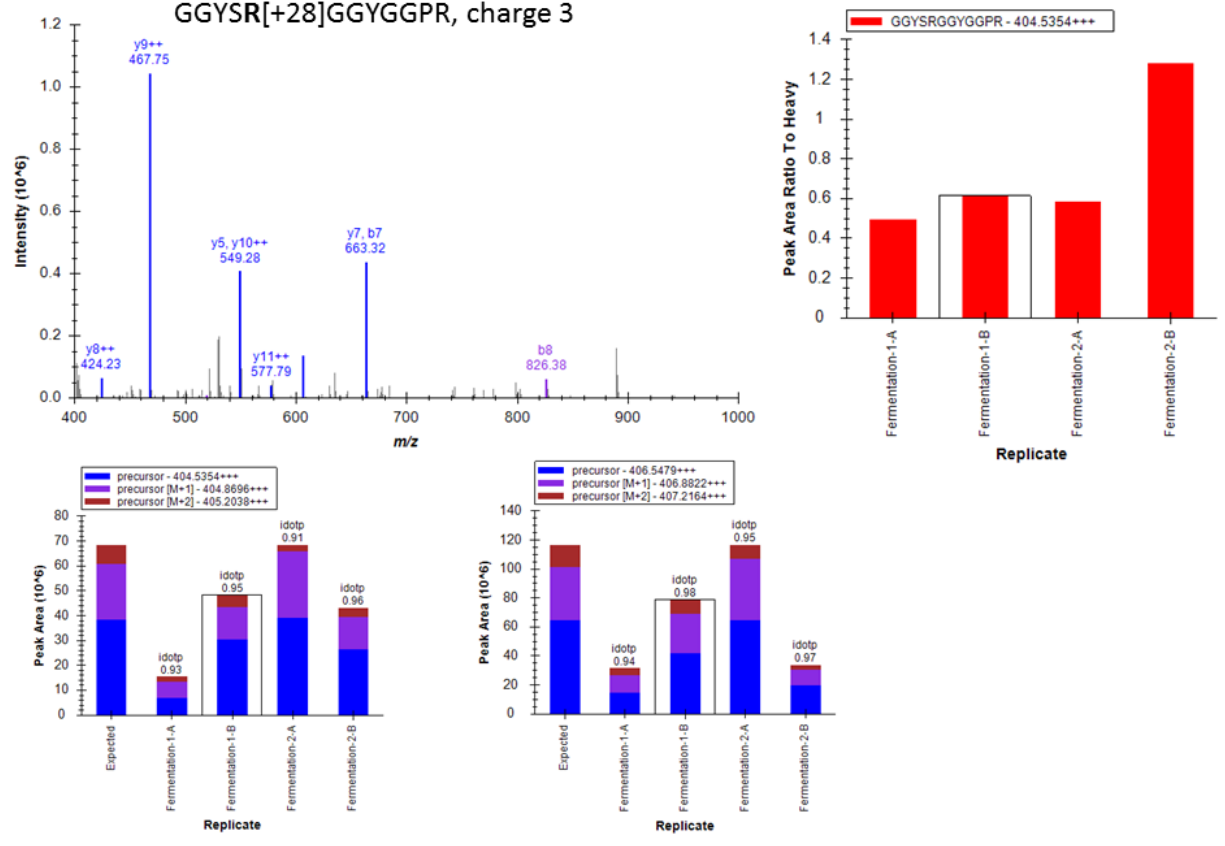
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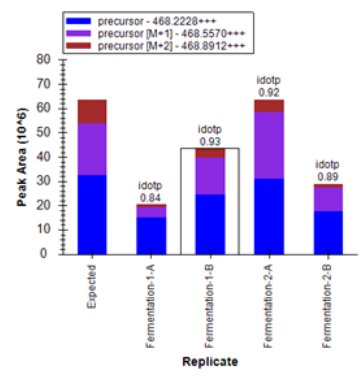
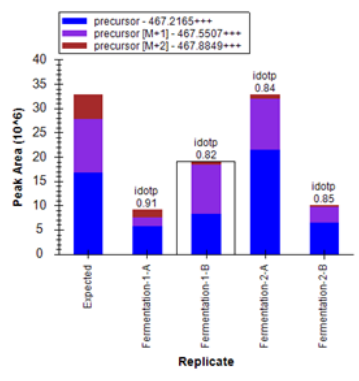
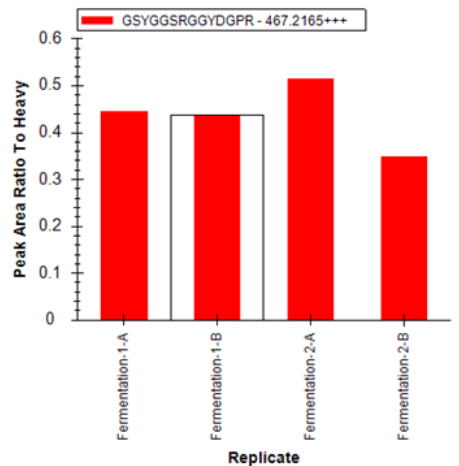
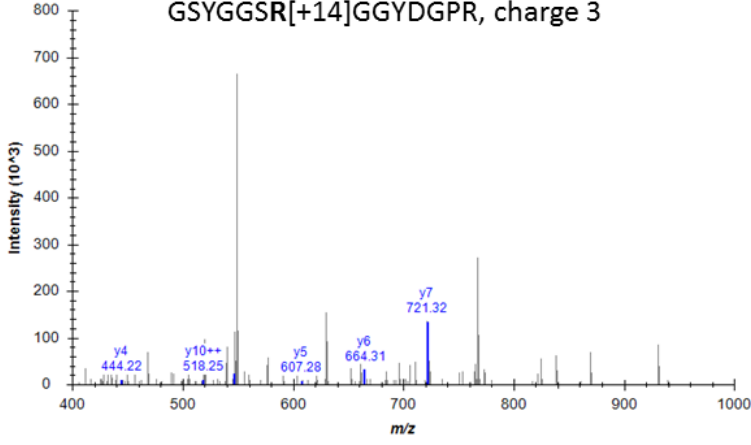
Fermentation: Rpl12b Fragmentation and Heavy-Light Doublet Confirmation



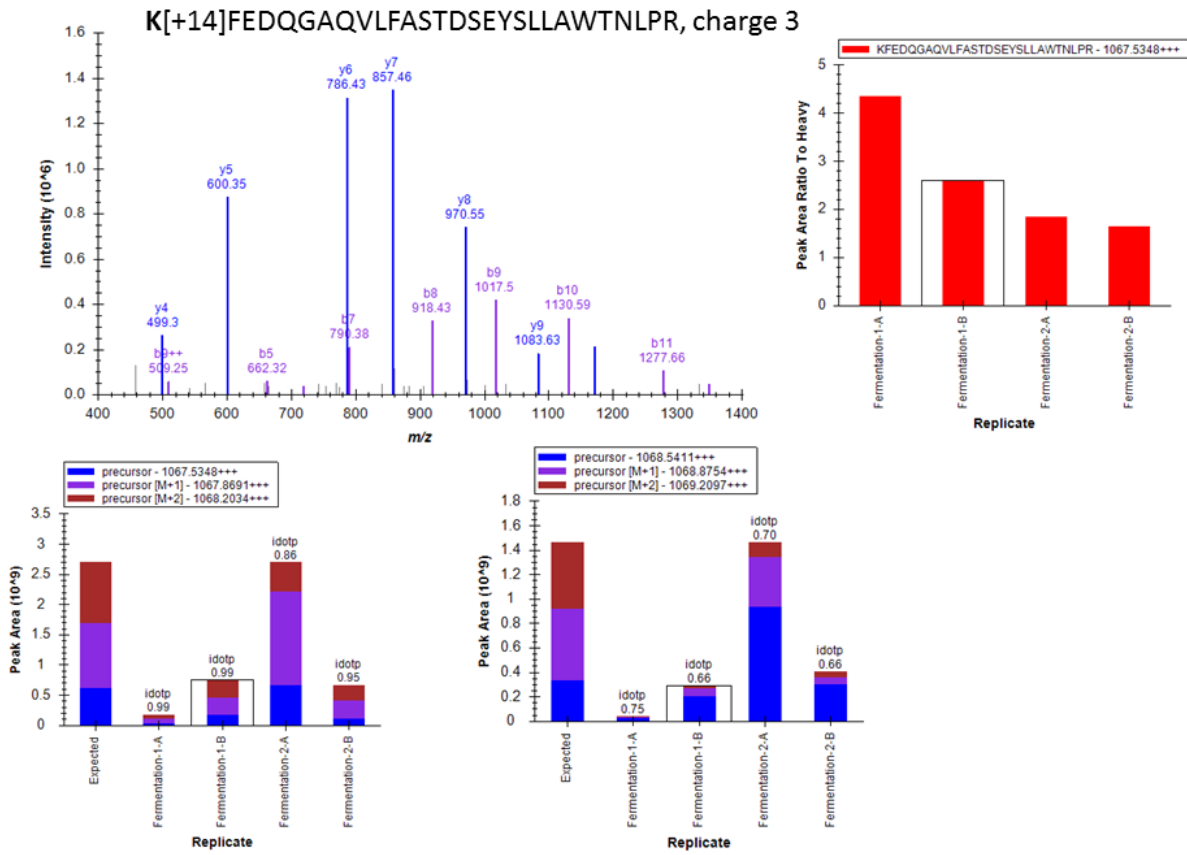
Fermentation: Npl3 Fragmentation and Heavy-Light Doublet Confirmation



Fermentation: Np13 Fragmentation and Heavy-Light Doublet Confirmation

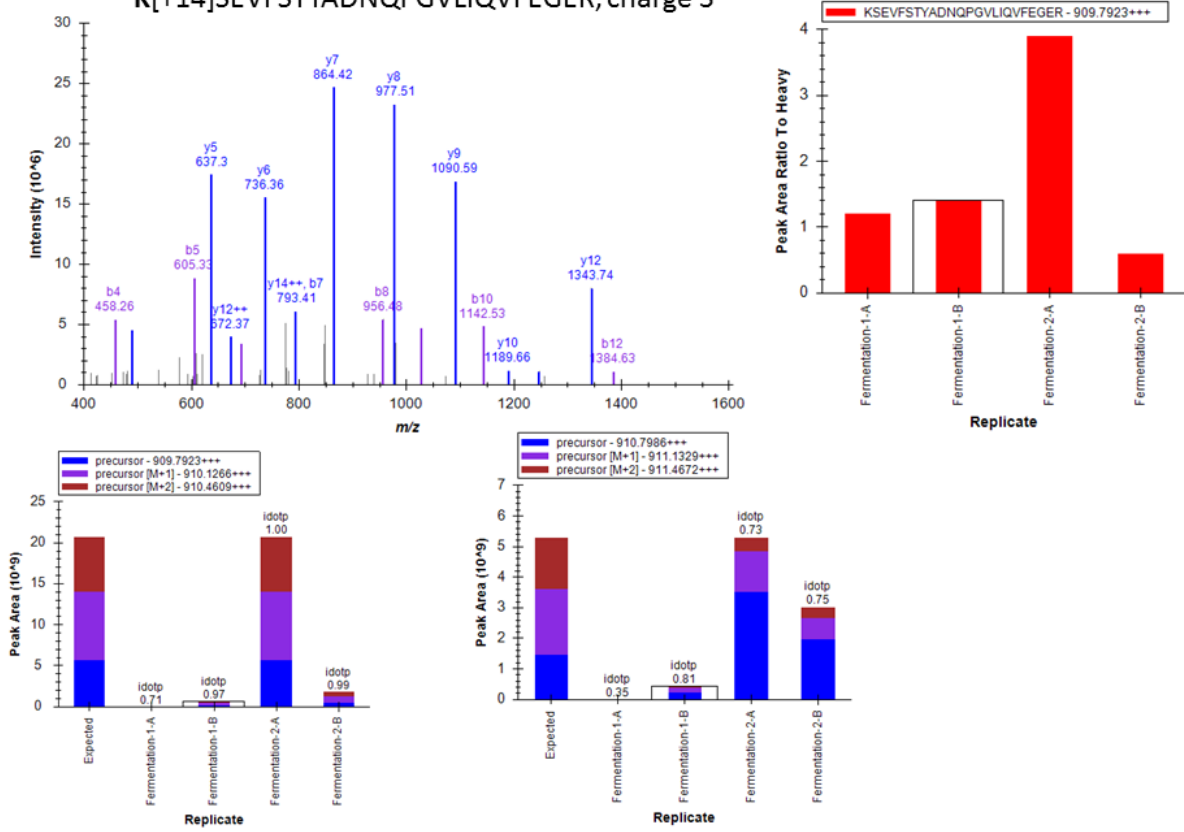


Fermentation: Tsa2 Fragmentation and Heavy-Light Doublet Confirmation

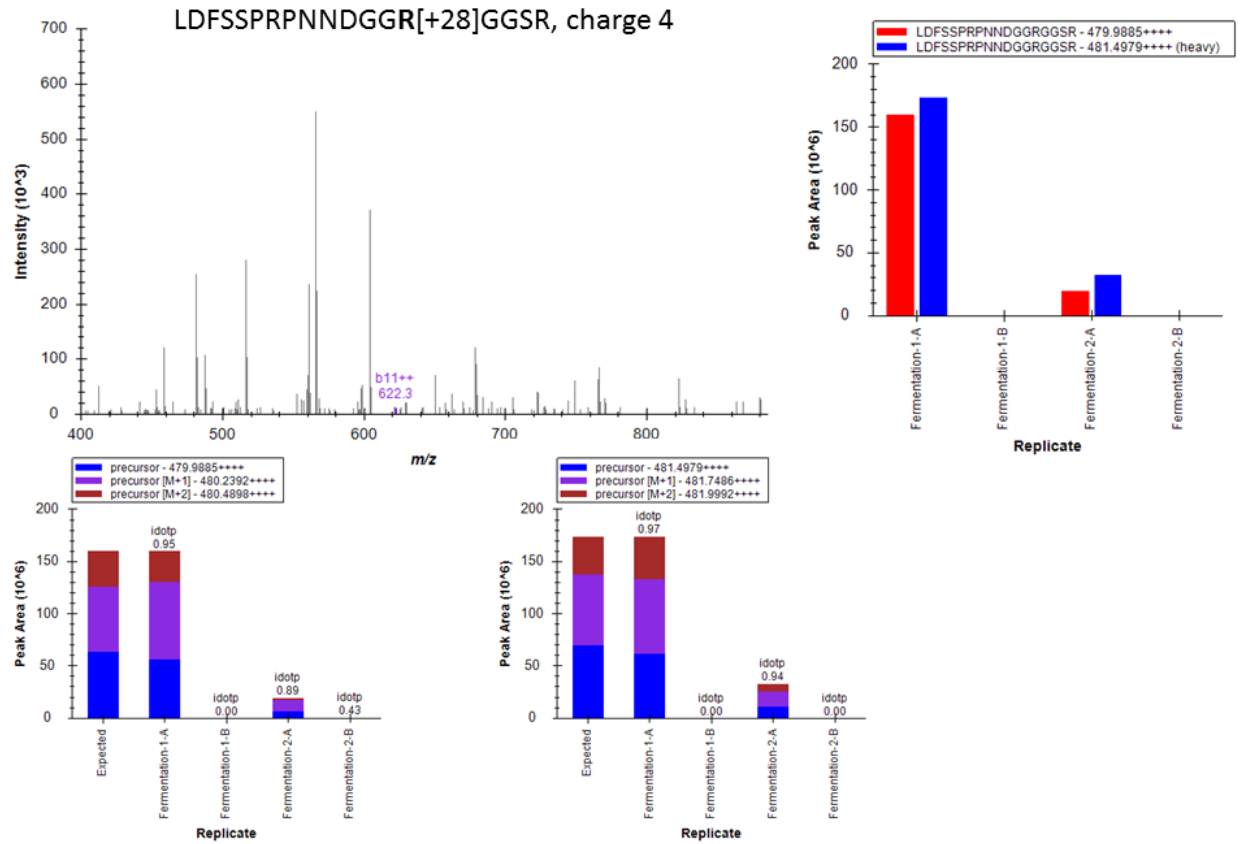


Fermentation: Ssa2/4 Fragmentation and Heavy-Light Doublet Confirmation

K[+14]SEVFSTYADNQPGLVLIQVFEGER, charge 3

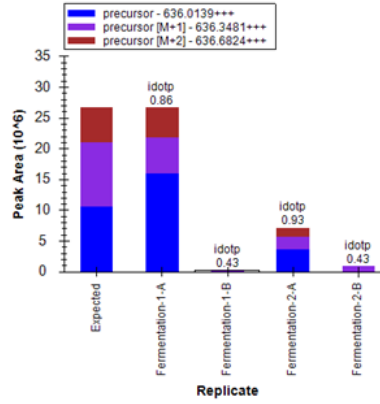
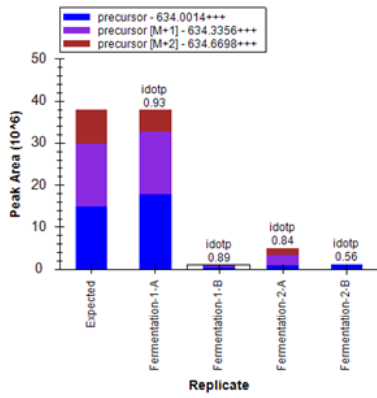
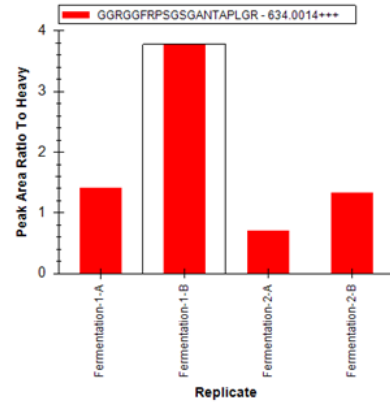
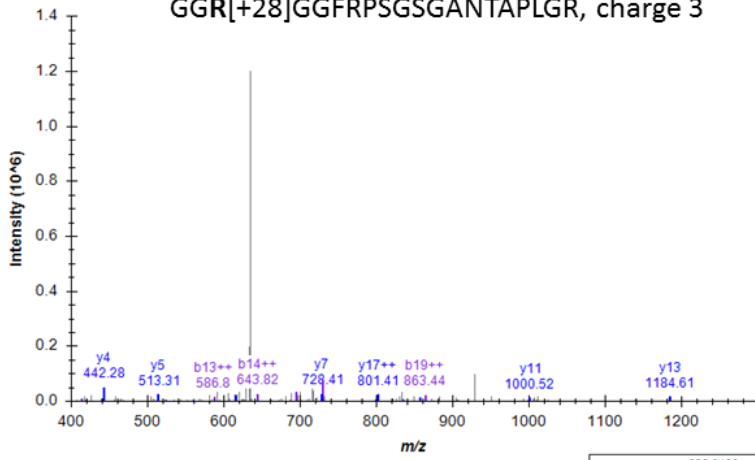


Fermentation: Nsr1 Fragmentation and Heavy-Light Doublet Confirmation

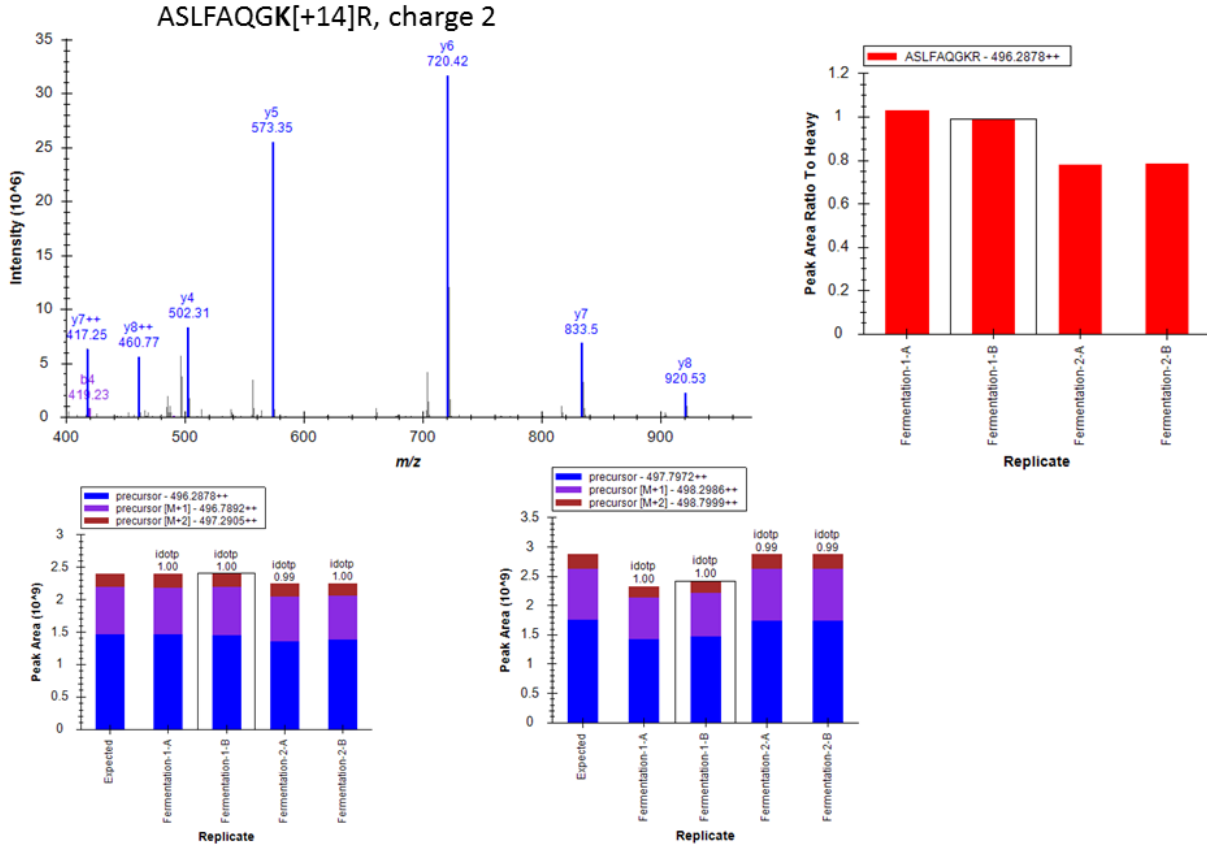


Fermentation: Nsr1 Fragmentation and Heavy-Light Doublet Confirmation

GGR[+28]GGFRPSGSGANTAPLGR, charge 3

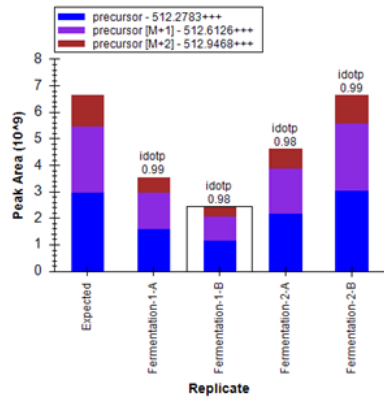
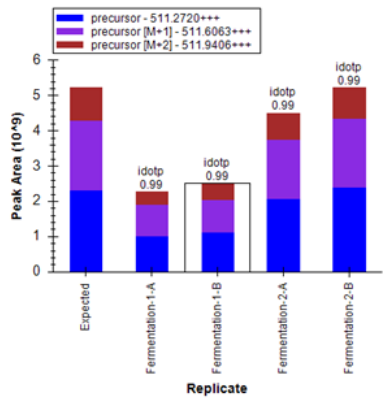
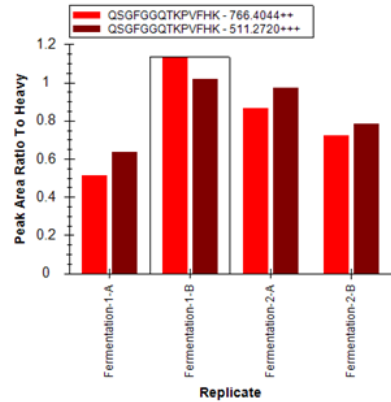
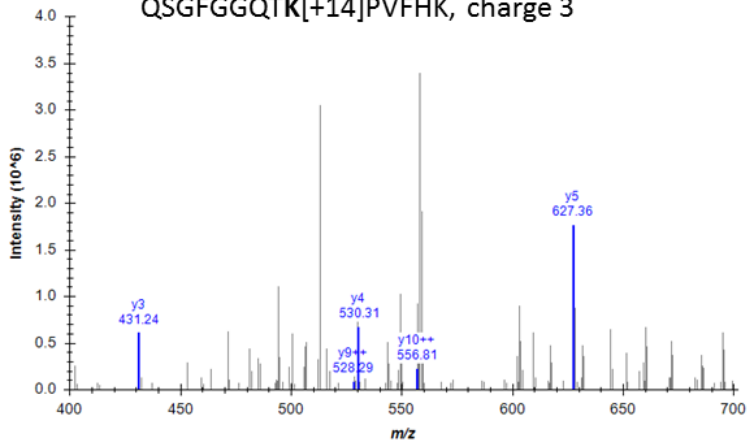


Fermentation: Rpl42b Fragmentation and Heavy-Light Doublet Confirmation

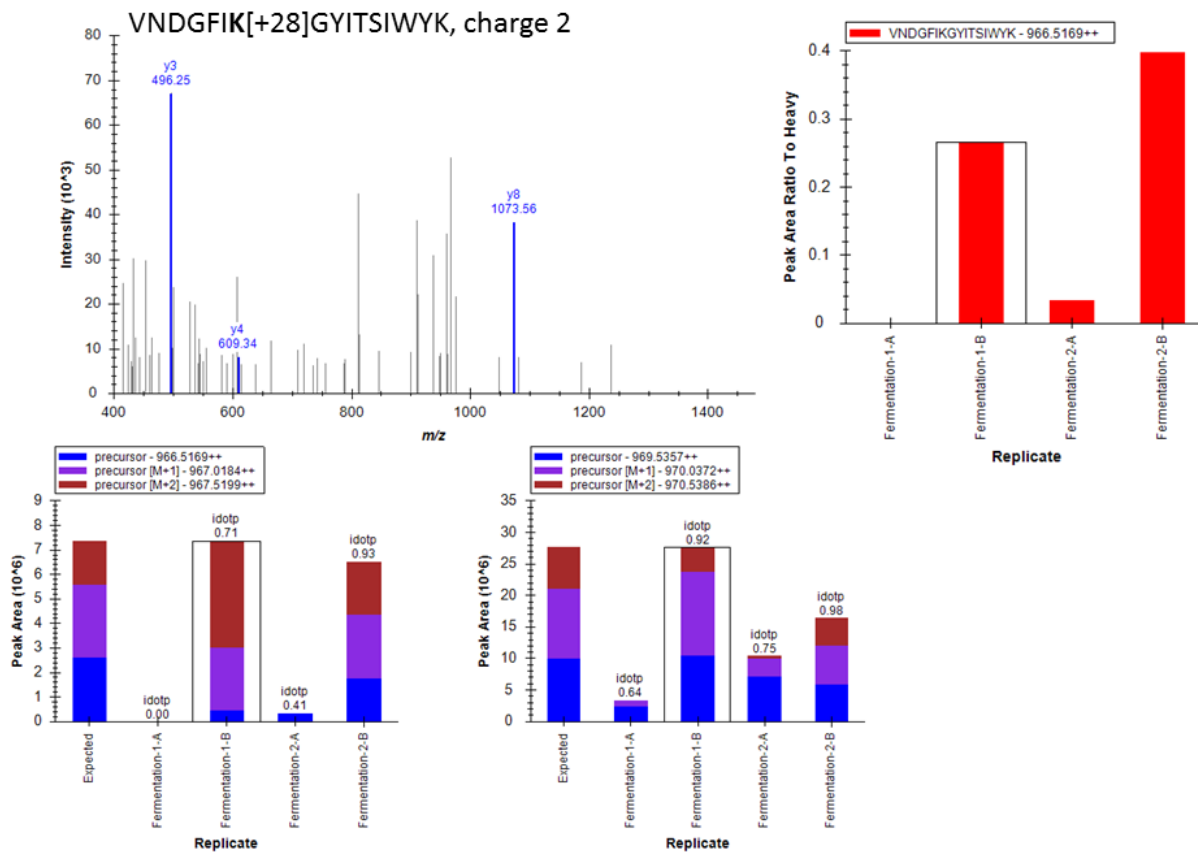


Fermentation: Rpl42b Fragmentation and Heavy-Light Doublet Confirmation

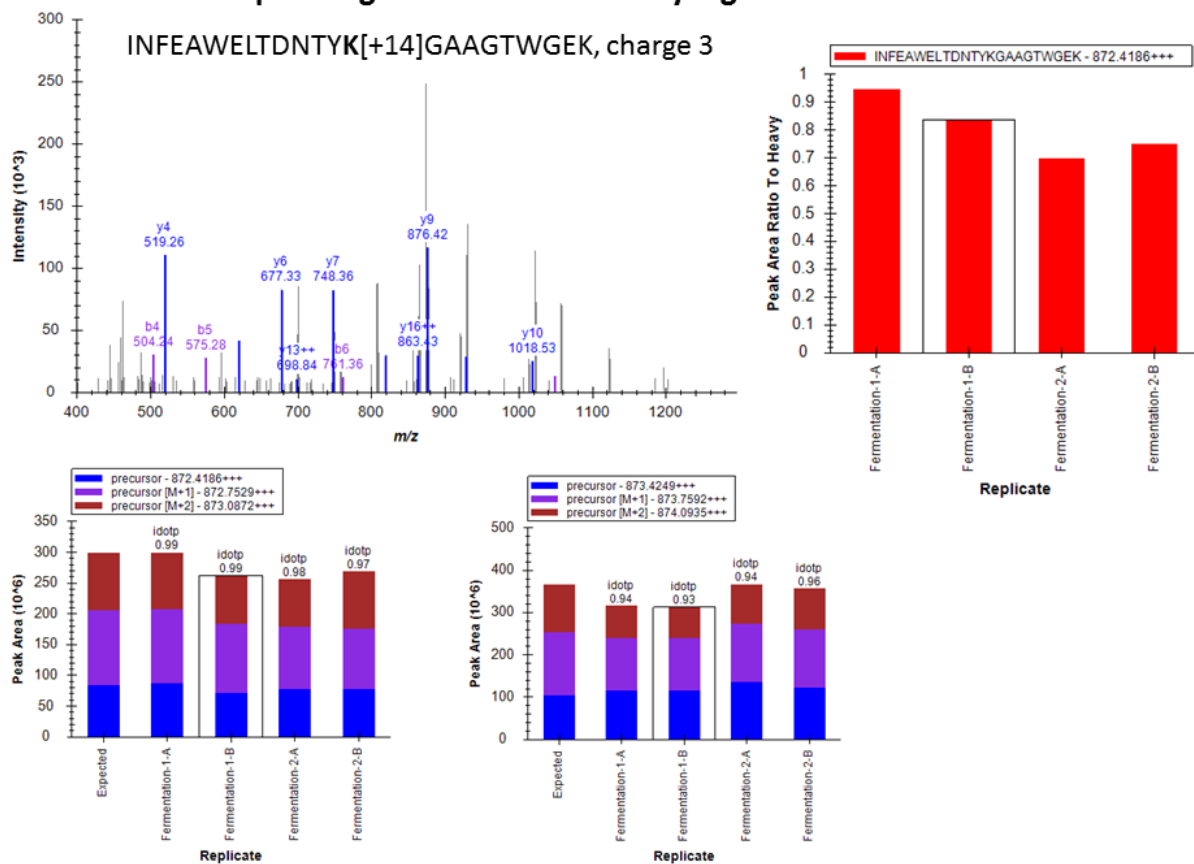
QSGFGGQTK[+14]PVFHK, charge 3



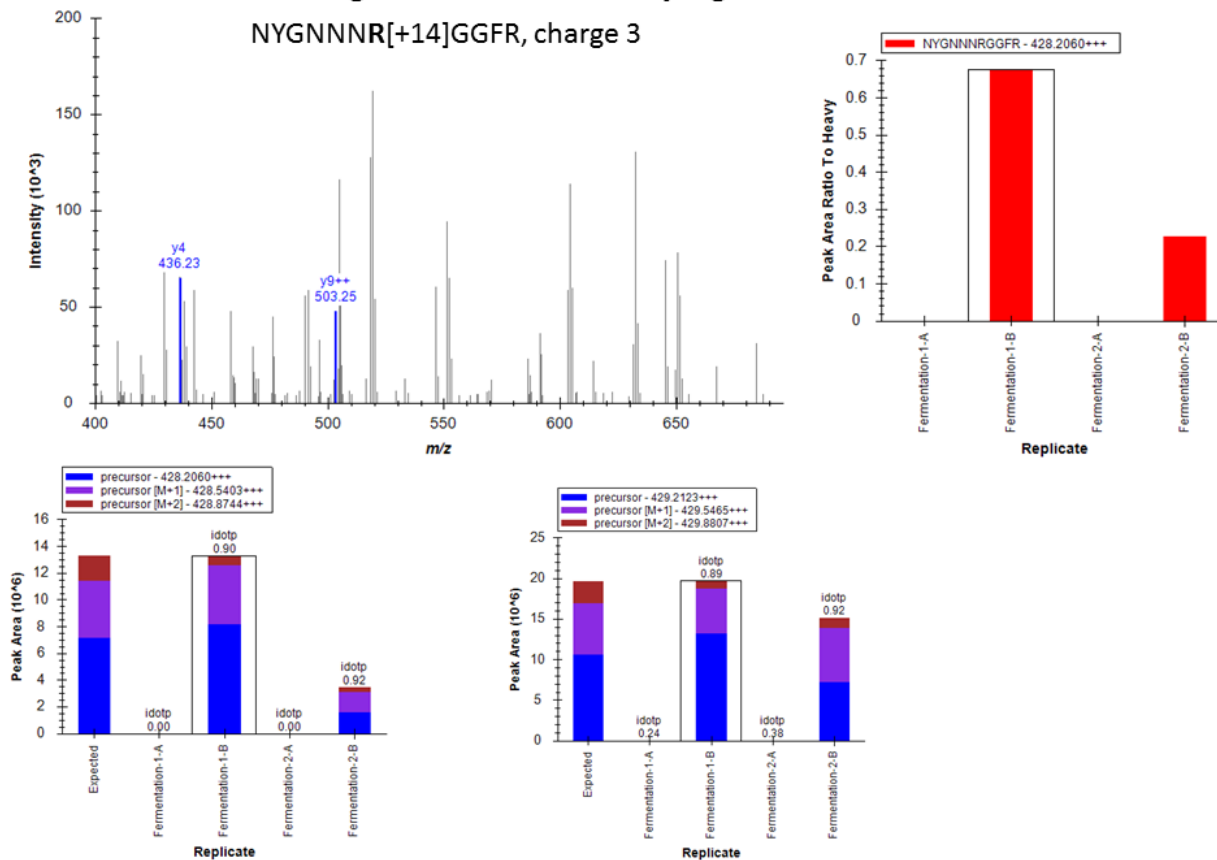
Fermentation: Kdx1 Fragmentation and Heavy-Light Doublet Confirmation



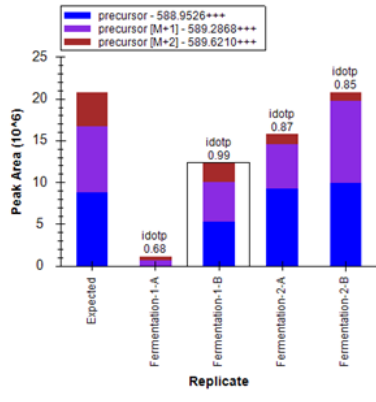
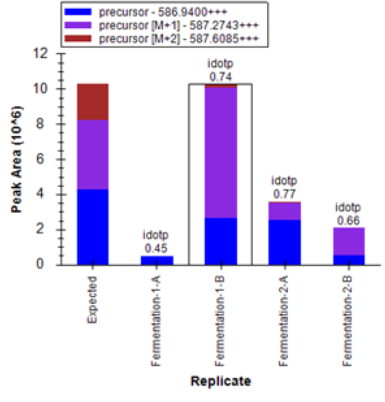
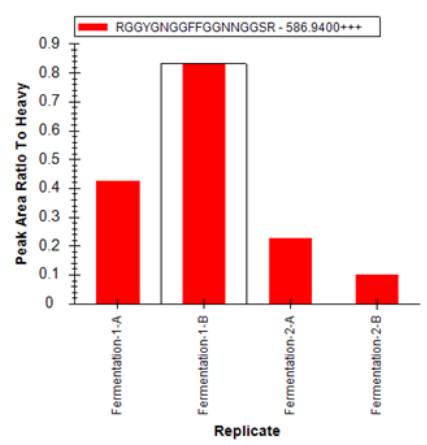
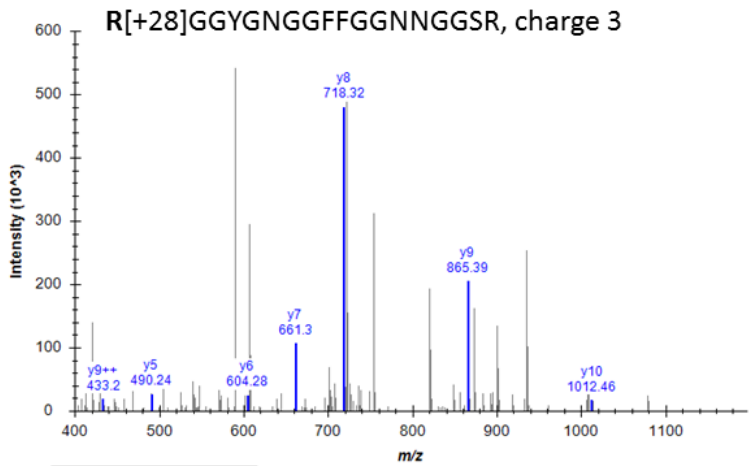
Fermentation: Srp40 Fragmentation and Heavy-Light Doublet Confirmation



Fermentation: Pub1 Fragmentation and Heavy-Light Doublet Confirmation

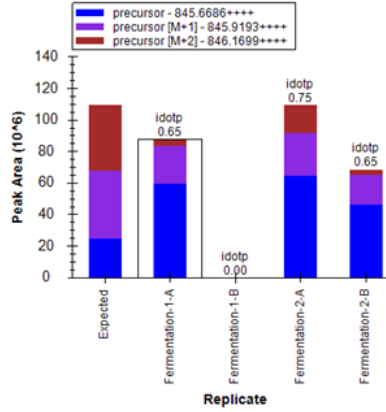
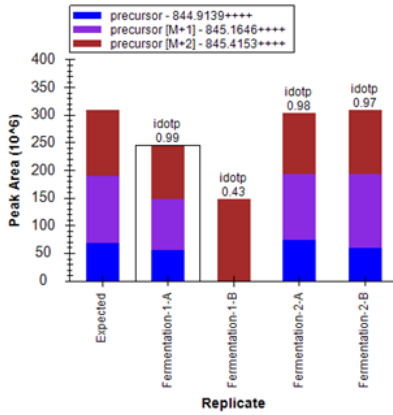
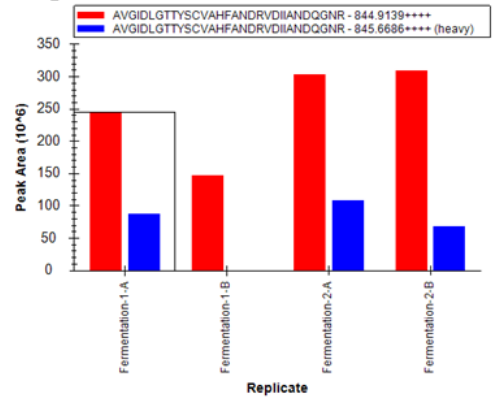
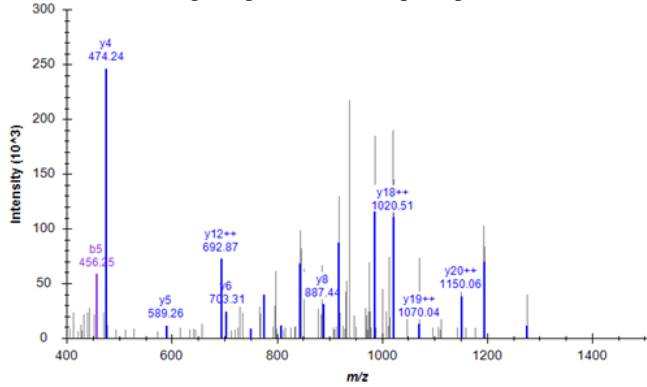


Fermentation: Ded1 Fragmentation and Heavy-Light Doublet Confirmation



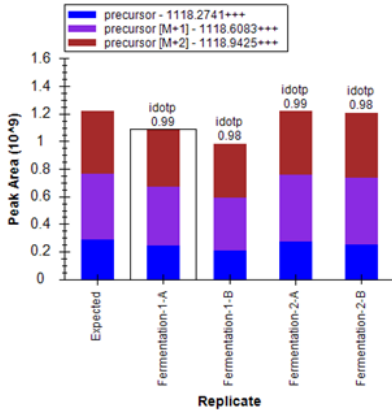
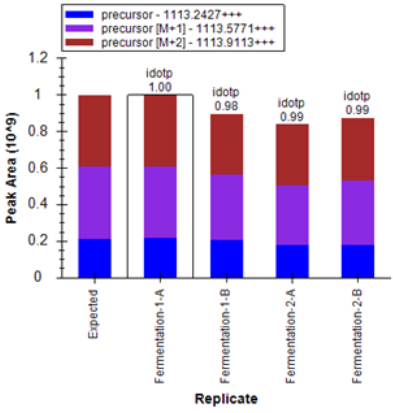
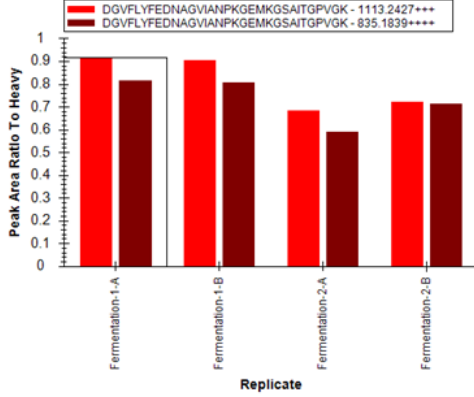
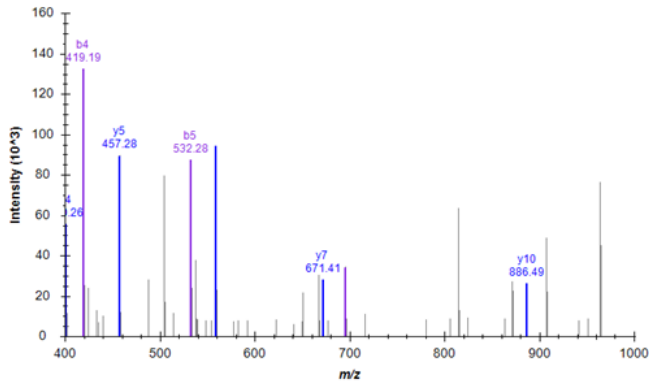
Fermentation: Ssa1 Fragmentation and Heavy-Light Doublet Confirmation

AVGIDLGTTYSC[+57]VAHFANDR[+14]VDIANDQGNR, charge 4



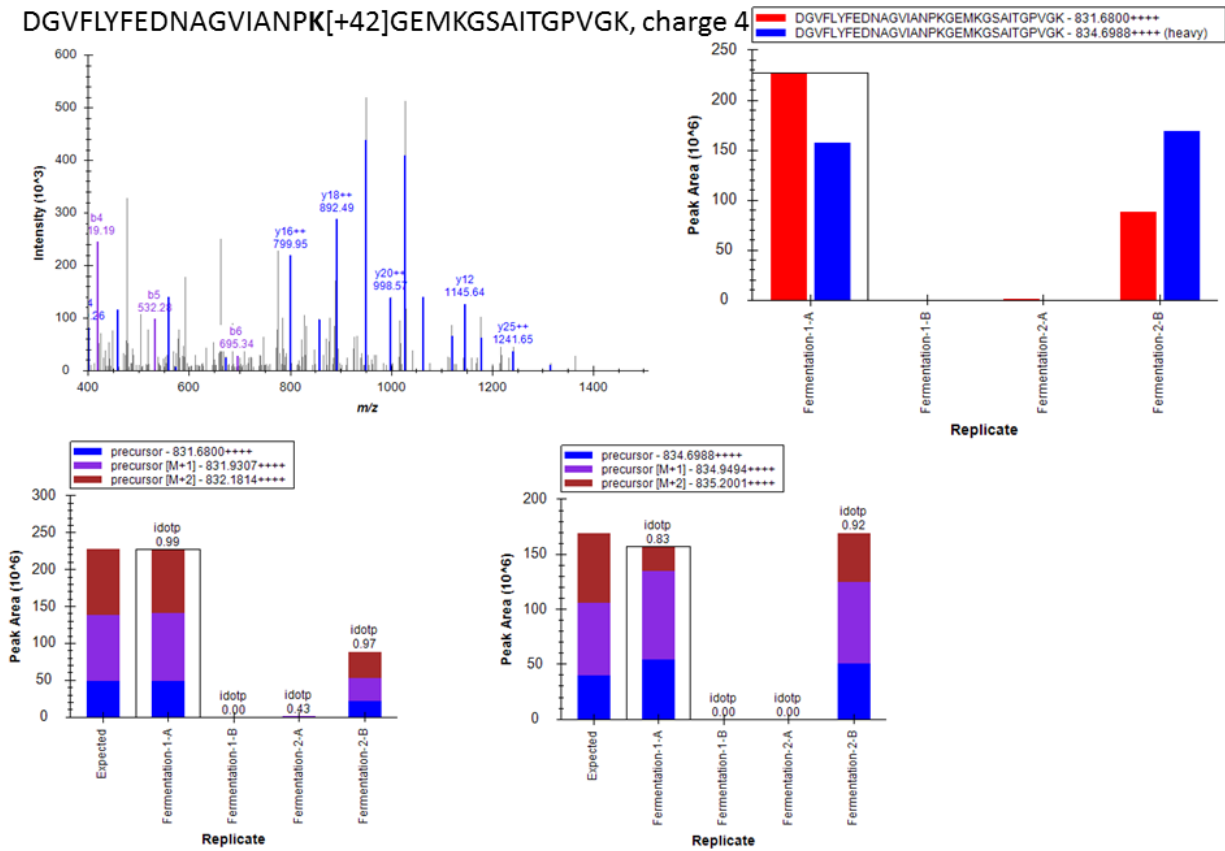
Fermentation: Rpl23a Fragmentation and Heavy-Light Doublet Confirmation

DGVFLYFEDNAGVIANPK[+28]GEMK[+28]GSAITGPVVGK, charge 3



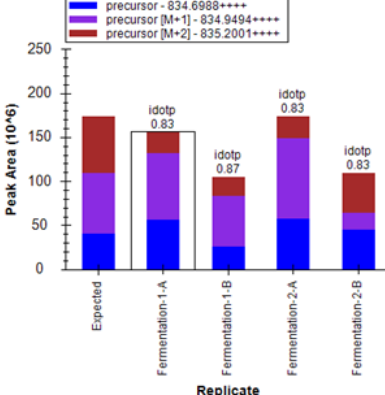
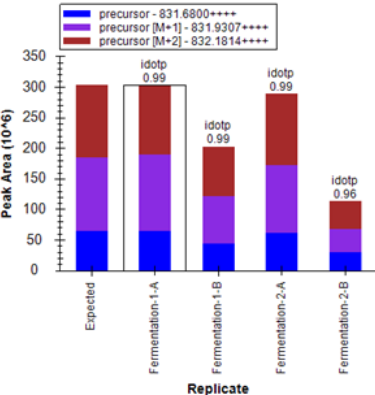
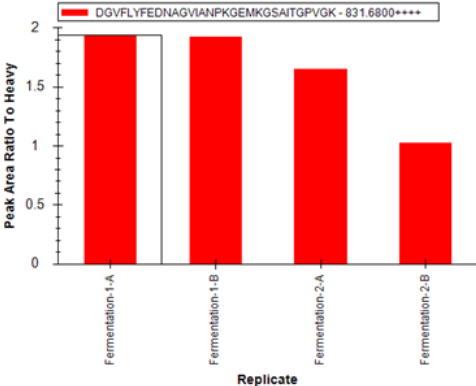
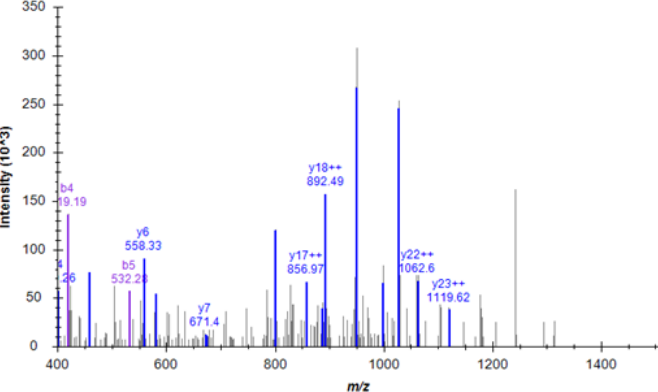
Fermentation: Rpl23a Fragmentation and Heavy-Light Doublet Confirmation

DGVFLYFEDNAGVIANPK[+42]GEMKGSAITGPV GK, charge 4

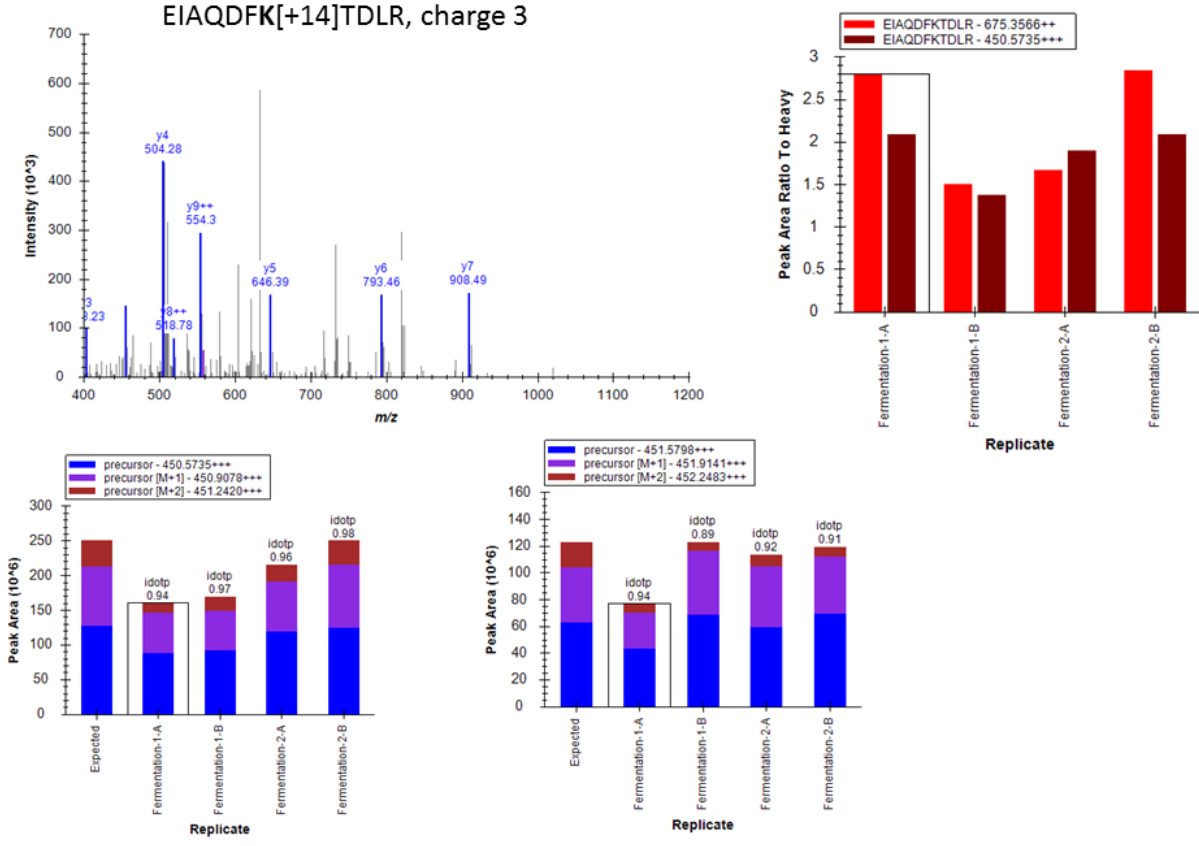


Fermentation: Rpl23a Fragmentation and Heavy-Light Doublet Confirmation

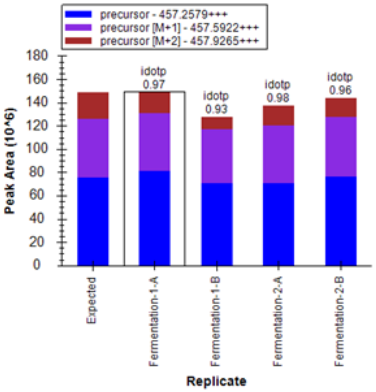
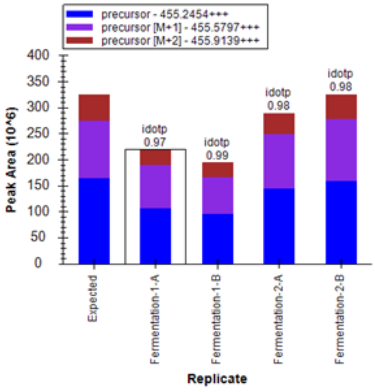
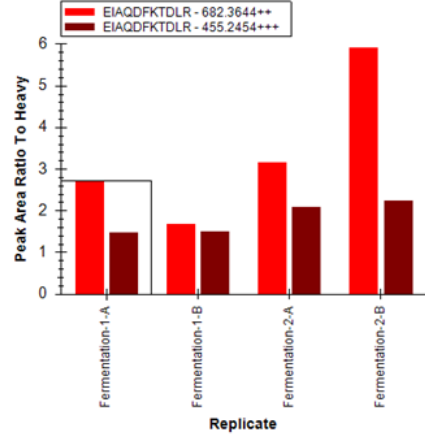
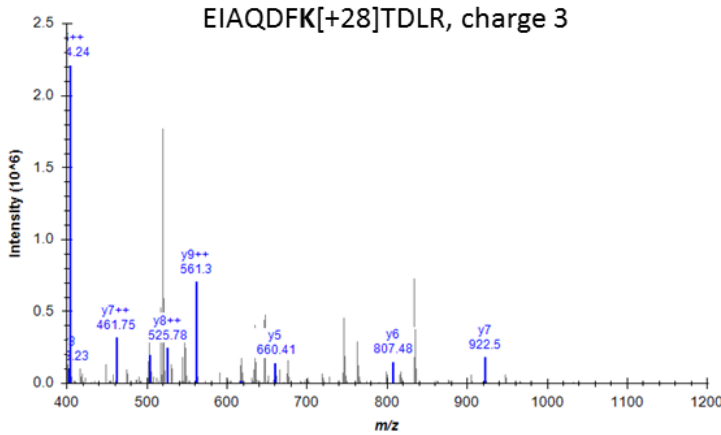
DGVFLYFEDNAGVIANPK[+28]GEMK[+14]GSAITGPVVGK, charge 4



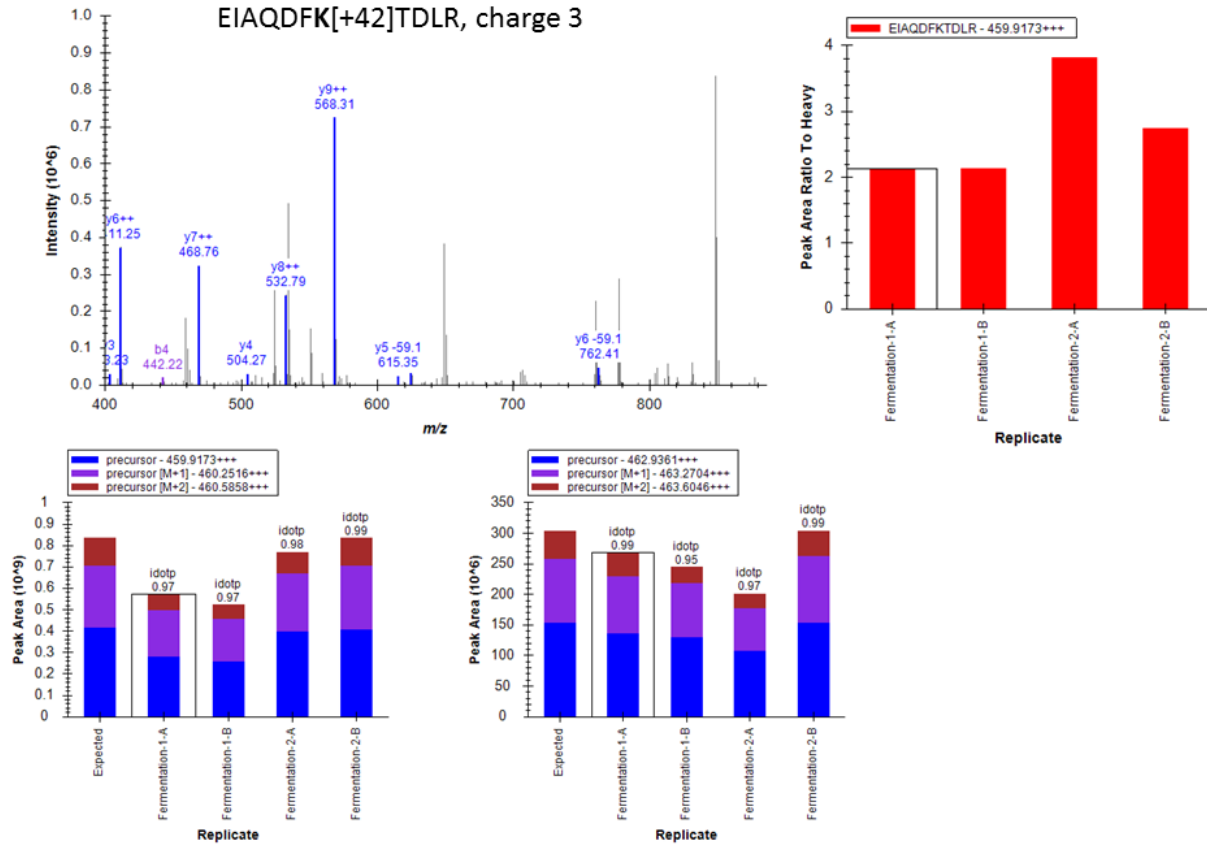
Fermentation: Hht1 Fragmentation and Heavy-Light Doublet Confirmation



Fermentation: Hht1 Fragmentation and Heavy-Light Doublet Confirmation

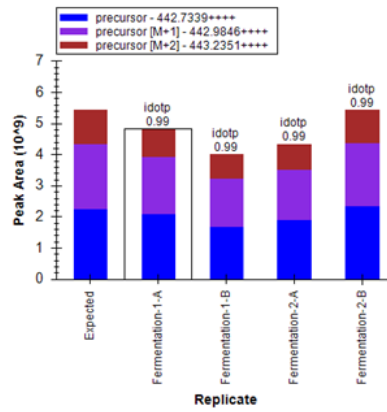
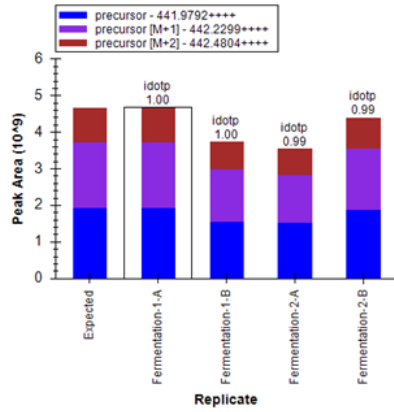
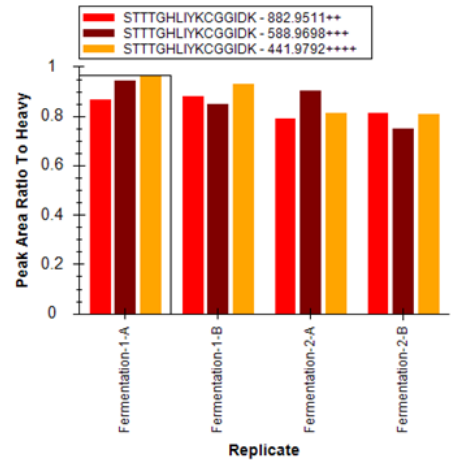
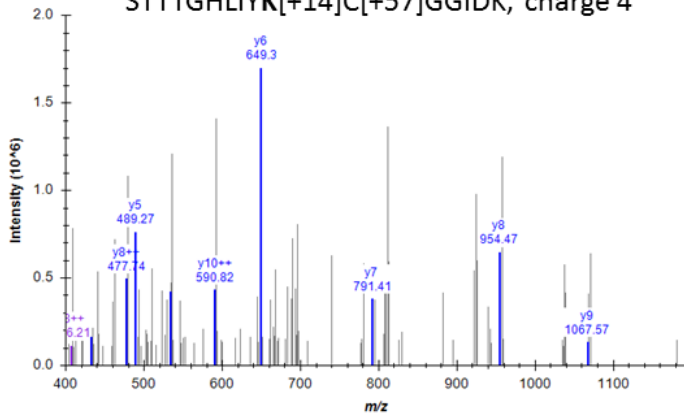


Fermentation: Hht1 Fragmentation and Heavy-Light Doublet Confirmation

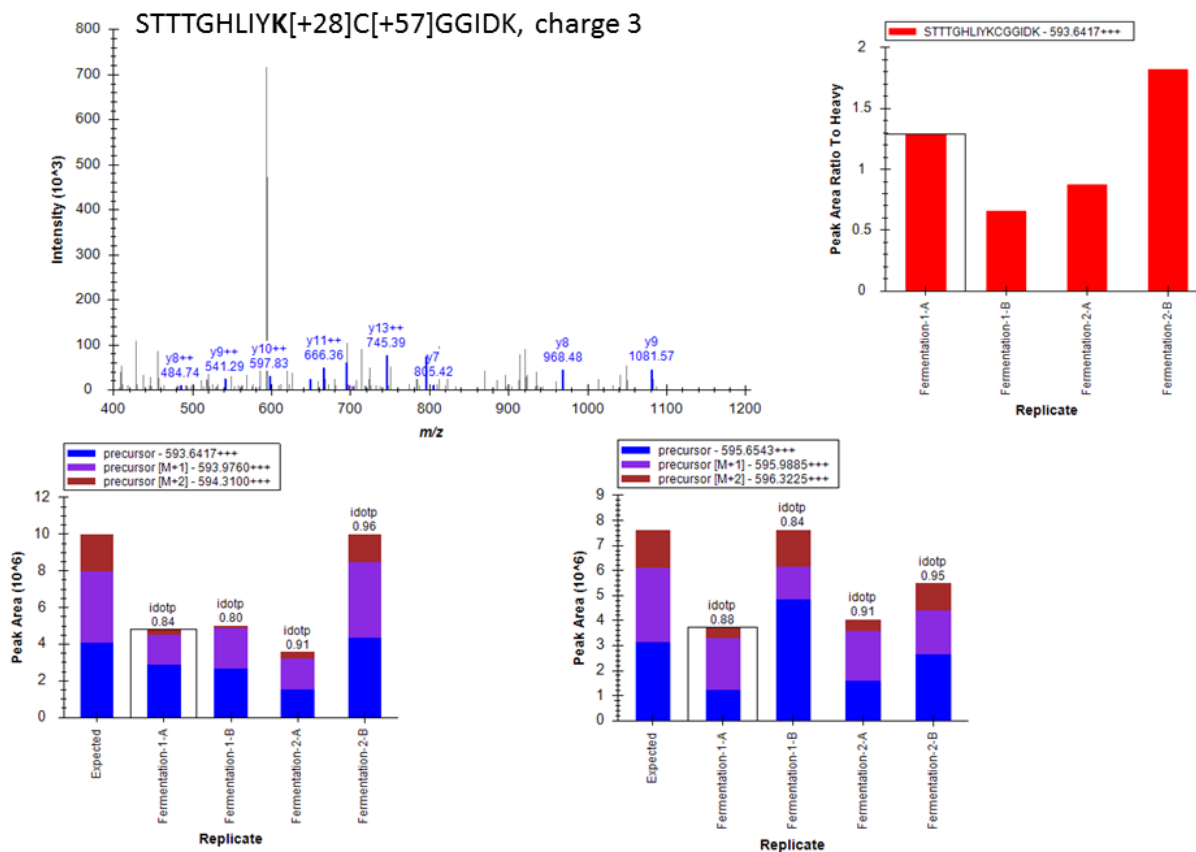


Fermentation: eEF1A Fragmentation and Heavy-Light Doublet Confirmation

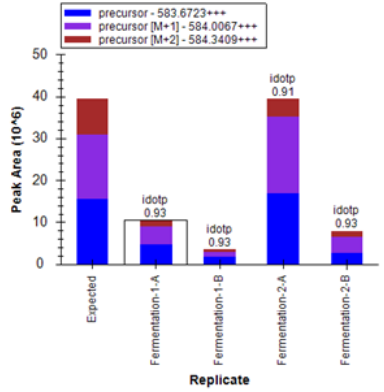
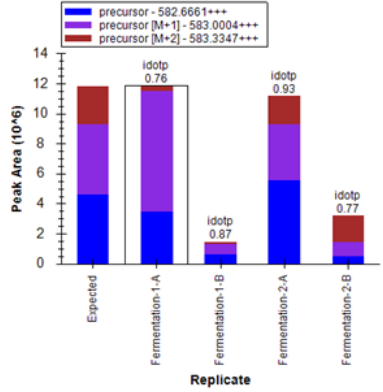
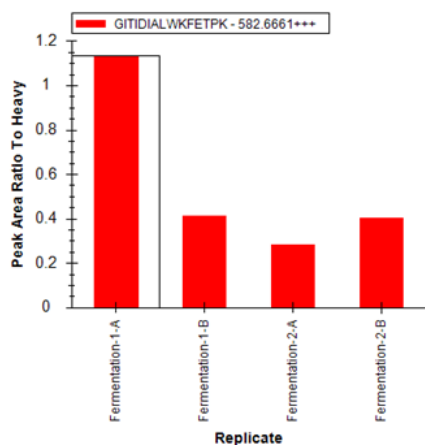
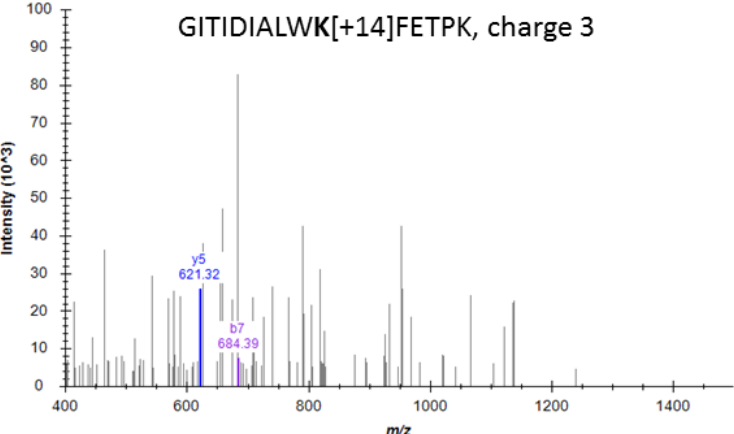
STTTGHLIYK[+14]C[+57]GGIDK, charge 4



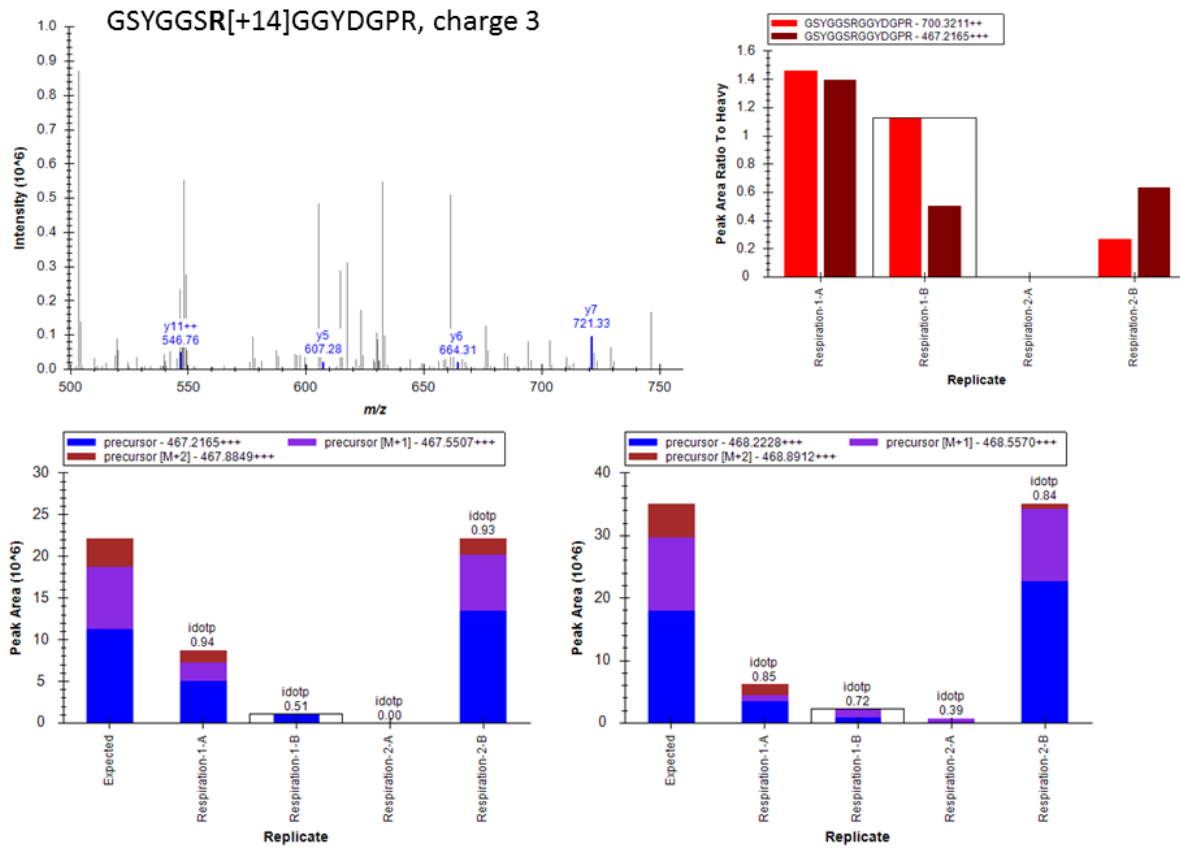
Fermentation: eEF1A Fragmentation and Heavy-Light Doublet Confirmation



Fermentation: eEF1A Fragmentation and Heavy-Light Doublet Confirmation

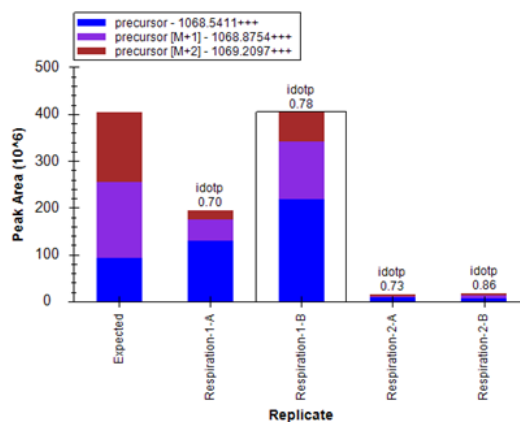
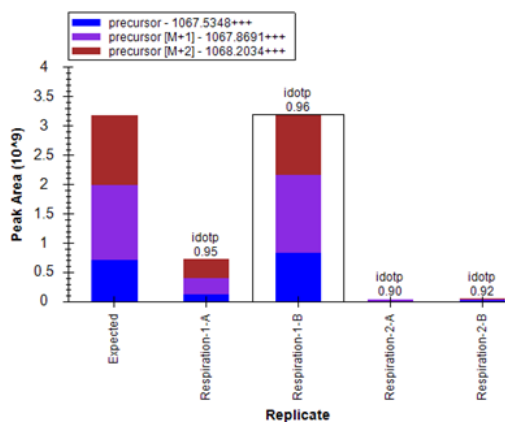
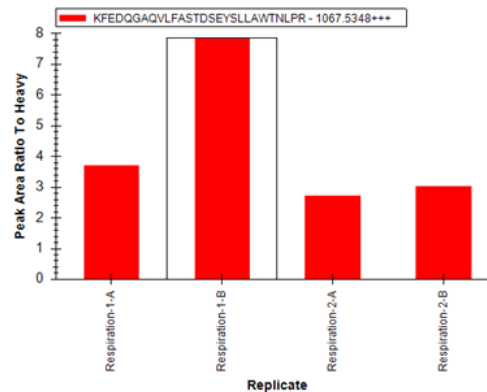
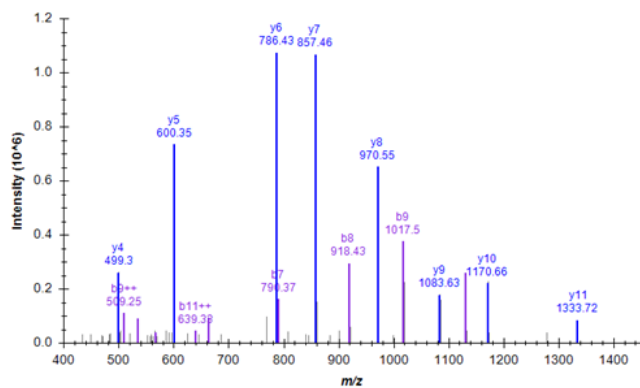


Respiration: Npl3 Fragmentation and Heavy-Light Doublet Confirmation



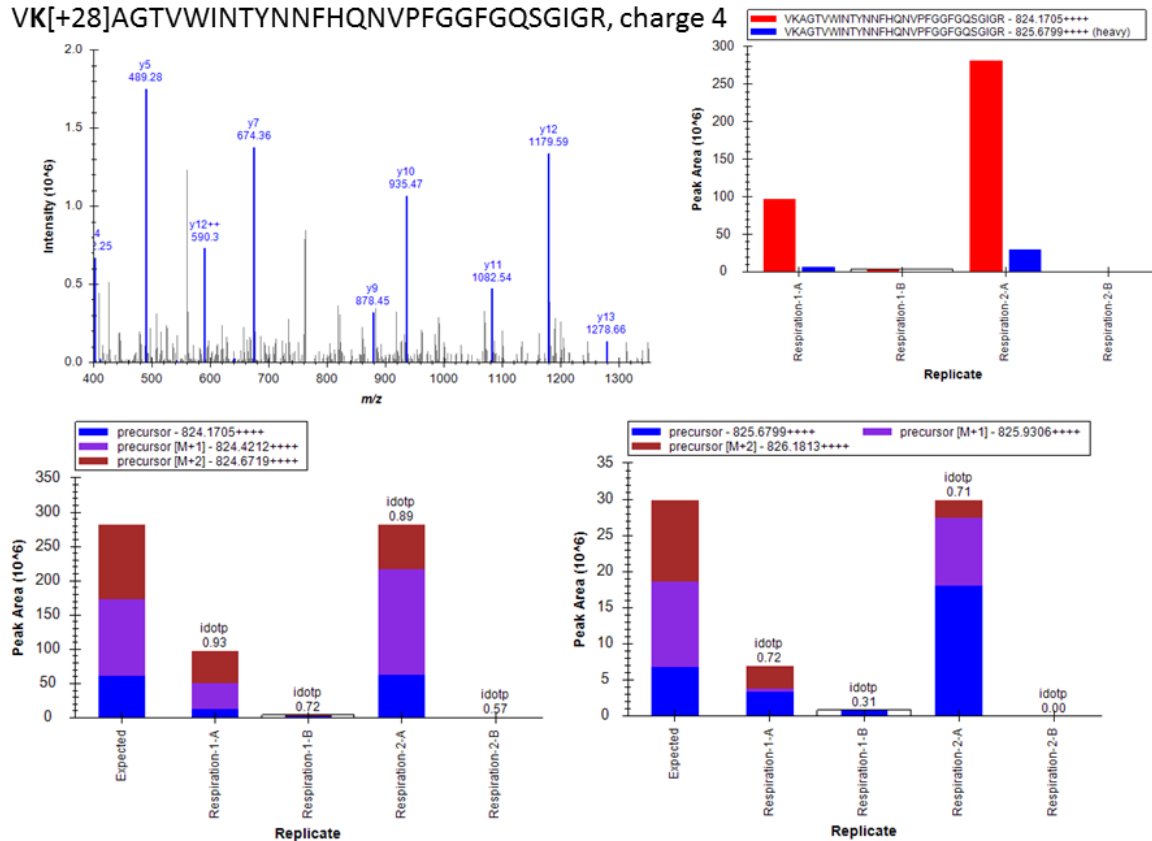
Respiration: Tsa2 Fragmentation and Heavy-Light Doublet Confirmation

K[+14]FEDQGAQVLFASTDSEYSLLAWTNLPR, charge 3

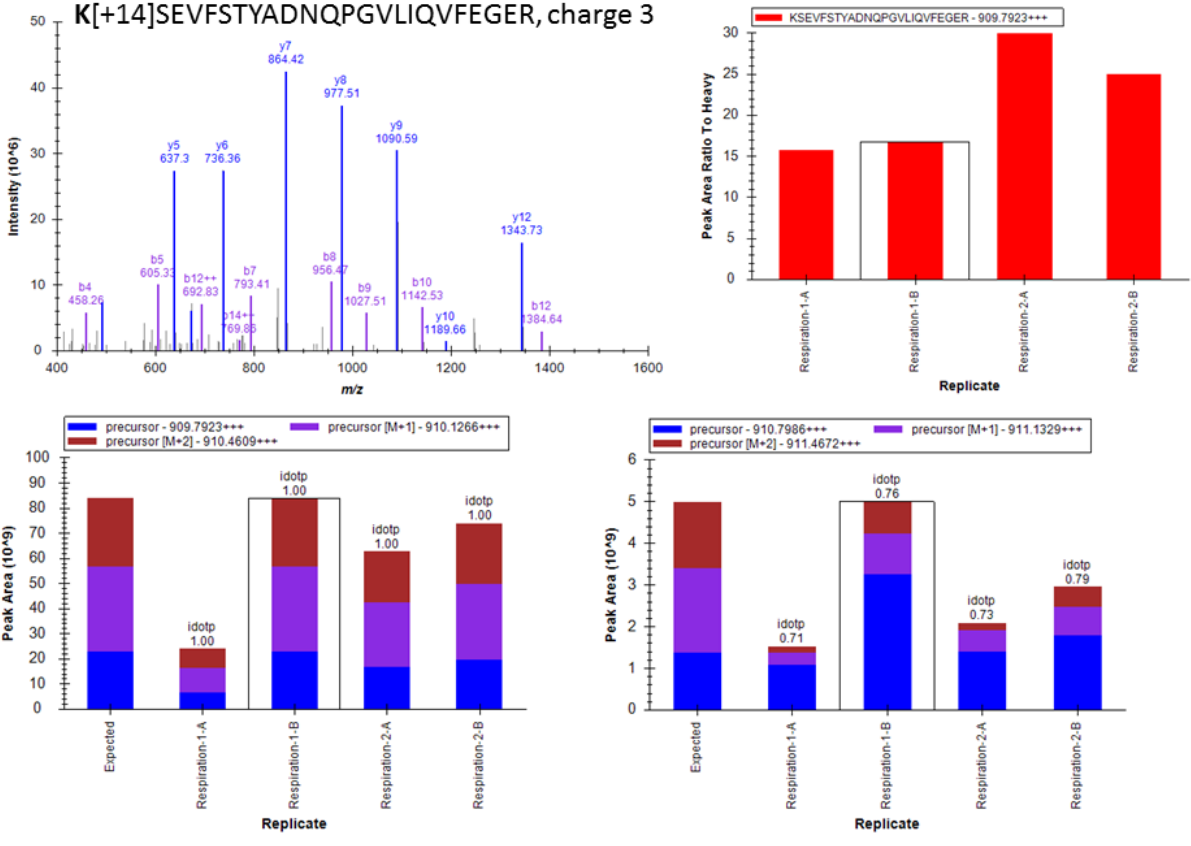


Respiration: Ald5 Fragmentation and Heavy-Light Doublet Confirmation

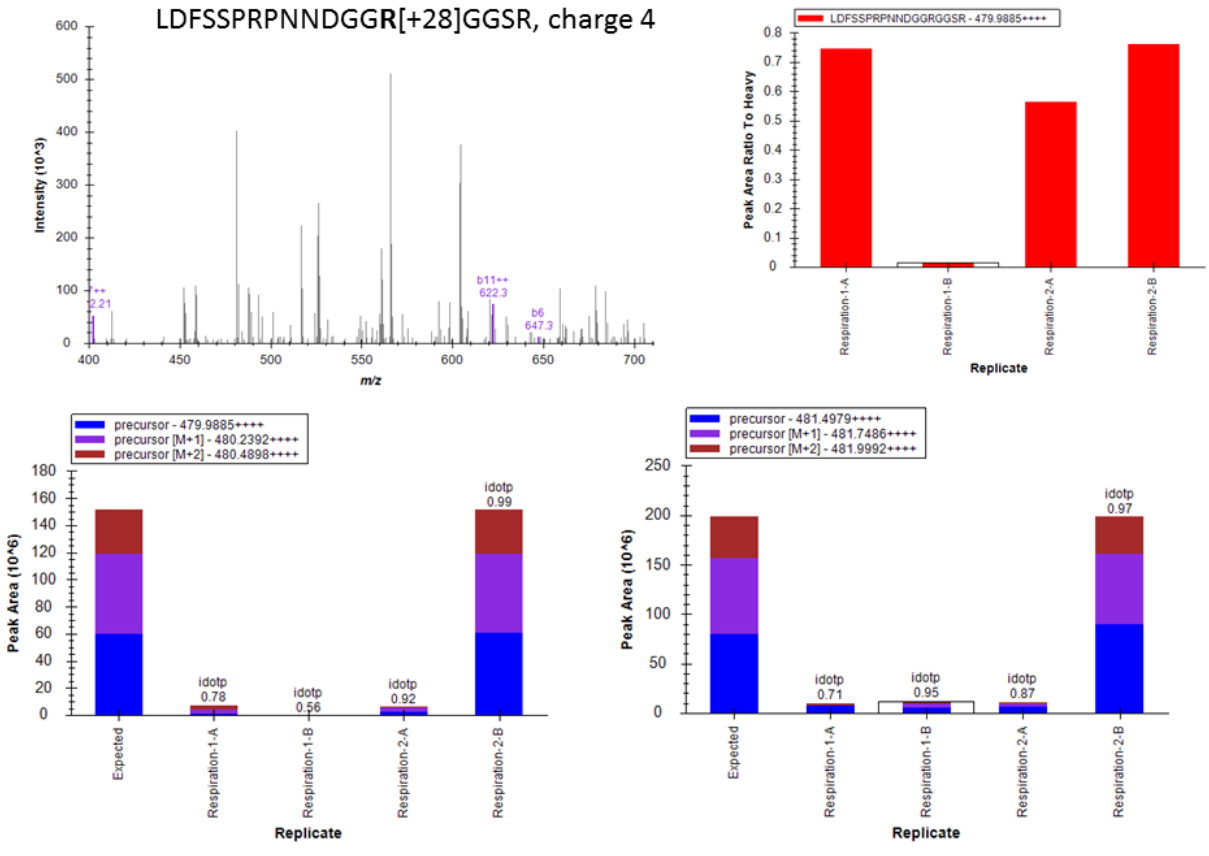
VK[+28]AGTVWINTYNNFHQNVFFGGFGQSGIGR, charge 4



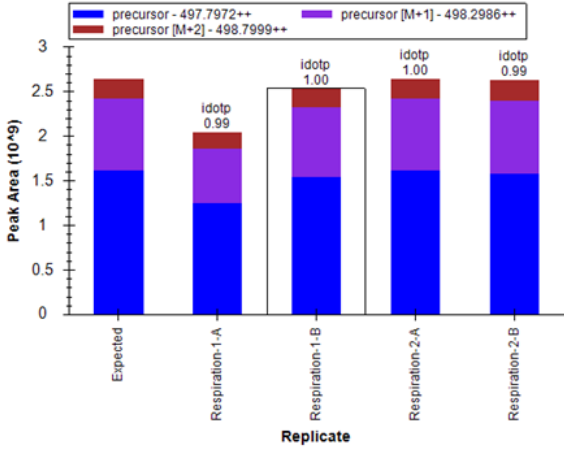
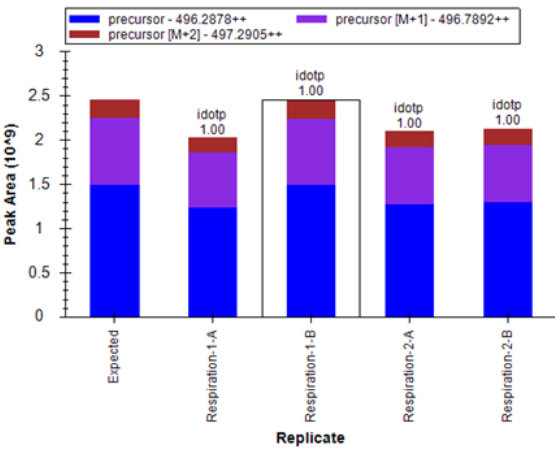
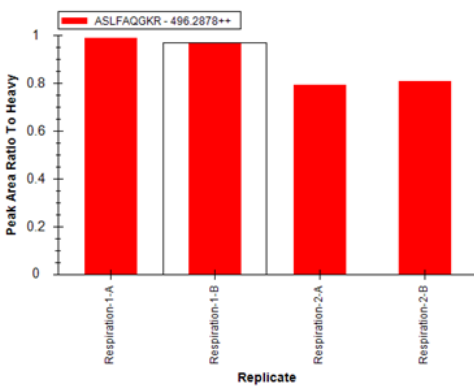
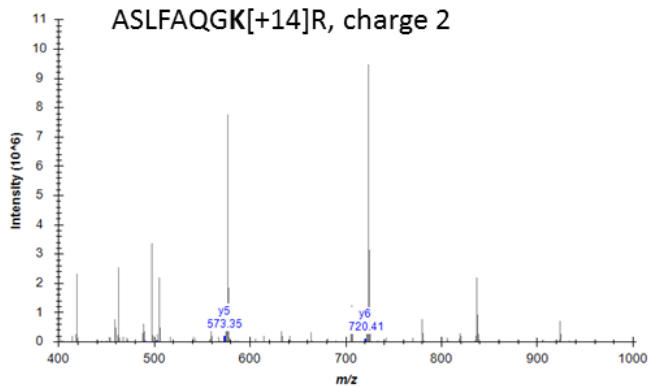
Respiration: Ssa2/4 Fragmentation and Heavy-Light Doublet Confirmation



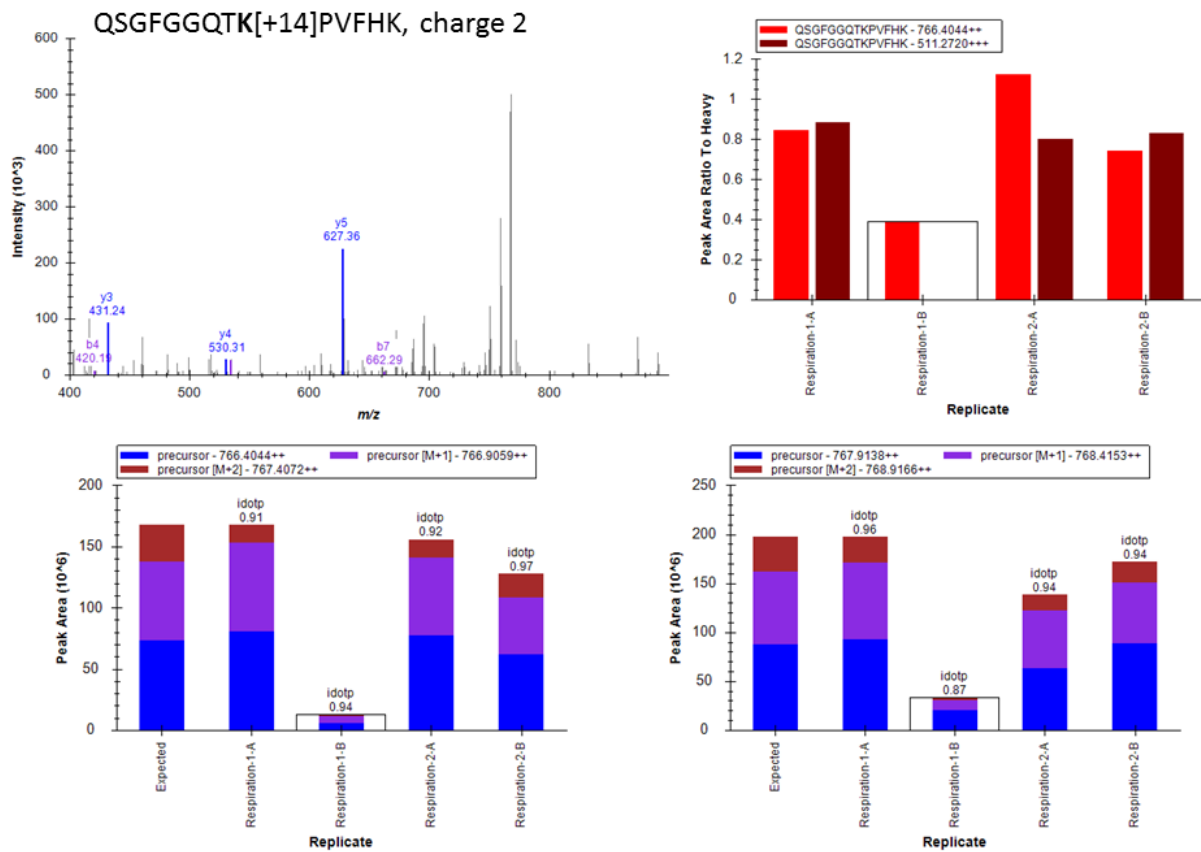
Respiration: Nsr1 Fragmentation and Heavy-Light Doublet Confirmation



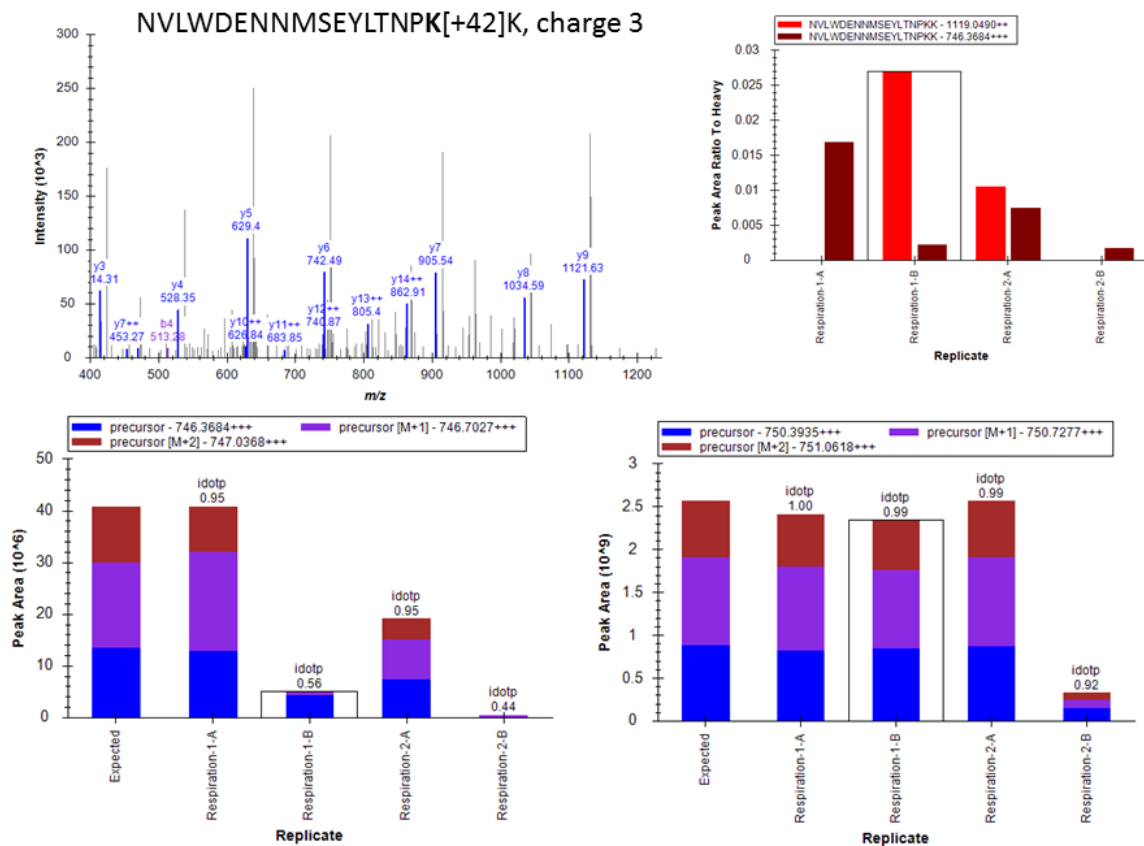
Respiration: Rpl42b Fragmentation and Heavy-Light Doublet Confirmation



Respiration: Rpl42b Fragmentation and Heavy-Light Doublet Confirmation

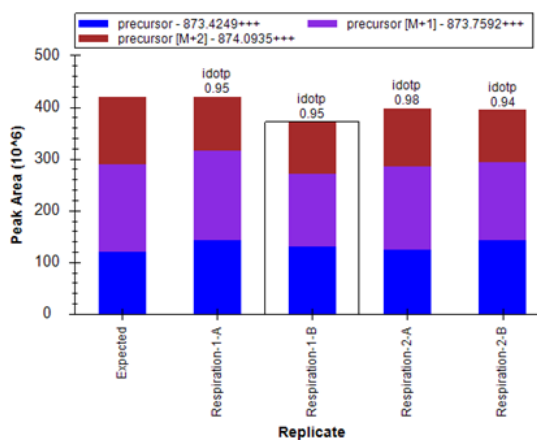
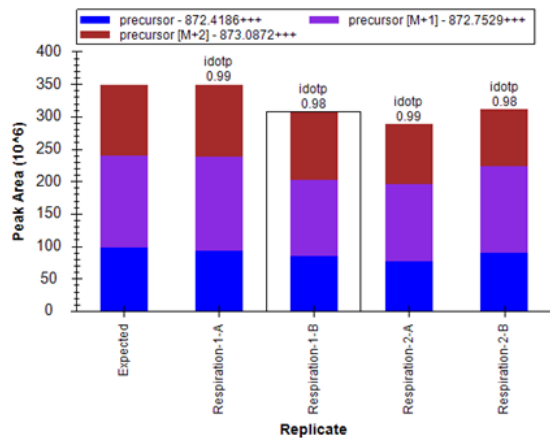
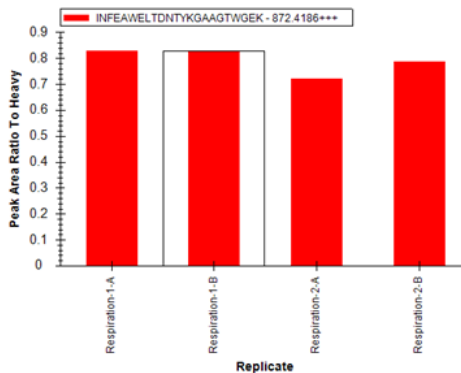
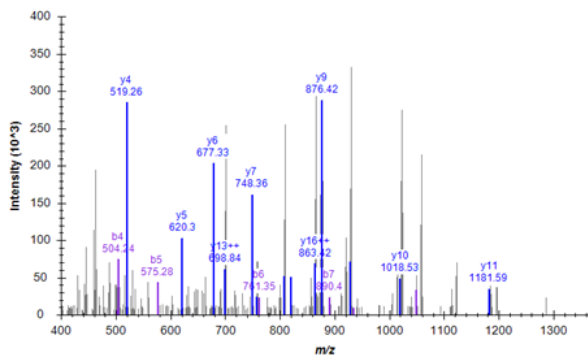


Respiration: Cyc1 Fragmentation and Heavy-Light Doublet Confirmation

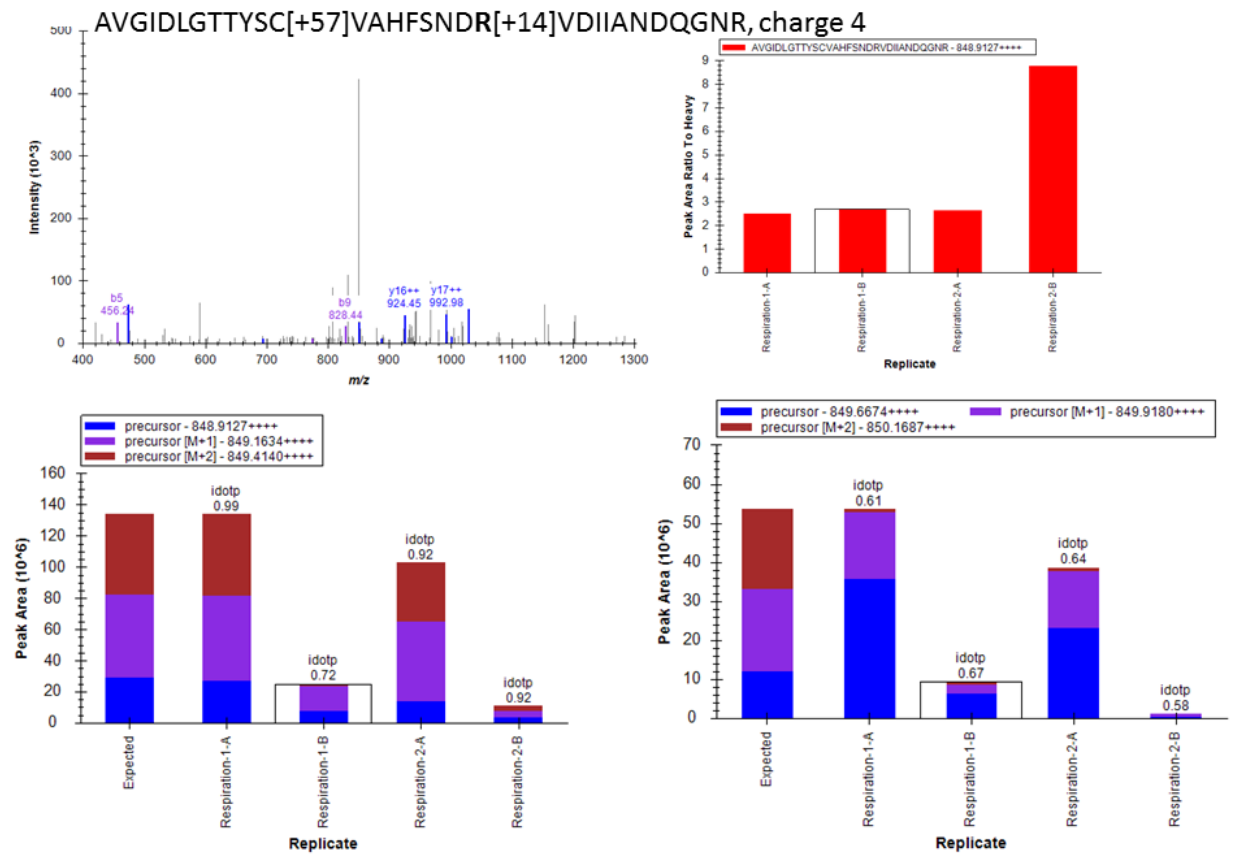


Respiration: Srp40 Fragmentation and Heavy-Light Doublet Confirmation

INFEAWELTDNTYK[+14]GAAGTWGEK, charge 3

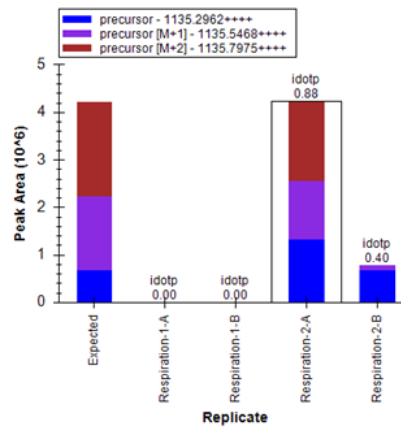
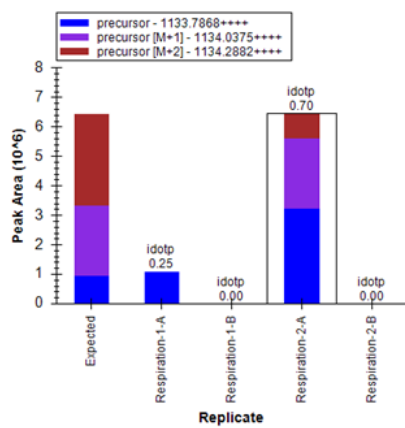
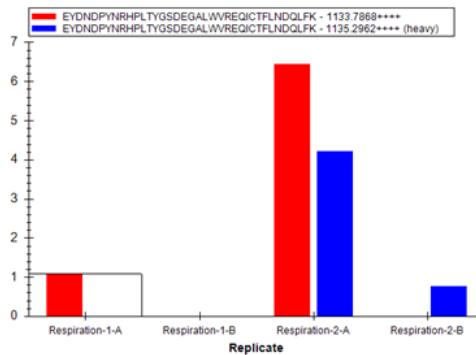
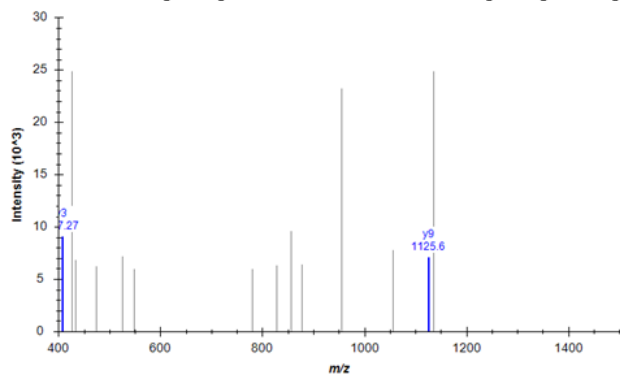


Respiration: Ssa2 Fragmentation and Heavy-Light Doublet Confirmation

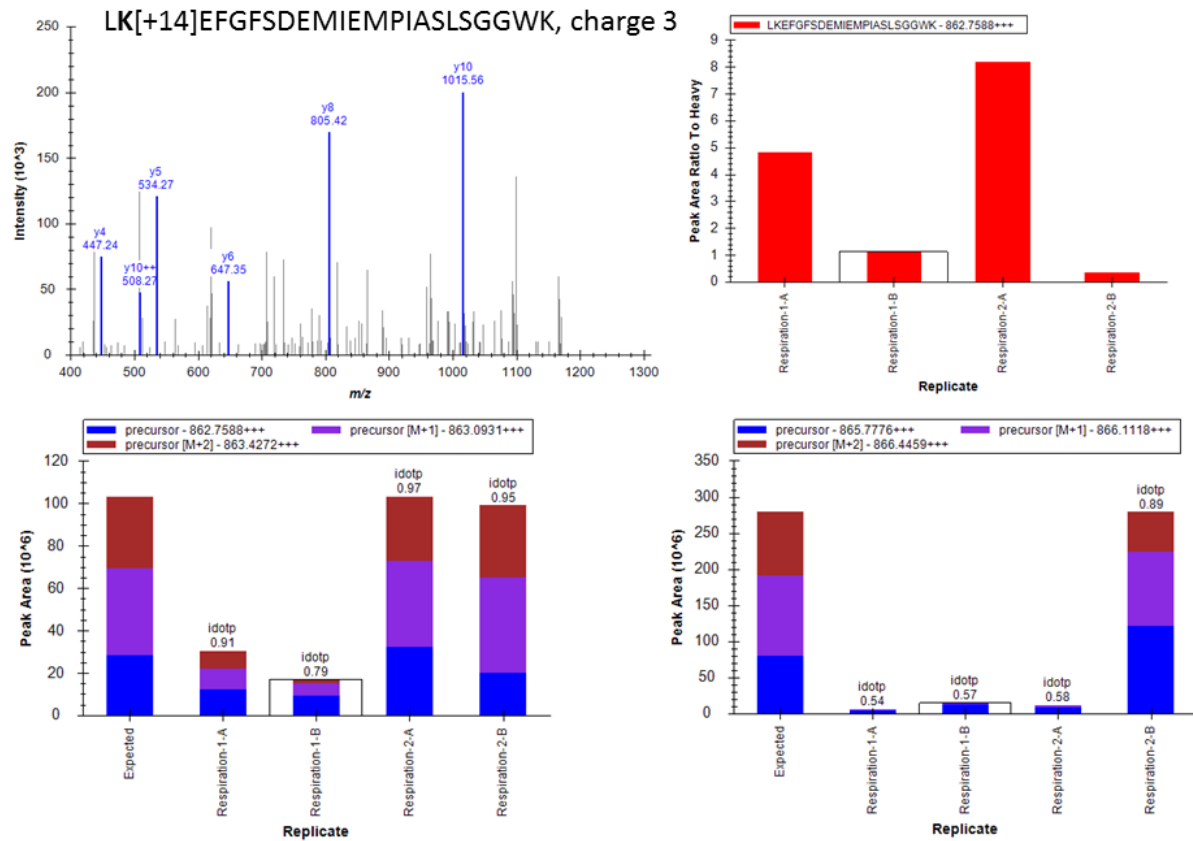


Respiration: YER152C Fragmentation and Heavy-Light Doublet Confirmation

EYDNDPYNR[+14]HPLTYGSDEGALWVR[+14]EQIC[+57]TFLNDQLFK, charge 4

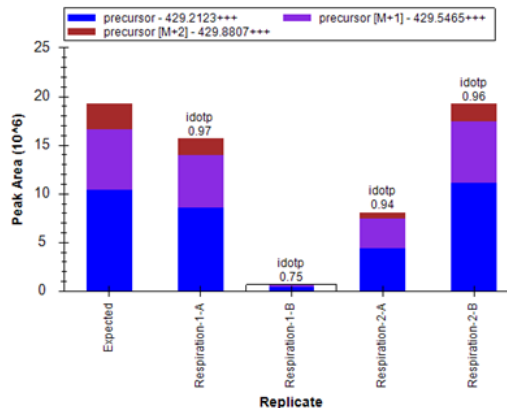
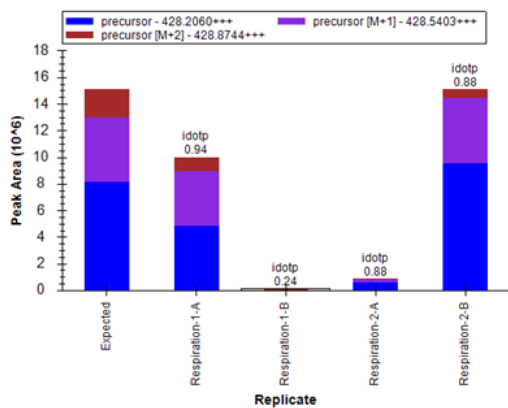
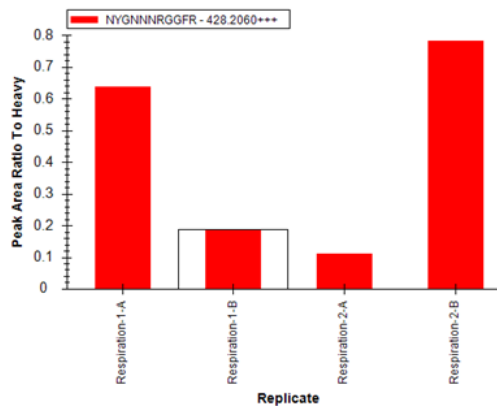
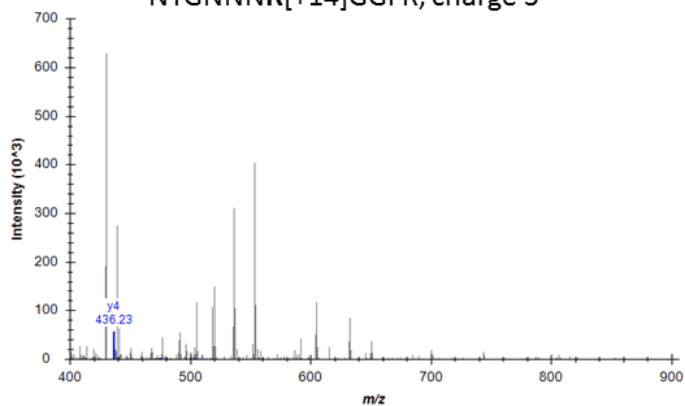


Respiration: Hef3 Fragmentation and Heavy-Light Doublet Confirmation



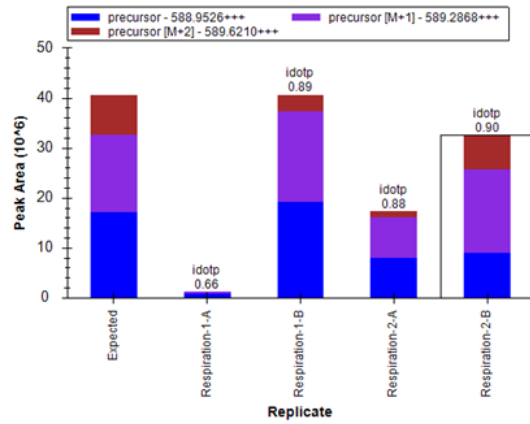
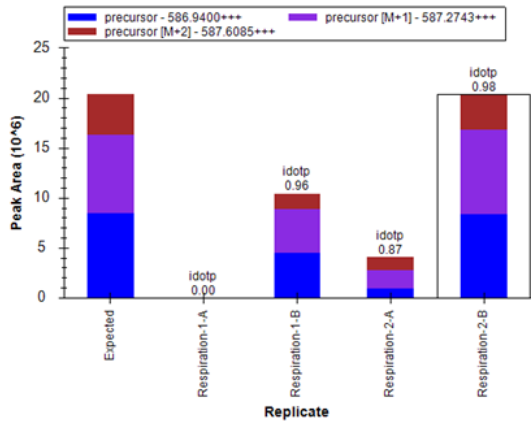
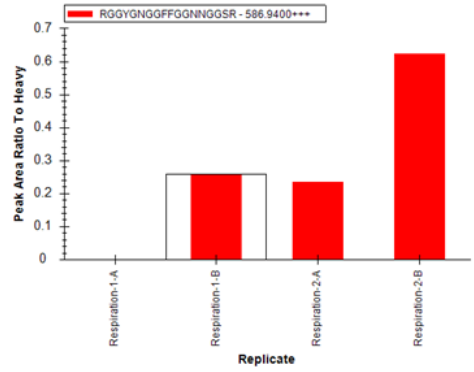
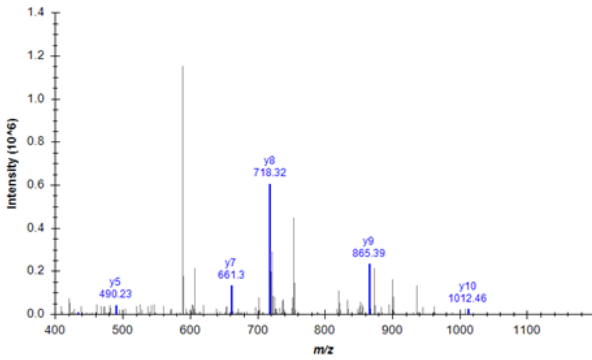
Respiration: Pub1 Fragmentation and Heavy-Light Doublet Confirmation

NYGNNNR[+14]GGFR, charge 3



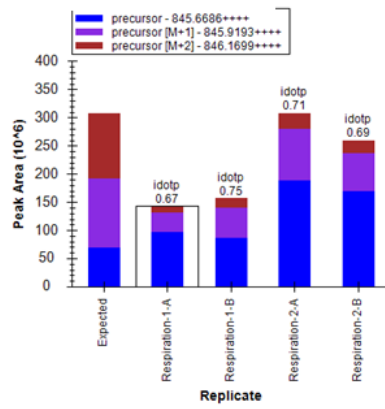
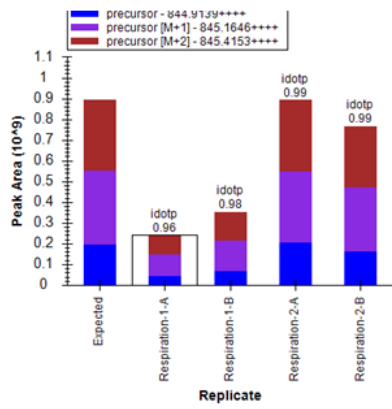
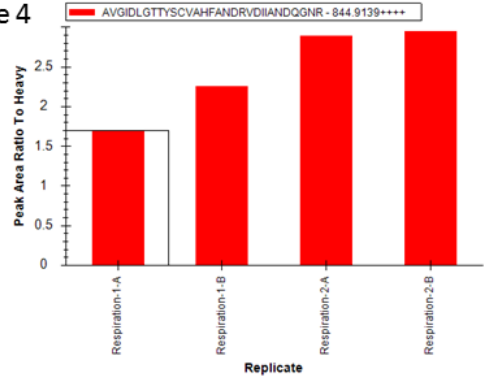
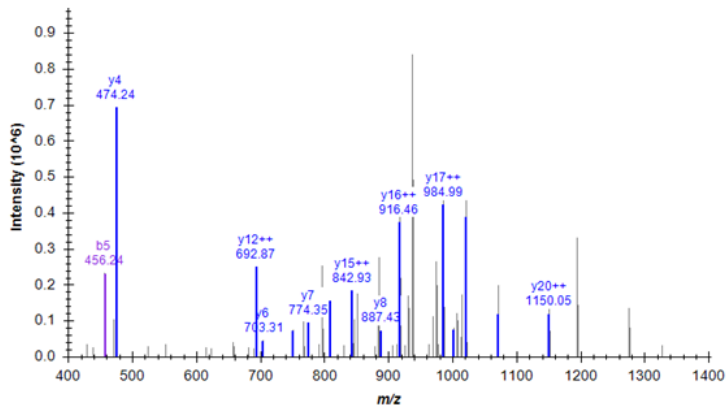
Respiration: Ded1 Fragmentation and Heavy-Light Doublet Confirmation

R[+28]GGYGNGGFFGGNNGGSR, charge 3



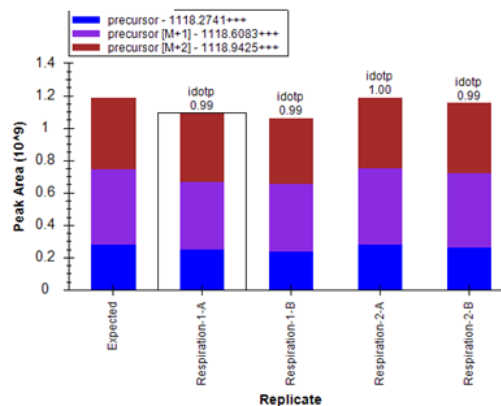
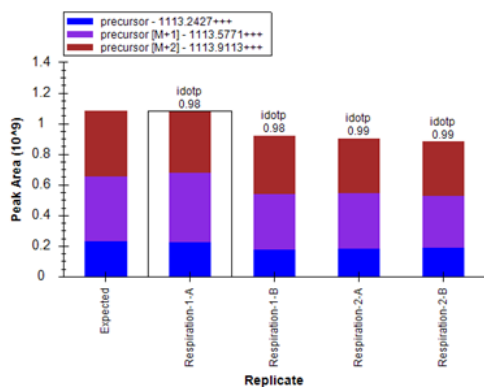
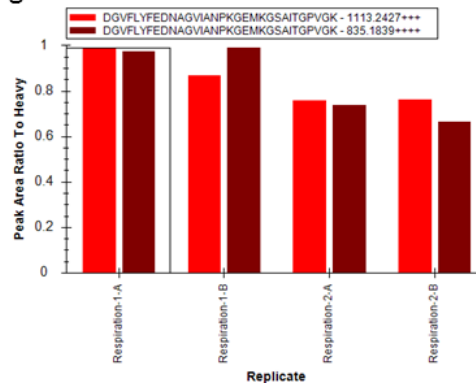
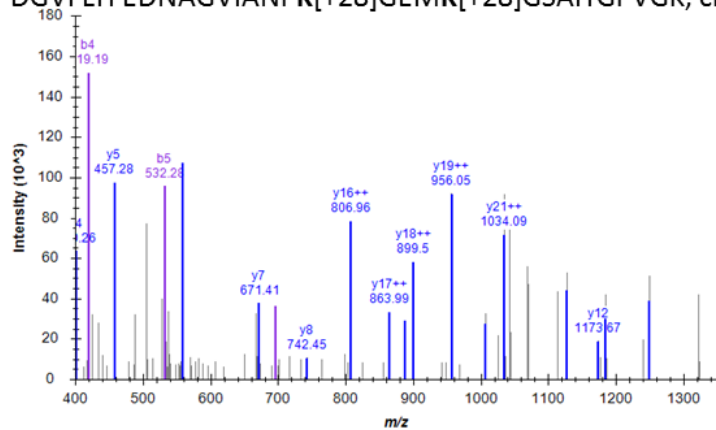
Respiration: Ssa1 Fragmentation and Heavy-Light Doublet Confirmation

AVGIDLGTTYSC[+57]VAHFANDR[+14]VDIANDQGNR, charge 4



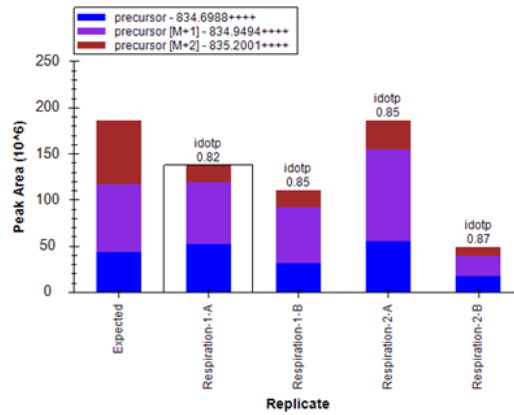
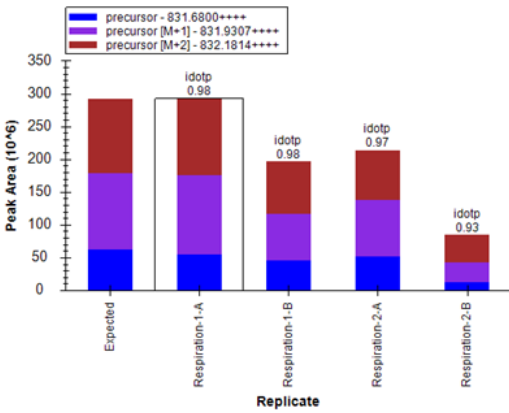
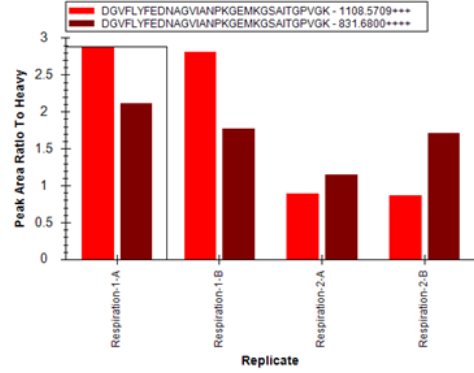
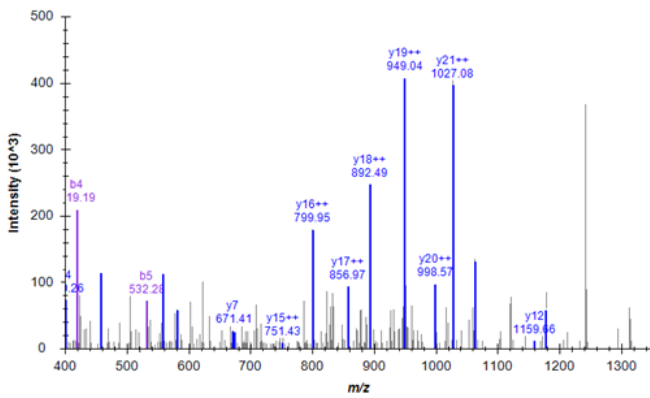
Respiration: Rpl23a Fragmentation and Heavy-Light Doublet Confirmation

DGVFLYFEDNAGVIANPK[+28]GEMK[+28]GSAITGPVVGK, charge 3

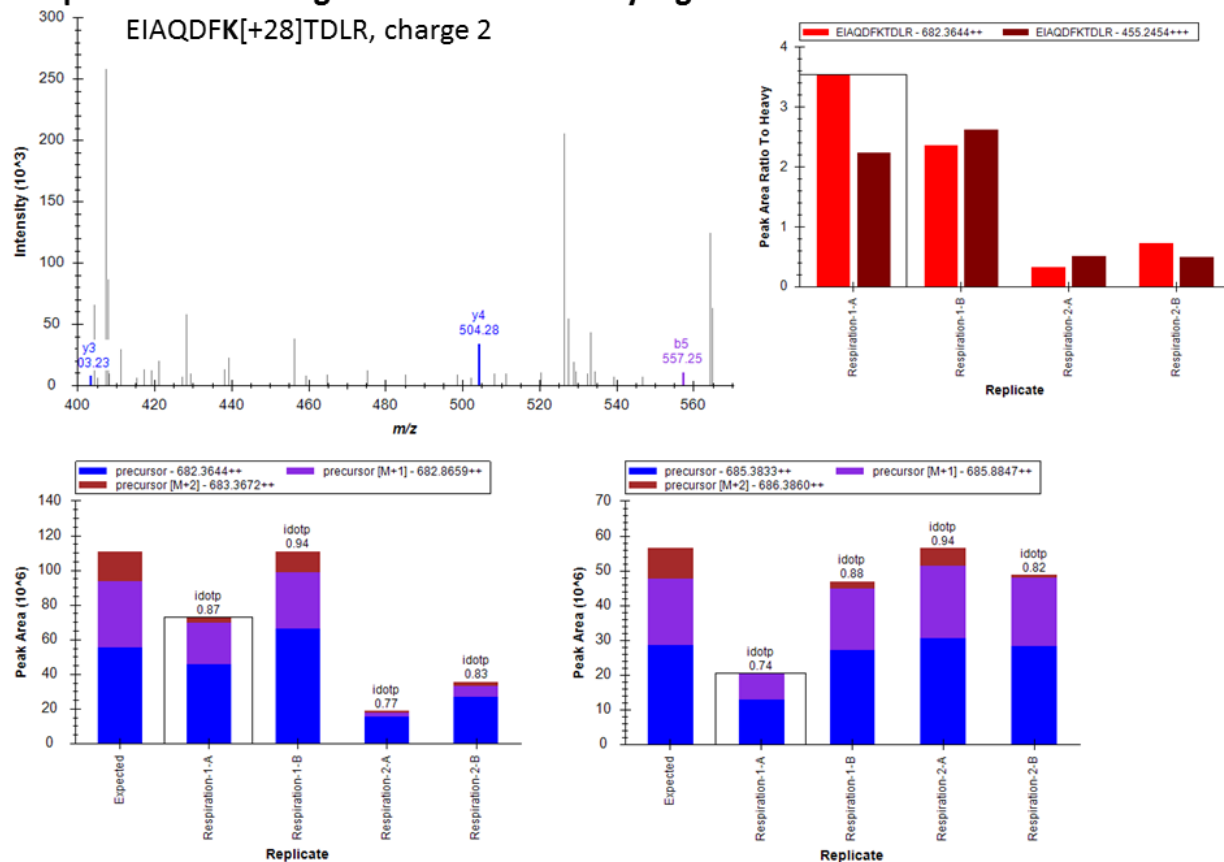


Respiration: Rpl23a Fragmentation and Heavy-Light Doublet Confirmation

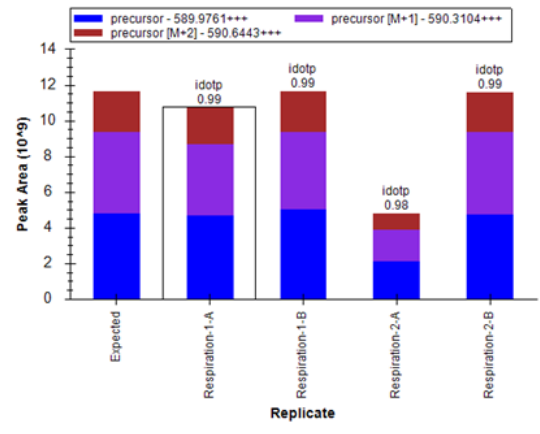
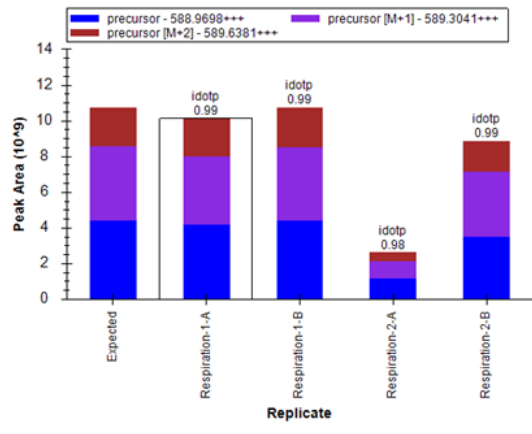
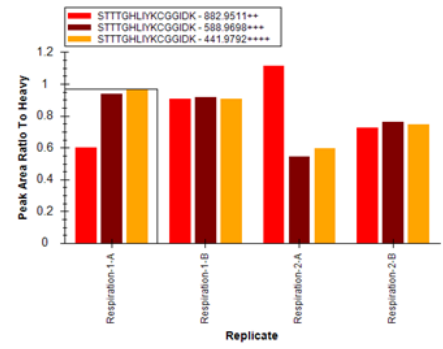
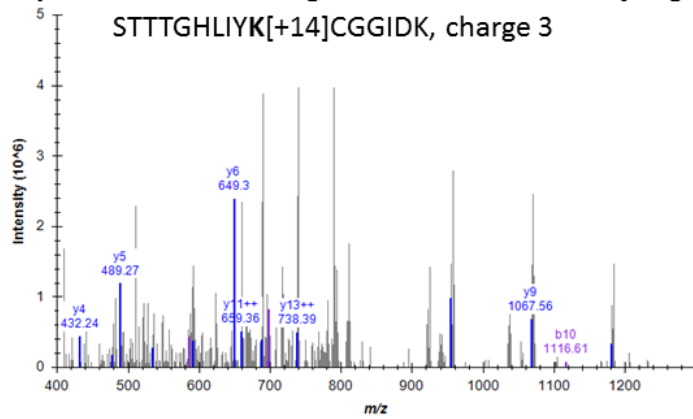
DGVFLYFEDNAGVIANPK[+28]GEMK[+14]GSAITGPVVGK, charge 4



Respiration: Hht1 Fragmentation and Heavy-Light Doublet Confirmation

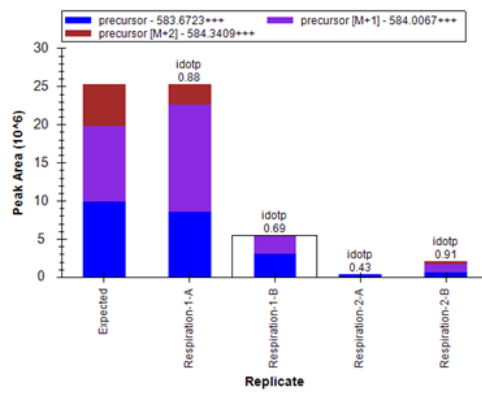
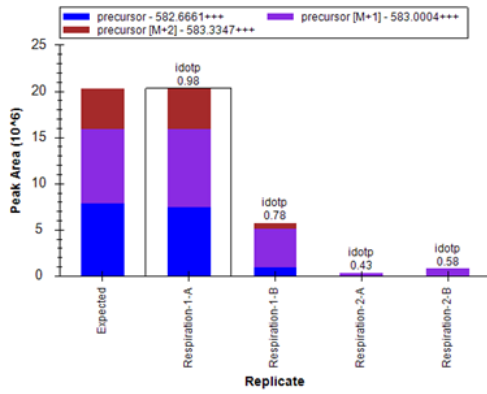
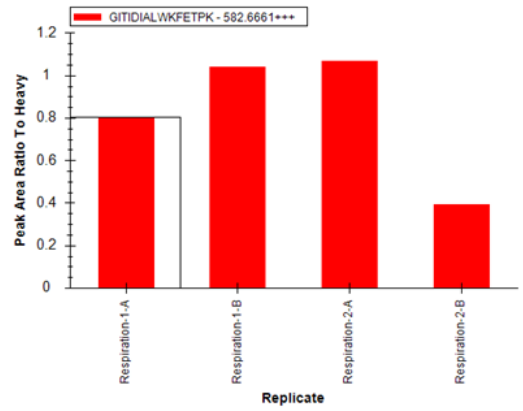
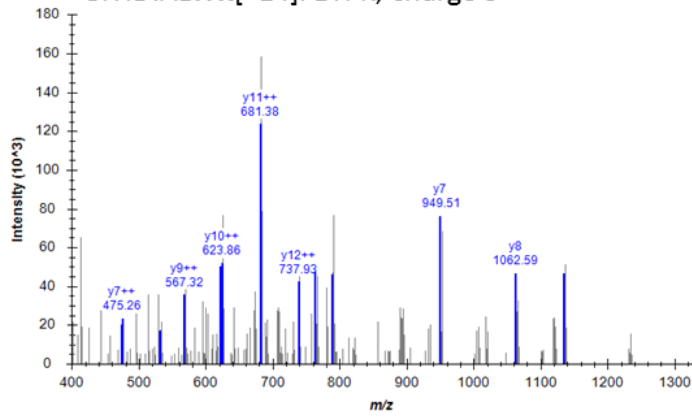


Respiration: eEF1A Fragmentation and Heavy-Light Doublet Confirmation

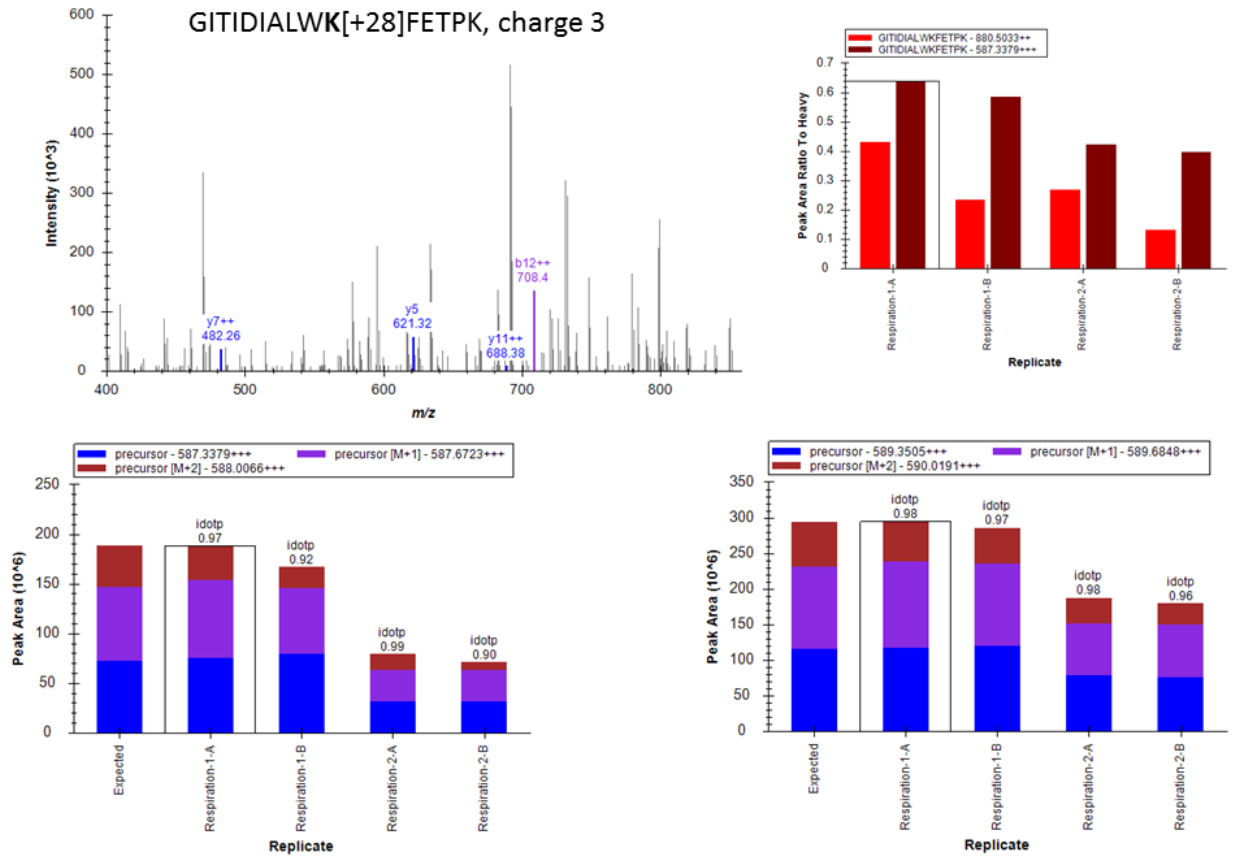


Respiration: eEF1A Fragmentation and Heavy-Light Doublet Confirmation

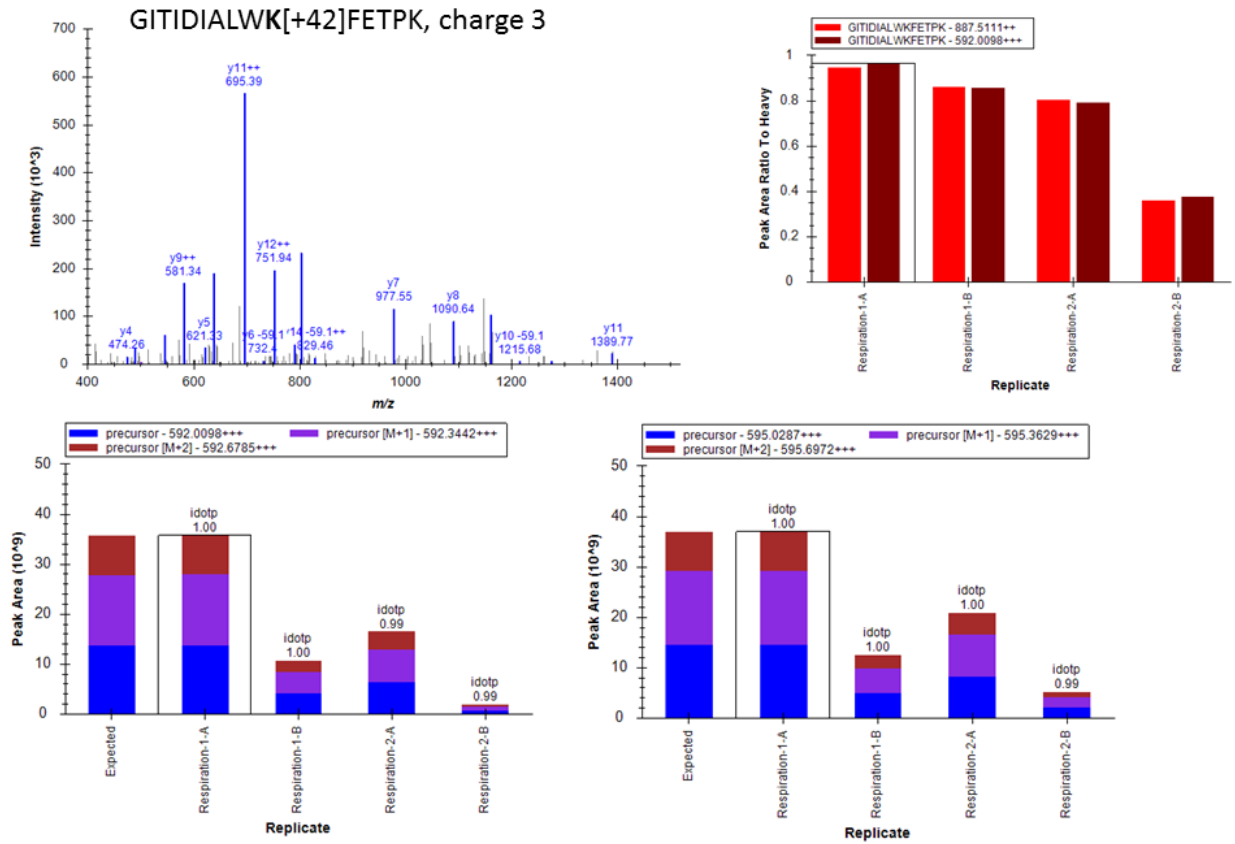
GITIDIALWK[+14]FETPK, charge 3



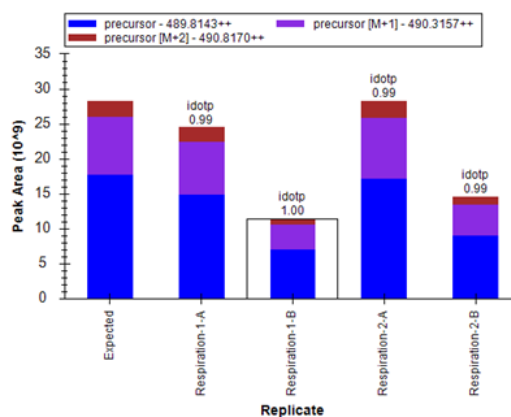
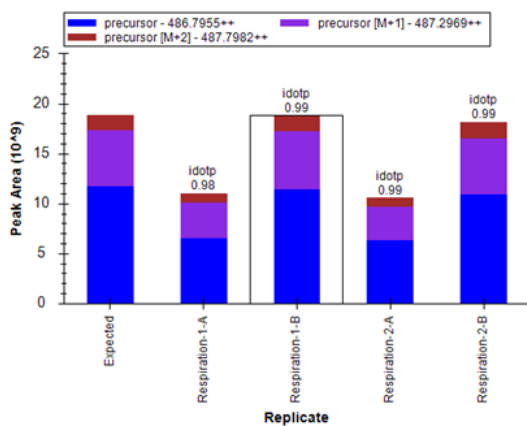
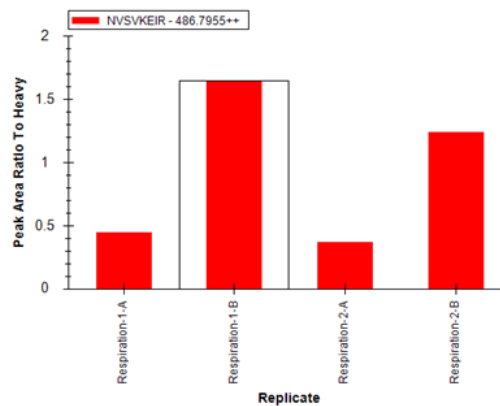
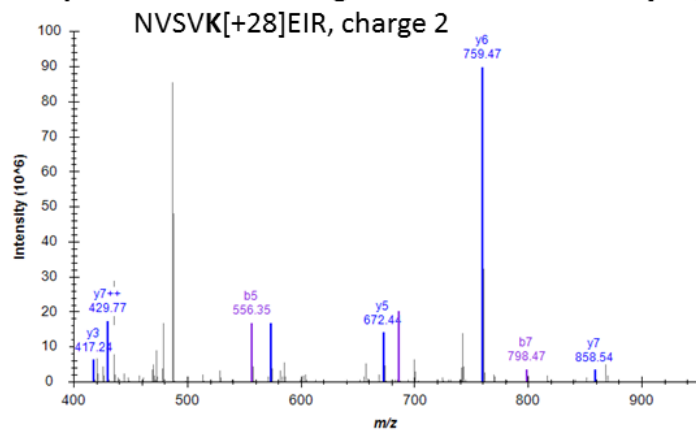
Respiration: eEF1A Fragmentation and Heavy-Light Doublet Confirmation



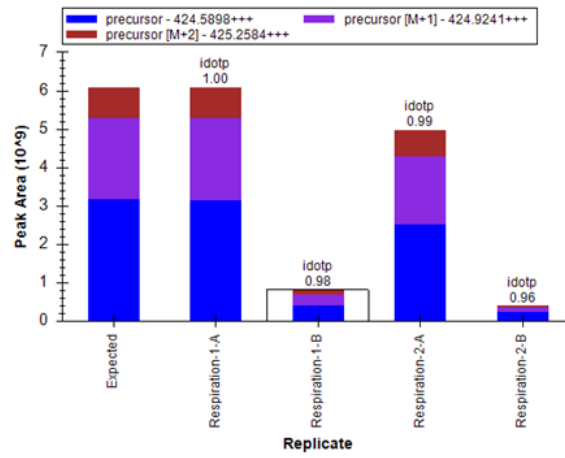
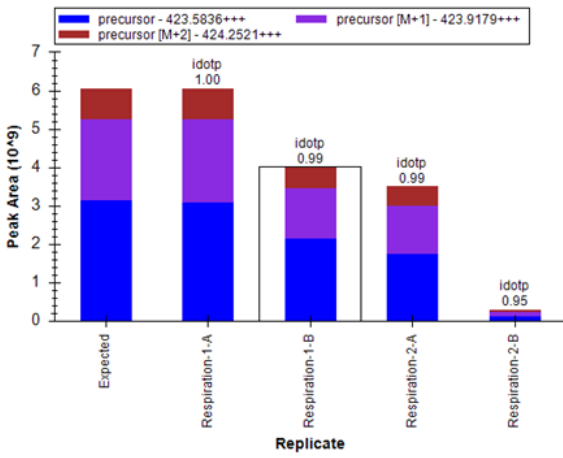
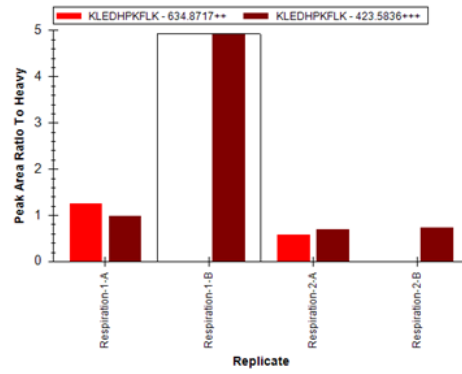
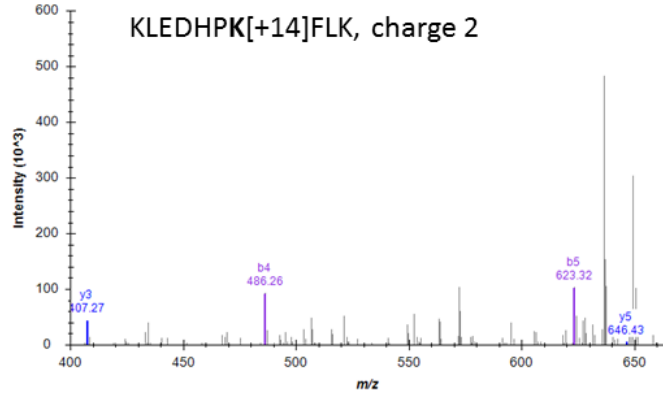
Respiration: eEF1A Fragmentation and Heavy-Light Doublet Confirmation



Respiration: eEF1A Fragmentation and Heavy-Light Doublet Confirmation

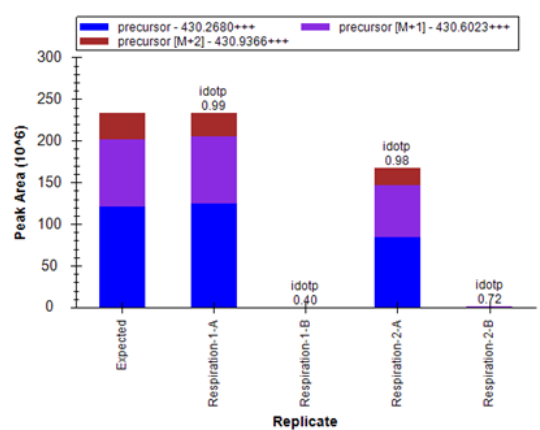
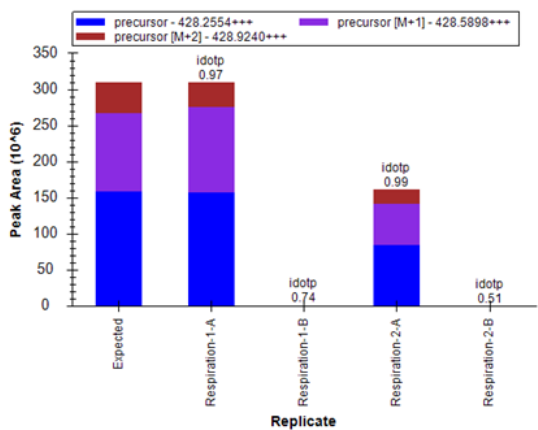
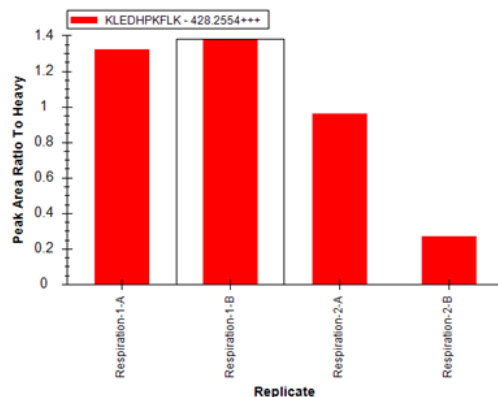
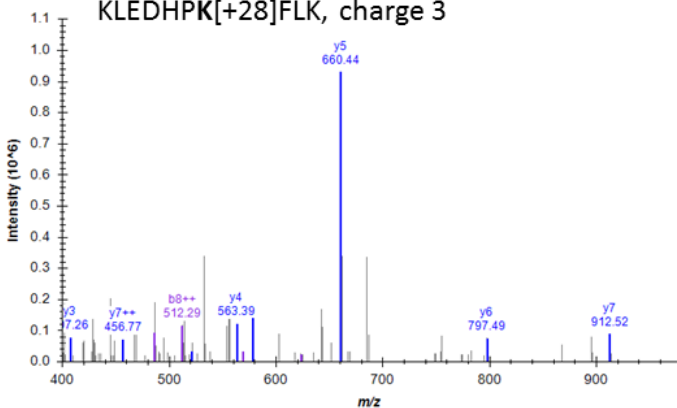


Respiration: eEF1A Fragmentation and Heavy-Light Doublet Confirmation



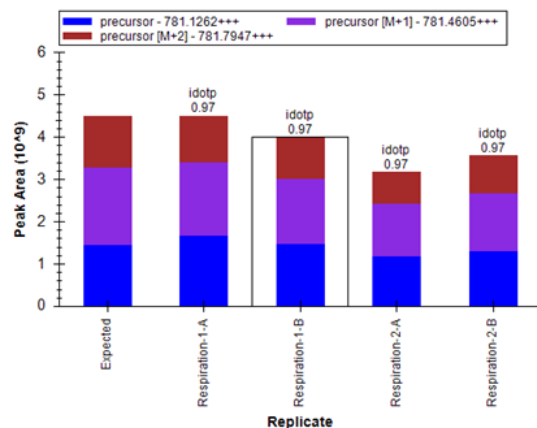
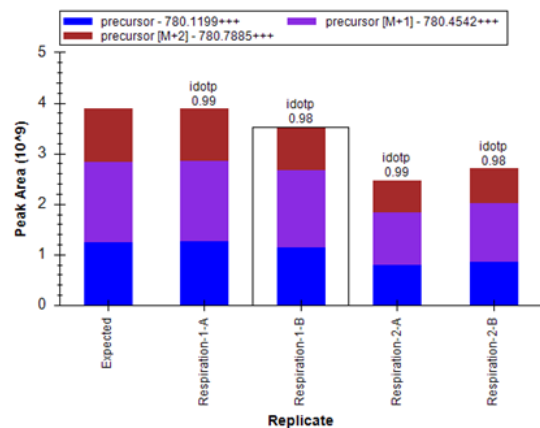
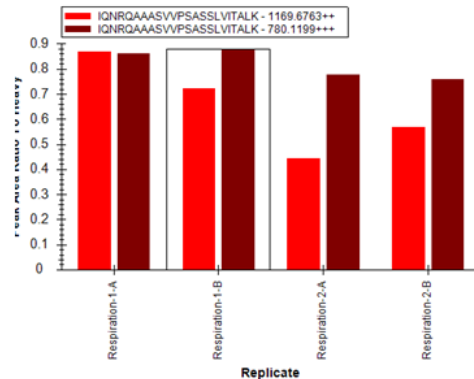
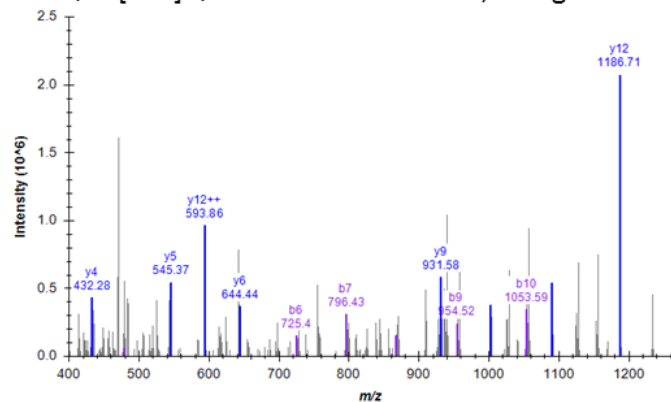
Respiration: eEF1A Fragmentation and Heavy-Light Doublet Confirmation

KLEDHPK[+28]FLK, charge 3

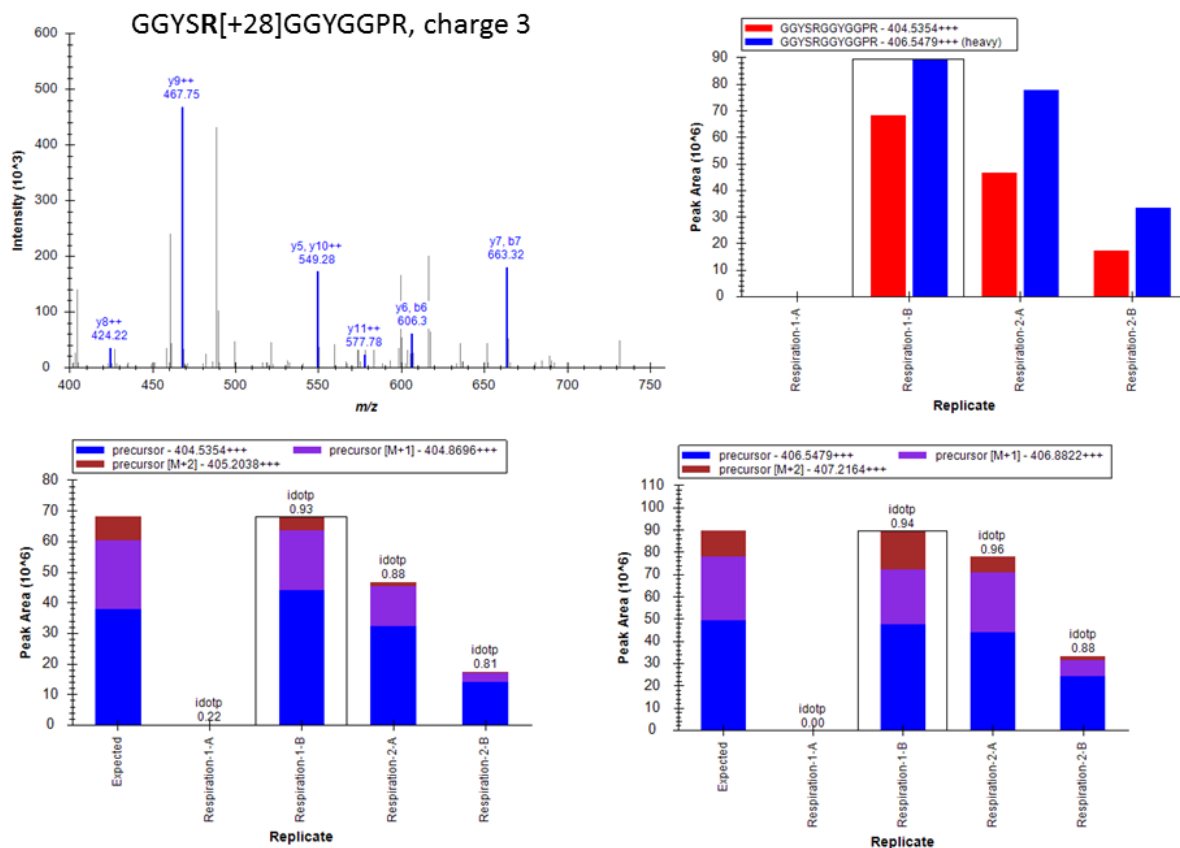


Respiration: Rpl12b Fragmentation and Heavy-Light Doublet Confirmation

IQNR[+14]QAAASVVPASSLVITALK, charge 3

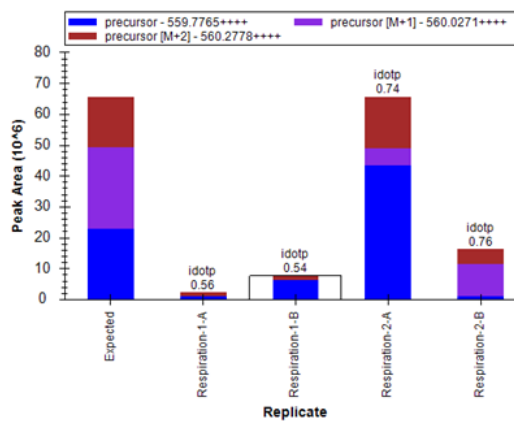
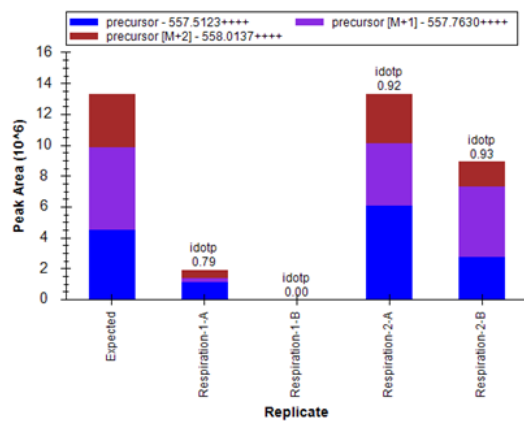
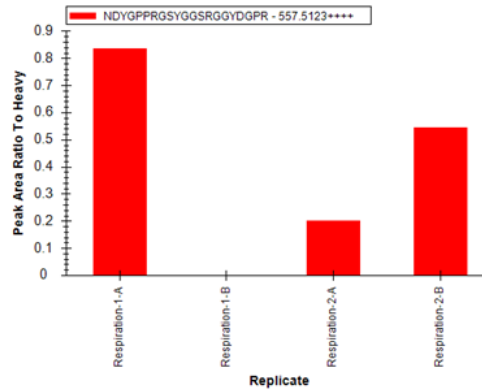
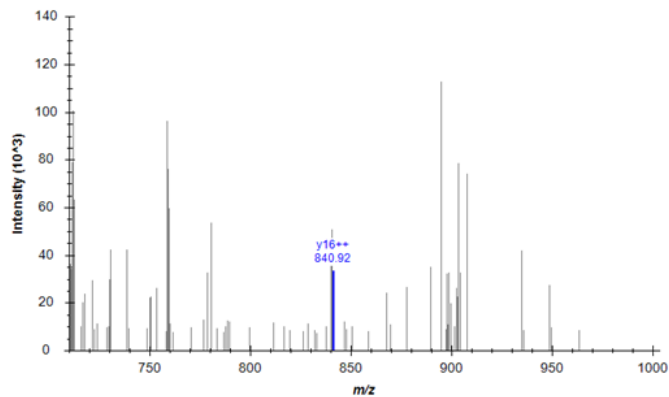


Respiration: Npl3 Fragmentation and Heavy-Light Doublet Confirmation



Respiration: Np13 Fragmentation and Heavy-Light Doublet Confirmation

NDYGPPR[+28]GSYGGSR[+14]GGYDGPR, charge 4



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