UNIVERSIDADE ESTADUAL DE CAMPINAS
Instituto de Biologia

Bradley Joseph Smith

PROTEIN SUCCINYLATION AND MALONYLATION IN SCHIZOPHRENIA

SUCCINILAÇÃO E MALONILAÇÃO DE PROTEÍNAS NA ESQUIZOFRENIA

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Dissertação apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestre em Biologia Funcional e Molecular na Área de Bioquímica.

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DEDICATION

I dedicate this work to my husband, who has supported me through every step of the way during my Masters degree. He has given me strength and focus after moving to a different country and has been with me during all the ups and downs, my normalness and frequent strangeness. Your steady presence has been both my driving purpose and my foundation and I couldn’t have done it without him.

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"Happiness can be found, even in the darkest of times, if one only remembers to turn on the light." — Harry Potter and the Prisoner of Azkaban, J.K. Rowling

RESUMO

A esquizofrenia é uma doença mental multifatorial que afeta até 1% da população mundial. Os pacientes são afetados negativamente pela presença de vários sintomas e não se sabe de uma cura para esta desordem. Vias associadas ao metabolismo energético estão desreguladas, e a desregulação metabólica é também um efeito colateral dos antipsicóticos, o tratamento principal para manejar os sintomas da esquizofrenia. Em 2011, duas modificações pós-traducionais de proteínas, a succinilação e malonilação de lisina, foram descobertas e devem existir em todos os domínios de vida. Os precursores dessas modificações – succinil-CoA e malonil-CoA – são parte de processos metabólicos centrais e a prevalência de ambas na célula pode variar por estímulos associados com condições metabólicas como hipóxia, que pode ser um gatilho ambiental para o desenvolvimento da esquizofrenia. Neste trabalho, a proteômica quantitativa em larga escala baseada em espectrometria de massas foi usada para determinar quais diferenças existem sobre várias condições. Tecido cerebral *post-mortem* de pacientes com esquizofrenia foram analisados em termos de malonilação e succinilação e comparados a tecido cerebral de pessoas mentalmente sadias. Também, culturas de precursores de oligodendrócitos humanos (linhagem MO3.13), tratadas com MK-801 e/ou um de 3 antipsicóticos foram analisadas. As diferenças descobertas aqui têm a capacidade para melhorar a compreensão da etiologia, a patofisiologia, os sintomas e o tratamento da esquizofrenia.

ABSTRACT

Schizophrenia is a multifactorial mental disorder that affects nearly 1% of the population worldwide. Patients are negatively affected in various ways; and there is no known cure for this disease. Pathways associated with energy metabolism are dysregulated, and metabolic disruption is also one of the side effects of antipsychotics, the principal way to manage the symptoms of schizophrenia. In 2011 two post-translational protein modifications, the succinylation and malonylation of lysine residues, were discovered to be widely present in likely all domains of life and furthermore have been observed on many proteins associated with glycolysis and metabolism. The precursors to these modifications, understood to be succinyl-CoA and malonyl-CoA, are also both a part of central metabolic processes, and their prevalence as a modification in cells can vary with metabolism-associated stimuli, such as hypoxia, a potential environmental trigger for developing schizophrenia. In this work, shotgun mass spectrometry-based quantitative proteomics was used to determine what differences in succinyllysine and malonyllysine profiles exist under various conditions. Postmortem brain tissue of schizophrenia patients was compared with tissue from mentally sound controls. Additionally, human oligodendrocyte precursor cell cultures (MO3.13 lineage) were treated with MK-801 and/or 3 antipsychotics and analyzed. The differences uncovered herein can potentially provide insight into the etiology, pathophysiology, symptoms, and treatment of schizophrenia.

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA – Analysis of Variance

BSA – Bovine Serum Albumin

CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

CID – Collision-Induced Dissociation

CoA – Cofactor A

DAVID – Database for Annotation, Visualization and Integrated Discovery

DIA – Data-Independent Acquisition

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl Sulfoxide

DTT - Dithiothreitol

ESI – Electrospray Ionization

FDR – False Discovery Rate

$HDMS^E$ – High-Definition MS^E

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IAA – Iodoacetamide

IMS – Ion Mobility Separation

MK-801 - Dizocilpine

MS – Mass Spectrometry

MS^E – MS/MS using alternating low-energy CID and high-energy CID

MS/MS – Tandem Mass Spectrometry

NMDA – *N*-Methyl-D-Aspartate

PCP – Phencyclidine

Q-TOF – Quadrupole Time-of-Flight

SCZ – Schizophrenia

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TCA – Tricarboxylic Acid

TEAB – Triethylammonium Bicarbonate

XIC – Extracted Ion Chromatogram

SUMMARY

INTRODUCTION

1.1 Schizophrenia

In the 21st century, many diseases and illnesses are now well-understood in terms of their causes and effects on a patient. A diabetic for example may have a dysfunction with insulin production and response; someone with anemia has a deficiency in iron intake or absorption; and a third patient with scarlet fever has an uncontrolled *Streptococcus* infection. With documented etiologies, disease prevention and treatment become simpler, safer, and more effective. However, in contrast, there are still some conditions that are much less understood.

Originally classified as a purely mental disorder, schizophrenia is a complex and multifactorial illness that even involves physical, observable changes in brain function and morphology (Karlsgodt et al., 2010; McDonald et al., 2005). There is thusly a physical cause, or a malfunction so to speak, in a human organ that allows for or induces the manifestation of this illness. However, despite decades of research, the scientific community is still in the dark about most of the fine details regarding schizophrenia. Current treatments are limited to symptom management; they are not a cure, and concrete knowledge about preventing the condition is limited.

Doctors diagnose the disorder based on the behaviors of a patient, not any physical or chemical changes in the body, and prescribe antipsychotics and/or psychosocial therapy to help them manage symptoms (Hasan et al., 2015). However, a clinical diagnosis such as this can easily be incorrect, mainly due to the complexity of – and overlap between – different mental disorders; constantly changing literature and misinformed doctors; and perhaps most importantly, the glaring lack of any definitive biological test with confirmed molecular biomarkers for this or many other mental disorders. Since a patient's symptoms do not always have an obvious biological cause, this can cause problems in diagnosis, treatment, and research of the disease. In fact, one study suggested that what is currently diagnosed as schizophrenia could actually be an umbrella term for eight separate biological dysregulations (Arnedo et al., 2014).

Despite all efforts, understanding a brain disorder is no menial task. The brain is one of the most complex systems known to man, a mesh of billions of individual units interconnected by trillions of connections, all with the goal of observing,

understanding, and reacting to the constantly changing environment. In this precariously balanced system, even a small change in a single protein could have widespread and potentially detrimental effects on the entire organism. Although the biochemistry of the symptoms of schizophrenia is slowly becoming more understood and therefore more treatable, the complexity of the disease hides its deeper roots, leaving researchers with a sprawling array of hypotheses as to its cause (Boison et al., 2012; Howes and Kapur, 2009; Hu et al., 2015; Owen et al., 2011; Selten et al., 2013; Timms et al., 2013; Watanabe et al., 2010).

Facing this frustrating lack of knowledge, a meta-study determined that schizophrenia affects between 0.3-0.7% of the population worldwide (Saha et al., 2005). Its onset is brought on by a culmination of biological and environmental factors (Tsuang et al., 2001), not all of which are known. These factors eventually lead to an individual experiencing an array of symptoms that negatively impact their ability to perceive and react to the world around them and this in turn causes deficits in social appropriateness and forms of hallucinations or psychosis (Andreasen, MD, PhD et al., 1995), among other symptoms. Even though schizophrenia affects nearly 1% of the worldwide population, there is still no comprehensive understanding of how it develops.

1.2 Causes of Schizophrenia

Over the decades that the illness has been documented, past and recent studies have not provided a definitive etiology of the disease; the current line of treatment nearly exclusively involves suppressing symptoms and assisting the patient to manage them (Hasan et al., 2015). Regardless, many risk factors for the disease have been documented and are grouped into two main categories: genetic and environmental.

Schizophrenia has a strong genetic influence, as proven by longitudinal studies in twins (Hilker et al., 2018). But this does not paint the whole picture, as many environmental factors can also change the risk factor for developing the disorder such as postnatal hypoxia, prenatal vitamin D deficiency, cannabis abuse as a teenager, a stressful childhood, certain viral infections, and diet (Davis et al., 2016).

Trying to link together these seemingly unconnected factors has proven to be a difficult task, and no current model for schizophrenia fully explains the disease and its symptoms. The most common hypotheses for the cause of schizophrenia –

although not an exhaustive list – are the dopamine hypothesis (Howes and Kapur, 2009), the glutamate hypothesis (Hu et al., 2015), myelination abnormalities (Mighdoll et al., 2015), and inhibitory neuron dysfunction with oxidative stress (Sullivan and O'Donnell, 2012). Many theories perform well when examining a specific part of the illness – for example treating schizophrenia using a medication that acts in line with the glutamate hypothesis relieves patients of the negative and cognitive symptoms; but does not remedy the positive symptoms (Tuominen et al., 2005).

One hypothesis has been developed that tries to link together these various observed changes, risk factors, and symptoms in their entirety called the neurodevelopmental theory. This theory posits that a cluster of risk factors during neurodevelopment set the stage for someone to develop schizophrenia later in life (Murray and Lewis, 1987) and has been since revisited many times (Chua and Murray, 1996, 1996; Fatemi and Folsom, 2009; Gupta and Kulhara, 2010; Owen et al., 2011). Regardless, this has not been entirely fleshed out and has its own unanswered questions, leaving a knowledge gap that not only makes preventing the disease more difficult, but also impedes a more effective treatment.

1.3 Treating Schizophrenia

As the etiology of schizophrenia is unknown, there is no developed cure. Instead, medications allow the patient to deal with their symptoms and lead a more fulfilling life. Treatment is primarily based on antipsychotics, which are not always effective in all patients (Lieberman et al., 2005). Antipsychotics fall into two overall categories: typical and atypical. Despite the beneficial reduction in positive symptoms, antipsychotic medication also has the potential to cause various debilitating side effects, especially with typical antipsychotics (Jones et al., 2006). The low efficacy and severe side effects lead to a high percentage of patients eventually abandoning treatment (Lieberman et al., 2005).

Typical antipsychotics are antagonists of the dopamine D_2 receptor (D_2R), and were first used in the 1950s (Shen, 1999). In patients with schizophrenia, they are administered to reduce positive symptoms by blocking dopamine's effects; however, not every patient will respond well to a particular antipsychotic, and less than half of patients are considered to be good responders to the first antipsychotic prescribed to them (Reynolds, 2012), requiring more time to find an optimal treatment, frustrating the

patient, and wasting resources. Complicating treatment further is the prevalence of tardive dyskinesia, a medication side effect that affects a patient's motor movements (Correll and Schenk, 2008).

Alternatives to typical antipsychotics have since been discovered, now called atypical antipsychotics. This class of medication binds to a different profile of receptors at different strengths, such as the serotonin 5-HT_{2A} receptor (Guenette et al., 2013); however how the differences in binding lead to different side effects and change therapeutic profiles is still undetermined (Sahlholm et al., 2014).

While atypical antipsychotics present a slightly lower risk for tardive dyskinesia – 3.9% compared to 5.5% (Correll and Schenk, 2008) – there is the chance of developing a blood condition called agranulocytosis, among other side effects. However in a study in Iceland on 611 schizophrenia patients, they found that a comparable number of patients treated with typical antipsychotics developed the condition (Ingimarsson et al., 2016). Additionally, atypical antipsychotics can also induce an array of side effects that are jointly called metabolic syndrome, but the incidence rate can vary between medications (Association, 2004). The main documented symptoms of metabolic syndrome are insulin sensitivity and weight gain (Riordan et al., 2011).

To better understand how to prevent and treat schizophrenia while minimizing the side effects of these treatments, various models have been developed and established, principally since the brain cannot be studied like saliva, blood, urine, or other less invasive samples.

1.4 Models for Schizophrenia

Studying schizophrenia at the cellular and molecular level cannot be achieved by simply taking the affected tissue for study especially since the brain is such a sensitive and enclosed organ. One of the main repercussions of this is the required use of postmortem brain tissue collected from donors or the development of other models altogether. In regards to postmortem tissue, studies based in genomics, transcriptomics, and proteomics have all been fruitful; but there are inherent issues with postmortem brain tissue such as confounding factors, macromolecule degradation, and the inability to manipulate variables experimentally (Harrison, 2011).

Nevertheless, when executed carefully and especially when integrated with other models, the results obtained from postmortem studies can – and have – provided important data in neuroscience, revealing proteins that are dysregulated and mitigated with antipsychotics (Chan et al., 2011), identifying targets for *in vitro* studies (Huang et al., 2008), and making progress towards biomolecular signatures of brain disorders (Martins-de-Souza et al., 2012). Due to the invariable nature of tissue, studies using tissue collected postmortem could be better suited to discovery-based projects for revealing potential targets for additional studies with controlled variables.

A more manipulatable model is cell culturing, a process that allows for the precise modification of specific variables and a comparison between conditions without nearly as many confounding factors. In schizophrenia, oligodendrocytes have been recurrently found to be associated with some of the dysregulations in and symptoms of schizophrenia (Hof et al., 2002; Martins-de-Souza, 2010; Takahashi et al., 2011; Tkachev et al., 2003; Uranova et al., 2004). This association is also in concordance with the myelin-associated protein dysfunction observed, and implicated, in schizophrenia (Karoutzou et al., 2008). Along these lines, oligodendrocytes hold promise to potentially uncover important information about the disease.

To develop oligodendrocytes in a laboratory setting, a human oligodendrocyte precursor cell line named MO3.13 has been shown to stay in an “arrested” immature development state (Buntinx et al., 2003). To simulate schizophrenia in these cells, they are treated with dizocilpine (hereupon referred to as MK-801), which has been established and used as a model via animal behavioral models. In rats, MK-801 induces negative symptoms (Rung et al., 2005), cognitive symptoms (Svoboda et al., 2015), brings about similar neurochemical changes to those that are seen in first-episode patients (Eyjolfsson et al., 2006), and potentially induce positive symptoms, although in a manner unlike what is seen in the PCP model (Rung et al., 2005; Seillier and Giuffrida, 2009). Translating this animal model to cells, a protocol was published that elaborates on the use of MK-801-treated cells as a model to study schizophrenia (Brandão-Teles et al., 2017).

Once a model has been established, it can be subjected to different conditions and studied using various tools such as genomics, transcriptomics, and proteomics. Due to the known existence of both genetic and environmental factors and

the dynamically responsive nature of the proteome, proteomics is an extremely useful tool to study illnesses such as schizophrenia.

1.5 Mass Spectrometry and Proteomics

In any living cell, proteins are found in high quantities, making up about 20% of a cell's total weight (Lodish et al., 2000). The proteins present and their levels of expression can provide a great deal of insight into a condition or disease since proteins are relatively quickly produced and degraded in response to internal and external stimuli. If these stimuli and responses can be manipulated, diseases can be better understood, treated, and even prevented. Studying which proteins are present – and to what degree – is the essence of proteomics, a term coined after genomics.

Originally, the methods to study protein expression involved stained 2-dimensional SDS-PAGE (Klose, 1975; O'Farrell, 1975) and Western blotting (Towbin et al., 1979). The turn of the century brought about an era of high-throughput mass spectrometry (Washburn et al., 2001), shortly thereafter ushering in quantitative mass spectrometry (Ong and Mann, 2005). As a result of constant developments, one method called shotgun proteomics has evolved, whose branching subcategories are able to indiscriminately identify thousands and quantify hundreds of proteins in a single, micro-scale sample (Bourassa et al., 2015). To identify proteins in a sample for experimental use, a mass spectrometer collects vast amounts of ion data, which specialized software uses to identify what proteins were present in the original sample.

1.6 Protein Identification

Different mass spectrometers work in different ways and have different strengths and weaknesses; but generally speaking, a sample is first prepared for analysis. In proteomics, this preparation consists of a reducing agent to break disulfide bridges in proteins and alkylation to cap off the cysteine residues. The resulting, less stable proteins are then digested with a protease to obtain smaller polypeptides which are more easily ionized during analysis (Resing and Ahn, 2005).

For more complex samples such as those used in shotgun proteomics, the samples are then fractionated to reduce complexity and increase the quantity and quality of the obtained data. This step is often done online with the mass spectrometer, which not only facilitates data collection, but automation reduces opportunities for experimental error. The peptides in the sample are then ionized by one of many

methods and is injected into a mass spectrometer. At this point especially, what happens next can vary greatly, depending on the configuration of the mass spectrometer. In the case of this study, the mass spectrometer used was a Waters Synapt G2-Si (see Figure 1).

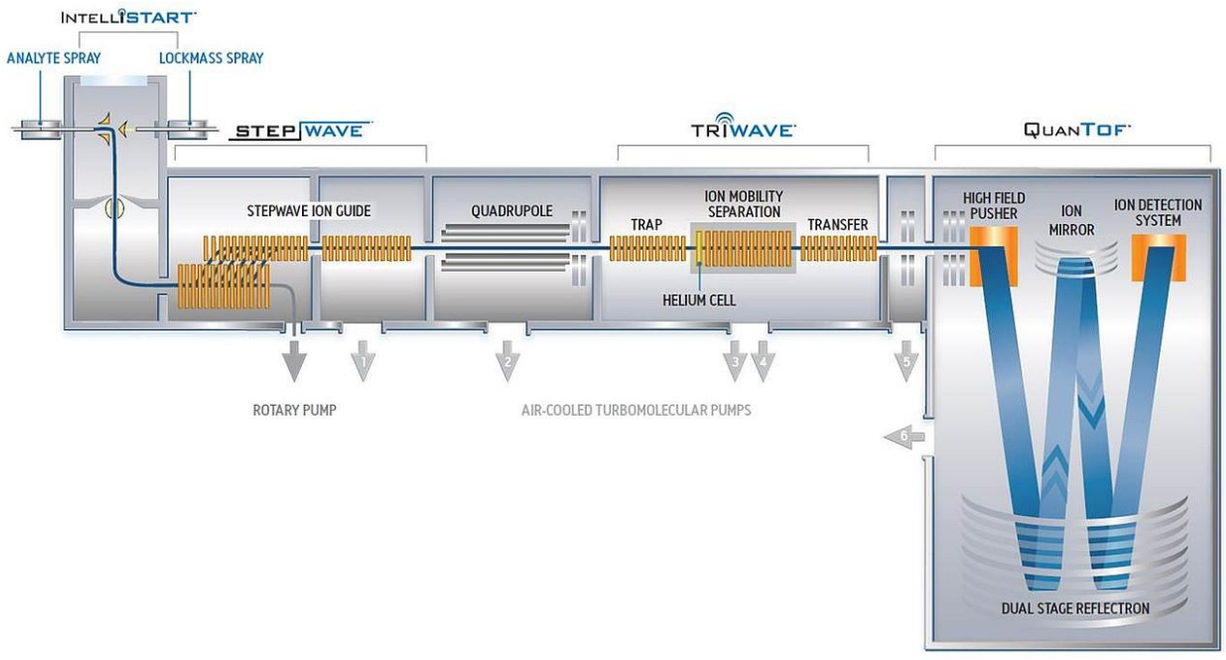

Figure 1 - Schematic of the Waters Synapt G2-Si (Waters, Co.)

In this study, peptides were ionized via nanoelectrospray injection in positive ion mode (nanoESI(+)) and passed through an ion guide, called a step wave, removing unionized particles. The resulting ion stream was passed through a quadrupole (Q), a collision chamber, and then a time of flight (TOF) analyzer. This setup is called a Q-TOF mass spectrometer. The machine was run in DIA (data-independent acquisition) mode, which allows for a high level of identification and quantitation without individual pre-programming for each sample (Hu et al., 2016).

The overall method used here was HDMS^E (high-definition MS/MS with alternating high- and low-energy collision), which first passes a group of peptide ions through the quadrupole and TOF analyzer (Bond et al., 2013) to record the mass of each peptide. An MS graph is created and once again, the stream of peptide ions is allowed to pass through the quadrupole; however, the peptides are fragmented via collision-induced dissociation (CID) in the collision chamber, which breaks apart the peptide ions by rapidly moving them ions through gas particles. The resulting peptide

fragments are analyzed by the TOF, obtaining an MS/MS spectrum. This alternation process is done rapidly and repeated until the entire sample has been run.

To assist in the resolution of the data, another molecular property was used: ion mobility separation (IMS) and related drift time. In this case, ions flowed against a chamber filled with helium and with an applied electric field, changing their eventual time of arrival at the analyzer in a constant manner based on their cross-sectional area, allowing for more precise ion separation and therefore identification (McLean et al., 2005).

The MS and MS/MS spectra were then imported into a specialized computer program that deconvoluted the MS/MS data, associating precursor ions with their fragment ions based on their chromatographic retention time and peak shape. In doing so, the software is able to piece together the fragments to not only identify which amino acids are in the peptide, but also in what likely order. Those peptide sequences are then cross-checked with a database of known proteins and their peptide ionization data to identify which proteins were present in the original sample. This form of ion-to-protein identification is a method called bottom-up proteomics. A scoring system is utilized by the software used to show how confident the identification is; and a false discovery rate (FDR) using a reversed dataset helps reduce the number of false positives. This system is the same way that peptides with post-translational modifications are identified. PTMs are not identified by adding the adduct mass to an identified peptide; rather the identification software treats it as a different and unique peptide. Therefore, the unmodified peptide does not need to be identified to register the modified version.

In addition to identification, if a sample is prepared and analyzed properly, software can be used to also quantify how much of the identified protein was present in the original sample, either relatively or absolutely.

1.7 Protein Quantitation

Once a peptide is (or multiple are) identified, data can be further processed to estimate (with varying degrees of accuracy) the amount of a protein present in the original sample. This can be done by various methods requiring different levels of preparation and analysis; but the software used in this study uses the Top N method

to relatively quantify peptides in different conditions and subsequently compare their parent proteins (Silva et al., 2006).

First, the chromatograms are aligned between samples to facilitate and improve comparisons. Variations in sample contents, temperature, and column age can slightly skew the observed elution times (Wandy et al., 2015). Once spectra are aligned, the software can then associate a peptide's identity with its LC elution profile. In TopN, a preset number of peptides – in this case 3 – is summed, where the area under their elution curves – or extracted ion chromatograms (XICs) – is compared with the value of another sample.

This provides a comparative – however not absolute – quantitation of the original protein. While this method does not provide the highest accuracy or precision of all methods available, it allows for the quantitation of up to thousands of proteins in a single sample without any type of labeling or manual peptide selection and still provides high-quality data (Gerber et al., 2003; Higgs et al., 2005; Koulman et al., 2009; Wang et al., 2008). In addition to identifying and quantifying the proteins in a sample, post-translational modifications such as phosphorylation can also be added to search parameters to identify and quantify modified proteins in a sample.

1.8 Post-Translational Modifications

Cells use many mechanisms to regulate the production, activity, binding, and degradation of proteins. One such mechanism that is found ubiquitously in life is the post-translational modification of proteins. Amino acid residues can be modified with various types of molecules, including small organic molecules, polysaccharides, other proteins, and fatty acids, as well as other forms of modification like cysteine disulfide bridges. New discoveries are constantly being made about additional modifications, such as the widespread addition of the acyl groups succinate and malonate to lysine residues (Peng et al., 2011; Zhang et al., 2011) (see Figure 2).

These two chemical groups are found naturally in cells in regards to metabolic processes (succinate in the TCA cycle and malonate in fatty acid synthesis) and their occurrence as a post-translational, protein modification has been observed in various prokaryotic and eukaryotic cells (Peng et al., 2011; Qian et al., 2016; Weinert et al., 2013; Zhang et al., 2017).

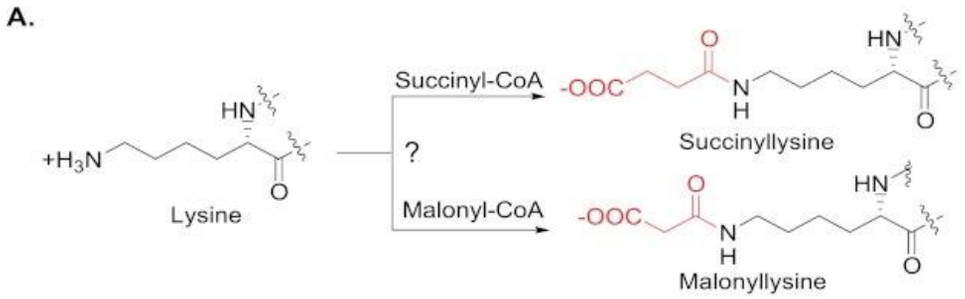

Figure 2-Addition of succinyl and malonyl groups. Adapted from (Xie et al., 2012)

Sites of modification have been discovered to be highly prevalent on metabolic proteins and other mitochondrial proteins, although the effects of these modifications are still mainly unknown (Bowman et al., 2017; Chen et al., 2017, 2017; Hirschey and Zhao, 2015; Qian et al., 2016; Rardin et al., 2013). In line with this data, there is substantial proof that metabolic dysregulation and oxidative stress occur in schizophrenia (Amorim et al., 2017; Khaitovich et al., 2008; Leonard et al., 2012; Marcelis et al., 2004; Martins-de-Souza et al., 2010; Nascimento and Martins-de-Souza, 2015). As such, there is great potential in researching a correlation between schizophrenia and these recently discovered post-translational modifications to perhaps uncover a new medicinal target or new research foci.

1.9 Succinylation

Succinylation was first found to be a protein modification less than a decade ago, and was confirmed to be a widespread PTM after a study with E. coli, S. cerevisiae, HeLa, and mouse liver cells (Weinert et al., 2013; Zhang et al., 2011). Its mechanism of addition is thought to be similar to acetylation via a succinyl-CoA moiety being added to a lysine residue via an acyltransferase protein; however, the actual mechanism of addition is still unconfirmed (Alleyn et al., 2017). Nevertheless, succinylation mechanisms are expected to be conserved between eukaryotes and prokaryotes (Weinert et al., 2013; Xie et al., 2012). Sites are unlikely to be random or unregulated, as software has been developed that helps predict which lysine residues are sites of succinylation (Dehzangi et al., 2018; Ning et al., 2018). The removal of a succinyl group is known to performed primarily by a sirtuin protein SIRT5 (Rardin et al., 2013).

Succinyl-CoA is an intermediate in the TCA cycle (see Figure 3), strengthening its link to metabolism and energy production. When investigating the sites of succinylation, one study found that a large number of histone proteins as well

as mitochondrial and metabolic proteins are modified with succinyl groups, discovering 2,572 sites on 990 proteins (Weinert et al., 2013) (see Figure 4). In addition, the protein succinylation profile has been shown to be extremely sensitive to certain external metabolic changes, even after stimuli as short as 20 minutes (Chen et al., 2017). These associations have opened new doors to study diseases with dysregulated metabolic processes (Alleyn et al., 2017) like schizophrenia, as well as Alzheimer's disease and aging, among others.

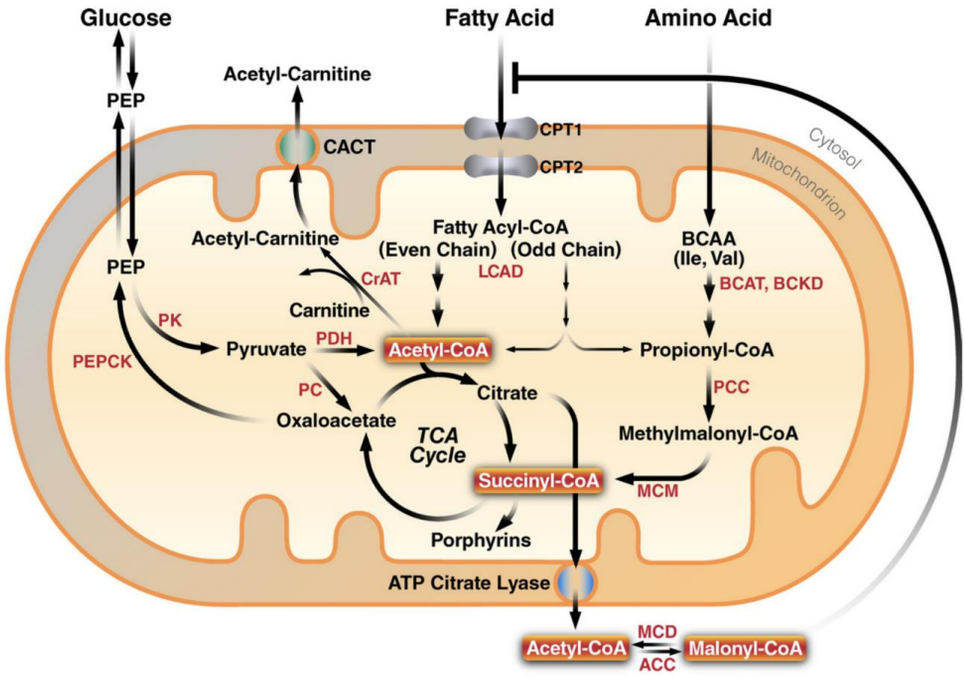

Figure 3-Position of succinyl-CoA and Malonyl-CoA in metabolism. From (Newman et al., 2012)

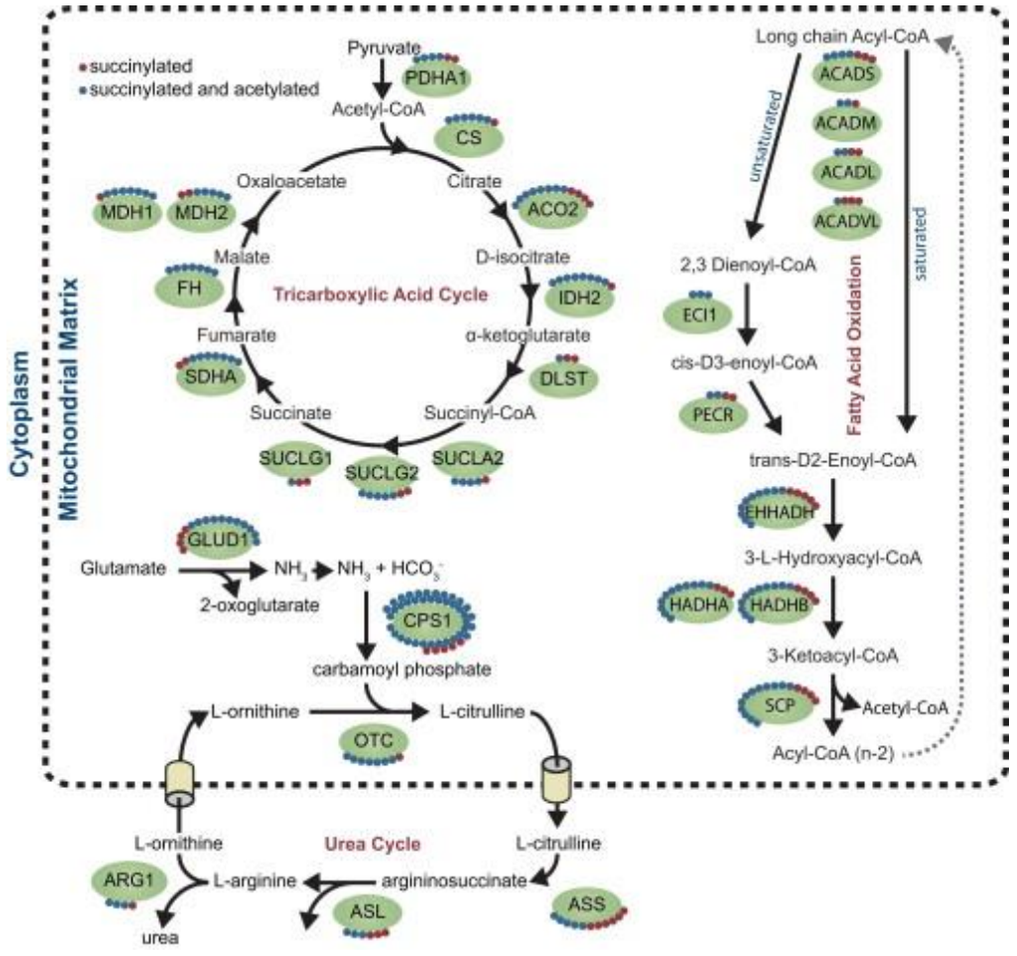

Figure 4-Sites of succinylation on metabolic proteins (blue dots are shared sites with acetylation). From (Weinert et al., 2013)

1.10 Malonylation

Similar to succinylation, malonylation is understood to be performed via a acyltransferase from malonyl-CoA, which was confirmed to be the PTM source after a knockout study of ACSF3 (Acyl-CoA Synthetase Family Member 3), an enzyme that converts malonate to malonyl-CoA (Bowman et al., 2017). Finer details about its mechanism of addition are not well understood; but, its removal is also thought to be regulated by the sirtuin SIRT5 (Nishida et al., 2015).

Since malonyl-CoA is an intermediate in fatty acid synthesis (see Figure 3), it is thought to also have strong ties with metabolism and energy regulation, a fact that has been confirmed in both analytical and knockout studies (Bowman et al., 2017; Nishida et al., 2015). One study linked weight gain from antipsychotics with perturbations in this branch of the metabolism, and specifically mentioned malonyl-CoA (Gonçalves et al., 2014). Like with succinylation, due to software being able to

predict sites of malonylation (Taherzadeh et al., 2018) this suggests a specificity and regulation for addition and removal.

In this work, the comparative locations and prevalence of these two post-translational modifications are studied in relation to schizophrenia using post-mortem brain tissue and MO3.13 human oligodendrocyte precursor cells treated with MK-801 and/or haloperidol, chlorpromazine, and quetiapine.

OBJECTIVES

1) What sites of protein malonylation and succinylation exist in cerebral tissue of patients with schizophrenia that do not exist in mentally sound controls and vice versa?

    Additionally, are there any differences in the prevalence of these modifications?

2) How does the schizophrenia-mimetic drug MK-801 affect the succinylation and malonylation profiles of oligodendrocyte precursor cells?

    Do any disturbances align with symptoms or the development of schizophrenia?

3) How do the antipsychotics haloperidol, chlorpromazine, and quetiapine affect the succinylation and malonylation profiles of oligodendrocyte precursor cells?

    Do any disturbances align with the side effects of these drugs?

4) Are any disturbances caused by MK-801 attenuated by the presence of antipsychotics?

    What pathways are affected and is there any relation to its ability to treat certain symptoms of schizophrenia and not others?

JUSTIFICATION

First, discovering new sites of modification will help the currently growing databases for these modifications, providing valuable data about conditional modifications. Comparing profiles in postmortem tissue could reveal dysregulations in metabolic pathways that are not observable through genomics, transcriptomics, or unfocused quantitative proteomics. These pathways could become the focus for further studies of the development, progression, or treatment of the condition.

Second, understanding the profile changes induced by MK-801 and antipsychotics could provide insight into their mechanisms of action in relation to schizophrenia. Moreover, this could uncover new lines of research to develop new medications and reduce the detrimental and potentially dangerous side effects of current treatments.

Third, understanding which pathways, if any, that antipsychotics balance when cells are disturbed with MK-801 could provide valuable understanding about how these medications function, and direct new studies to determine how to manage the cognitive and other symptoms of schizophrenia that are left unchecked by antipsychotic-based treatment.

CHAPTER 1

A Guide to Mass Spectrometry-Based Quantitative Proteomics

Bradley J. Smith, Daniel Martins-de-Souza, and Mariana Fioramonte

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Abstract:

Proteomics has become an attractive science in the post-genomics era, given its capacity to identify up to thousands of molecules in a single, complex sample and quantify them in an absolute and/or relative manner. The use of these techniques enables understanding of cellular and molecular mechanisms of diseases and other biological conditions, as well as identification and screening of protein biomarkers. Here we provide a straightforward, up-to-date compilation and comparison of the main quantitation techniques used in comparative proteomics such as *in vitro* and *in vivo* stable isotope labeling and label-free techniques. Additionally, this chapter includes common methods for data acquisition in proteomics and some appropriate methods for data processing. This compilation can serve as a reference for scientists who are new to, or already familiar with, quantitative proteomics.

Chapter 1

A Guide to Mass Spectrometry-Based Quantitative Proteomics

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Abstract

Proteomics has become an attractive science in the postgenomic era, given its capacity to identify up to thousands of molecules in a single, complex sample and quantify them in an absolute and/or relative manner. The use of these techniques enables understanding of cellular and molecular mechanisms of diseases and other biological conditions, as well as identification and screening of protein biomarkers. Here we provide a straightforward, up-to-date compilation and comparison of the main quantitation techniques used in comparative proteomics such as in vitro and in vivo stable isotope labeling and label-free techniques. Additionally, this chapter includes common methods for data acquisition in proteomics and some appropriate methods for data processing. This compilation can serve as a reference for scientists who are new to, or already familiar with, quantitative proteomics.

Key words Quantitative proteomics, Label-free, Mass spectrometry, Stable isotope labeling

Abbreviations

AIF	All-ion fragmentation
AQUA	Absolute quantification
CAD	Collision-activated dissociation
CE	Collision energy
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
dNSAF	Distributed normalized spectral abundance factor
emPAI	Exponentially modified protein abundance index
FT-ARM	Fourier transform-all reaction monitoring
$HDMS^E$	High-definition MS^E
iBAQ	Intensity-based absolute quantification
ICPL	Isotope-coded protein label
IMS	Ion mobility separation
LRP	Labeled reference peptide
MRM	Multiple reaction monitoring
MS^E	DIA method from Waters Co.
MSX	Multiplexed MS/MS

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mTRAQ	Mass-differential tags for relative and absolute quantitation
NSAF	Normalized spectral abundance factor
PSAQ	Protein standard absolute quantification
pSILAC	Pulsed stable isotope labeling of amino acids in cell culture
QconCAT	Quantitative concatemers
QQQ	Triple quadrupole
SID	Standard isotope dilution
SILAM	Stable isotope labeling of amino acids in mammals
SILIP	Stable isotope labeling in planta
SIN	Normalized spectral index
SPS-MS3	Synchronous precursor selection MS/MS/MS
TMT	Tandem mass tags
$UDMS^E$	Ultra-definition MS^E
XDIA	Extended data-independent acquisition
XIC	Extracted ion chromatogram

1 Introduction

Generally, in large-scale proteomics experiments, the identification of proteins in a sample is just the first step. Protein quantitation is an important, additional part of many protocols and mass spectrometry combined with liquid chromatography (LC-MS) has found its way into becoming a crucial tool in both biological and clinical research settings [1–3]. Protein levels are often compared across different cell conditions, types, compartments or over time. While several well-known techniques have excelled in the quantitation of mixed protein samples as a whole for decades, measuring the levels of individual proteins in a complex mixture has proven to be a more difficult task.

A major challenge of quantitation is due to the varying rates of peptide ionization in a mass spectrometer; the quantity of a molecule in a sample is not universally related to the intensity of the ions measured by the mass spectrometer. The efficiency of a peptide's ionization depends on many characteristics including but is not limited to peptide size, basicity and hydrophobicity [4]. The composition of the solvent and other peptides present can additionally confound results, to the point of having a varying ionization efficiency of a single peptide over its elution peak [5]. This makes it impossible to compare the abundance of two different peptides based on their intensities alone in a mass spectrum.

Due to this challenge, many mass spectrometry (MS)-based quantitation methods rely on relative quantitation, comparing the intensities of individual peptides across different samples under the same acquisition conditions. In recent decades, multiple methods for the relative quantitation of proteins have been proposed and brought into mainstream use, each with their own benefits and

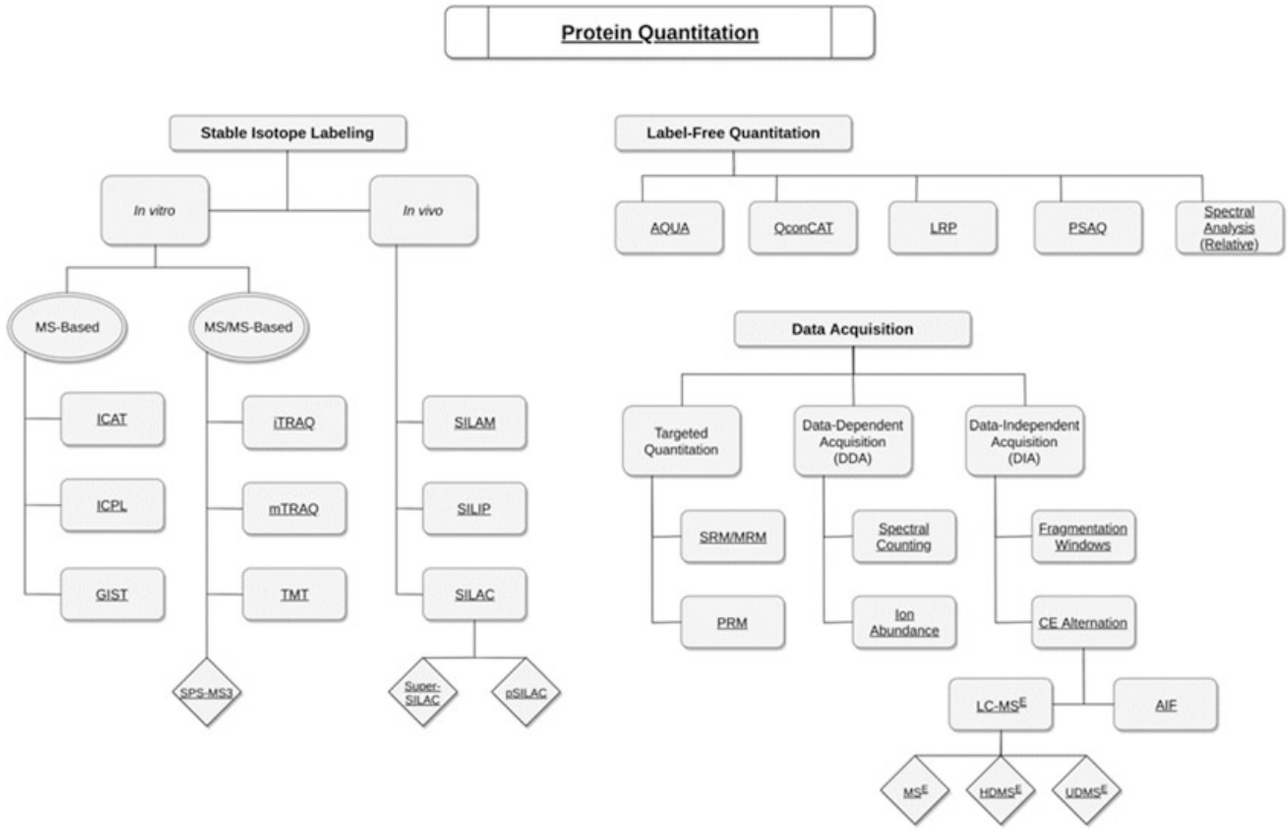


Fig. 1 Categorical representation of the individual methods for protein quantitation, separated into the three main branches: stable isotope labeling, label-free and methods for data acquisition. Standard isotope labeling is divided into in vitro MS-based and MS/MS-based, and in vivo. Abbreviations: *ICAT* isotope-coded affinity tag, *ICPL* isotope-coded protein labeling, *GIST* global internal standard technology, *iTRAQ* isobaric tag for relative and absolute quantitation, *mTRAQ* mass-differential tags for relative and absolute quantitation, *TMT* tandem mass tags, *SPS-MS3* synchronous precursor selection MS/MS/MS, *SILAM* stable isotope labeling of amino acids in mammals, *SILIP* stable isotope labeling in planta, *SILAC* stable isotope labeling of amino acids in cell culture, *pSILAC* pulsed SILAC, *SRM* selective reaction monitoring, *MRM* multiple reaction monitoring, *PRM* parallel reaction monitoring, *CE* collision energy, *LC-MS^E* liquid chromatography MS^E, *HDMS^E* high-definition MS^E, *UDMS^E* ultra-definition MS^E, *AIF* all-ion fragmentation, *AQUA* absolute quantification, *QconCAT* quantitative concatemers, *LRP* labeled reference peptide, *PSAQ* protein standard absolute quantification

drawbacks. Current methods for protein quantitation fall under two main categories: stable isotope labeling, which marks different conditions with various "heavy" components, and label-free quantitation, which uses peptide or peptide fragment signals alone to obtain quantitative data (Fig. 1).

2 Sample Preparation Methods

Stable isotope labeling employs markers containing non-radioactive isotopes of common atoms in proteins such as ^{2}H, ^{13}C and ^{15}N. These isotopes cause a mass shift for the peptide with no significant changes in physicochemical properties. Additionally, excluding deuterated isotopic labels, peptides will coelute in liquid

chromatography (LC) separation [6, 7]. The peaks of the "heavy" and "light" peptides can then be compared, providing relative quantitative data. Adding a condition with a known protein quantity can additionally provide absolute quantitative data. This can all be performed both in vivo and in vitro.

The labeling itself can be achieved by various means, such as using modified tags covalently bound to specific residues and/or peptide termini. Stable isotope labeling (SIL) can be subdivided into multiple groups depending on the type of tag used.

2.1 SIL Techniques In Vitro

SIL in vitro has great flexibility, capable of accepting virtually any sort of sample source and condition. Complexity of the experimental setup is not too high but specific, commercially obtained tags must be used to mark the conditions before analysis, which can increase costs. These tags, depending on their composition, are either identified at the MS or the MS/MS level.

2.1.1 Quantitation at the MS Level

The first methods developed for quantitation by SIL rely on data obtained from the MS level of acquisition; and peptides are fragmented further only for identification purposes. Use of only MS data for quantitation reduces the time required for data analysis [8].

2.1.2 ICAT

Isotope-Coded Affinity Tag (ICAT) MS [9] was created in an attempt to both mark peptides and purify them from a more complex mixture. The label consists of: (a) a sulfhydryl-reactive group that covalently binds to cysteinyl residues, (b) a cross-linker with originally either hydrogen (the light chain) or deuterium (^{2}H, the heavy chain with +8 to its mass), and (c) an affinity tag, such as biotin, which allows for the enrichment of peptides with the tag and assists in the detection of peptides with lower abundances. Hydrogen and deuterium were later replaced with ^{12}C and ^{13}C as a recommended protocol modification from the same laboratory upon discovering that deuterium causes small peak shifts in liquid chromatography [7, 10].

The heavy and light tagged samples are then combined, digested, and incubated with resin containing immobilized avidin (or streptavidin), which binds to the biotin end of the tags and greatly enriches the target peptides and reduces sample complexity. Due to the cysteine selectivity of the tag and the fact that not every peptide contains a cysteine residue, sample complexity is further reduced. However, this is true to such an extent that data may be lost. For example, in *Escherichia coli*, 14% of open reading frames (ORFs) do not code for a single cysteine residue [11].

During analysis, MS/MS data is used for peptide sequence identification, while the relevant LC-MS peak intensities are compared (Fig. 2a) for relative (or absolute with a standard) quantitation.

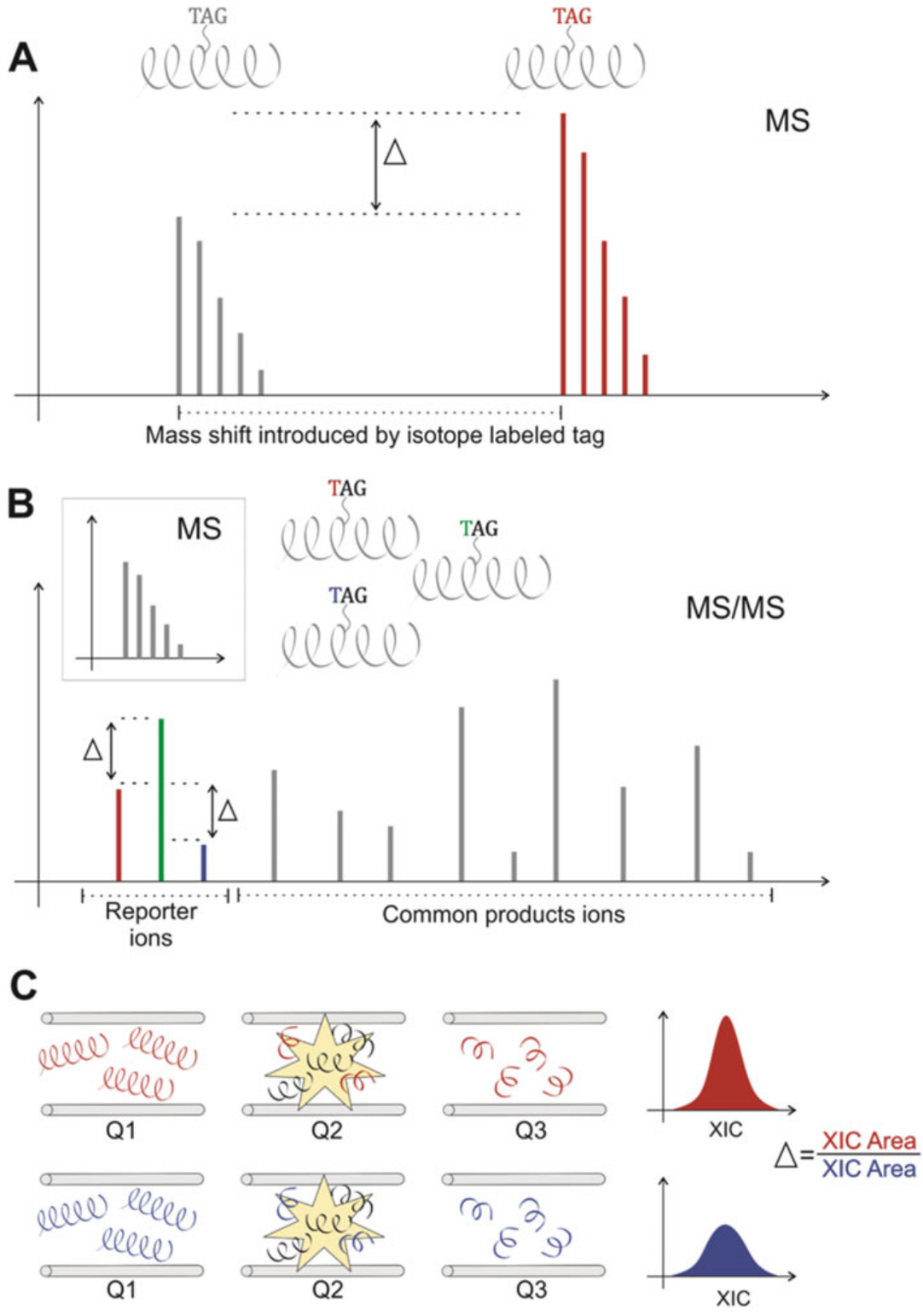


Fig. 2 (**A**) Representation of quantitation at MS level as described for ICAT (isotope-coded affinity tag) and ICPL (isotope-coded protein labeling) techniques. The relative quantitation is based on the difference of precursor ion intensities. (**B**) Representation of quantitation at MS/MS level as described for iTRAQ (isobaric tag for relative and absolute quantitation) and TMT (tandem mass tag). For these techniques, the MS spectrum presents a single precursor for each peptide, and the quantitation is performed based on the difference of intensity of reporter ions, represented in red, green and blue on the MS/MS spectrum. The gray fragments represent the sequence identification ion fragments, which are common for all tagged peptides. (**C**) Schematic representation of SRM (selective reaction monitoring) methodology. Q1 and Q3 represent quadrupoles and Q2 could represent either a quadrupole or any other kind of collision cell. In the scheme, the target peptide is selected in Q1, fragmented in Q2 and a specific fragment is selected in Q3. The quantitation is then performed using the ratio between the XICs (extracted ion chromatograms) of the different conditions

ICAT is best suited, only possible, when comparing two sample conditions since there are only heavy and light tags and it works well with relative quantitation. However, as referenced above, not every protein target will pair well with this technique due to the relative rarity of cysteines in protein ORFs. This means extra care must be taken to not exclude potentially valuable proteins if performing a global study, since a lack of protein identification does not inherently mean a lack of its presence.

2.1.3 ICPL

To combat the nearly tenfold reduction in protein coverage caused by the cysteine-based selectivity in ICAT, Isotope-coded protein labeling (ICPL) was developed [11]. While the less complex samples from ICAT do reduce spectrum convolution, this comes at a potential price of reducing the quantity and certainty of data obtained during protein identification. As protein identification is rooted in unique peptide matching, the more unique peptides present during acquisition the better. Moreover, the need to reduce sample complexity is not as vital in recent times, due to the ever increasing performance of mass spectrometers.

ICPL remedied the drastic reduction by instead labeling lysine residues and N-termini of intact proteins with light or heavy tags. This modification allows for identification and quantitation of a larger number of proteins, as lysine residues in proteins are often more abundant than cysteines [12], with the additional consideration that most peptides cleaved by trypsin have one at their N-terminus. As there is no biotin tag like in ICAT, depending on sample complexity, it may be necessary to employ a fractionation method. All currently available fractionation methods including gel electrophoresis and LC are compatible with ICPL, and can be performed either before or after digestion.

ICPL is, like ICAT, is a chemical labeling method and is feasible in all types of cell lines and tissues. ICPL is capable of up to 4-sample multiplexing by using different combinations of ^{2}H and ^{13}C. An additional benefit of this technique lies in the use of intact proteins to interact with the tags. This allows all proteins to be labeled and combined before digestion, fractionation, and other preparatory techniques, reducing the chance of error propagation.

One downside to this technique is that the covalent modification of lysine destroys a tryptic site of digestion. As a result, trypsin is only effective at cleaving at arginine residues, which requires the inclusion of additional missed cleavage sites, increasing peptide length and data processing time. Some ways to remedy this are to use a different digestion enzyme that cleaves at other residues, or combine trypsin with a second digestion enzyme.

2.1.4 GIST

Continuing to expand the methods for selectivity as MS power has increased, a branch of tagging called Global Internal Standard Technology (GIST) was classified and categorized [13]. The goal was to create a general method that could label any and all peptides, independent of their sequence or any posttranslational modifications. That goal has been attained in more than one way, each with its benefits and drawbacks.

Three example methods for GIST protocols are (1) the acylation of all primary amines after protein digestion [14], (2) the incorporation of isotopically labeled amino acids in vivo [15, 16], and (3) selectively using $H_2{}^{18}O$ during proteolysis [17] or deglycosylation [18]. In all of the cited methods above, only two conditions are used: labeled and unlabeled.

This disadvantage is further compounded by the potential for unintended or incomplete labeling. For example in $H_2{}^{18}O$ incorporation methods, certain carboxyl groups can be unintentionally replaced with ^{18}O [13], and peptide sequence can vary the rate of incorporation of labels [17]. One main benefit to GIST however, is the ability to employ multiple labeling types to increase the quality of data without having undesired interactions between labeling techniques, as each of the aforementioned modifications affect different (yet universally present) sites.

In general, GIST methods are a good choice for comparative quantitation of protein expression between two samples, especially in conditions where posttranslational modifications are present or when studying proteins with few of the normal reactive sites like lysine and cysteine, since the labels affect sites of cleavage instead of side groups.

2.1.5 mTRAQ

Originally a modification of isobaric Tag for Relative and Absolute Quantitation (iTRAQ, *see* below) is mass-differential tags for relative and absolute quantitation (mTRAQ) (Applied Biosystems, Inc., Foster City, CA, USA). This protocol differs from iTRAQ in that it uses a nonisobaric tag, whose utility is maximized using multiple reaction monitoring (MRM) to obtain a high number of different transitions [19], which are the pairs of precursor and product ions that are created during fragmentation. The protocol can be modified to perform absolute quantitation.

In mTRAQ, a Global Internal Standard (GIS) can also be used. This standard allows for direct comparison between samples in the GIS, regardless of when and on what machine a sample is measured. Another option is to use a Reference Internal Standard (RIS). Using an RIS as the standard then allows for comparison of samples with a Time 0 reference sample. Lastly, a single or small group of peptides can be used as the internal standard. In doing so, a known quantity of peptide is labeled and injected, which allows for the absolute quantitation of a desired list of proteins.

Some reasons why mTRAQ would be selected over iTRAQ are: first, the accuracy of the quantitative data obtained is increased by the use of a global comparative standard; and second, proteins of interest can be better quantified and identified due to the non-isobaric tags and MRM selection method. This means that mTRAQ is able to compare samples across multiple runs since a single, internal standard is used for all experiments [19].

This is in contrast with iTRAQ, where quantitative data is compared to each run's standard before it is able to be compared with a different run. However, in a comprehensive study on global proteomics and phosphoproteomics, although iTRAQ had less accuracy in quantitation, it was found to be less variable than mTRAQ, and also identified a distinctly larger number of proteins and phosphopeptides [20]. mTRAQ is available only in triplex, where one label is used for the internal standard and two are used for experimental samples.

2.2 Isobaric Tagging and Quantitation at the MS/MS Level

All the methods described thus far are based on the mass shift of precursor ions by adding tags to proteins/peptides and, as such, quantitation is performed at the MS level. When this is done, a labeled peptide becomes slightly heavier than its unlabeled counterpart, making it possible to differentiate between them. However, this can cause three main problems: (1) a labeled peptide may have the same mass-to-charge ratio (m/z) as a different, unlabeled peptide, causing peak overlap of unrelated peptides, complicating quantitation, (2) signal dilution, as peptide MS signals are being divided into two or more signals, and (3) the extra mass added onto the peptide and potential changes in chemical properties modifies a labeled peptide's LC elution profile. The increased mass bound to the peptide leads to a later elution time, not only separating LC peaks, but also presenting different ionization conditions for a peptide.

To remedy this, another way to tag the peptides is to use an isobaric tag. Isobaric tags are labels that have the same intact mass and confer the same chemical properties as tags from other conditions during LC and MS analysis. However, during further ionization of the peptide and tag, MS/MS spectra reveal two types of product ions: (1) the peptide fragment ions, common for the peptides from all the different conditions, which are used for peptide identification; and (2) fragments called reporter ion peaks, which will be specific for each sample condition. Relative quantitation is then performed by comparing the intensity of reporter ions (Fig. 2b).

The main advantage of isobaric tags is that peaks of precursor and reporter ions of the same peptide found in different conditions are not spread out. This is because precursor ions have the same m/z, regardless of which tag is bound, eluting together during LC. Also, this technique avoids overlap of the isotopic patterns of

precursor ions, as is seen in the case of other SIL techniques, providing cleaner MS m/z peaks.

Isobaric reagents are, in their most basic form, composed of four parts. A protein-reactive terminal chemically binds to side chains or N-termini. An isobaric group, different for each condition, allows MS/MS measurement of peaks, providing relative quantities. A mass-normalizing group is included to ensure that tags all have the same mass, elute at the same time, and can be selected together in the mass spectrometer. Lastly, there is a cleavable linker region, which releases the isobaric group during ionization.

During collision-induced dissociation (CID), when the linker region is cleaved, an ion with a specific and known m/z is released. As all peptide–tag pairs migrate together chromatographically, the signals will coincide, increasing accuracy of the comparison. Also increasing accuracy is the ability to selectively use only the MS/MS spectra, allowing for the reduction of background noise due to unlabeled peptides [21].

In addition to the standard, bottom-up approach for identification, a top-down approach has also been proven to function with these techniques, showing the liberation of ions over a wide dynamic range [22].

2.2.1 iTRAQ

One such isobaric tagging technique is iTRAQ [23]. iTRAQ is a commercially available kit, available for multiplexing with 2, 3, 4, and 8 samples, all of which react with primary amine groups of tryptically digested peptides.

This protocol is adaptable to different machine types and data processing methods, and is additionally able to be modified for absolute quantitation and comparisons between runs. Using one reporter tag for a known quantity of protein, the remaining reporter peaks can be compared to that signal, allowing for relative comparison to that known quantity and gaining absolute data.

The main downside general to isobaric tag methods is the requirement to purchase ready-made kits which can quickly increase costs, depending on experiment size. Since this technique labels peptides at amine groups, every peptide will have at least one labeling site at its N-terminus, making it compatible with any source of protein. iTRAQ has kits for multiplexing of 2, 3, 4, and 8 samples.

2.2.2 TMT

Tandem Mass Tag (TMT) labeling is another isobaric method for relative quantitation, similar in function to iTRAQ (binding to primary amine groups) and is available in many different sets: 2-plex, 6-plex, 10-plex, and 11-plex, allowing for a higher number of sample condition comparisons within the same run. Alternatively, TMT is also available in 6-plex sets capable of reacting with cysteine or carbonyl groups instead of primary amines.

Comparing iTRAQ with TMT has not delivered any definitive results on which gives higher-quality data and one study claims no difference in performance between the two methods [24]. Additional contrasting studies have been published on a possible but not certain reduction of quantitation accuracy when performing higher-plexed experiments [25–28].

2.2.3 TMT Isobaric Isotopologues

An additional set of labels for TMT have been developed that use ^{15}N instead of ^{13}C, causing a change in label mass on the order of milliDaltons (mDa). This extremely small mass difference is able to be detected by high-resolution mass spectrometers, allowing for the addition of several more conditions in what are called TMT isobaric isotopologues [29]. This new technology has allowed TMT experiments to expand to the previously mentioned 10-plex and 11-plex reagent sets (noting that the proof of concept cited above used a maximum of 8-plex).

2.3 SIL Techniques In Vivo

Instead of linking tags to proteins or peptides in solution, it is also possible to label proteins in vivo, in what is referred to as metabolic labeling. When performing in vivo SIL, isotopically labeled constituent subunits of proteins are introduced to the growth environment. Since there are conversion pathways that can convert one amino acid to another, the labeled amino acids are specially selected to reduce the amount of unintended labeling. Further benefits and downsides have been researched for specific methods.

When using SIL, nearly all proteins are then inherently labeled with high incorporation, and the samples can be combined much earlier in the protocol—before cell lysis, protein digestion and fractionation, avoiding the possibility of error propagation.

2.3.1 SILAC

In 1999, Oda et al. described a method for whole-cell stable isotopic labeling [15], which utilized ^{15}N-labeled ammonium persulfate as the only source of nitrogen in a yeast culture, leading to the labeling of every amino acid. A few years later, two laboratories nearly simultaneously published an extension to that method by using unlabeled and deuterated leucine, an essential amino acid (D10-Leu) [30] (D3-Leu) [31]. Ong et al. built upon this method, which they had named Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC). Since then, the use of leucine [31], lysine [32] and methionine [33] has all been documented in SILAC experiments.

Later, the use of $^{13}C6$-lysine and/or $^{13}C6$-arginine has been suggested as practical options in SILAC [34] due to their relationship with trypsin cleavage sites, ensuring any peptide generated will be quantifiable except for final, C-terminal peptides [35]. Additionally, ^{13}C-labeled proteins have less of an effect on reverse-phase chromatography elution times than deuterium labels [36].

When using SILAC, high incorporation rates of the labeled amino acids have been measured (more than 90% after 6–8 cell passages [31]) and it allows for accurate quantitation of proteins, even with small differences in expression. Three conditions have been successfully combined in a single experiment, giving peak trios for each peptide in MS, while still being able to identify sequences using both the labeled and unlabeled peaks in MS/MS, which is especially important if a protein is not found in the unlabeled condition [34]. Upon completion, relative quantitation can calculate the fold change of protein expression between the samples.

Despite SILAC's high accuracy and usefulness in cell cultures, it has several drawbacks, making its employment oftentimes prohibitive. The drawbacks are led by the high cost of isotopically labeled amino acids and associated buffers. Other, lesser complications include the conversion of arginine to proline in some cell types [34, 37–40] which has been solved with additional preparation steps [41–43], otherwise providing unexpected increases in peaks for peptides, and the fact that some cell types do not respond well to changes in medium, or simply cannot be kept growing for the required number of passages for sufficient incorporation.

2.3.2 pSILAC

A direct derivation from the SILAC protocol called pulsed SILAC (pSILAC) [44] globally labels proteins at a specific point in time, to mark changes in protein expression due to a specific stimulus. This technique was developed to fill in the knowledge gap of protein translation rates, as previous expression quantitation techniques could measure mRNA levels and protein turnover rates, but not the rate of protein translation.

In pSILAC, an unlabeled cell line is split into two conditions, each one with a different medium. The cells are left to grow for a short period of time before they are lysed, and the extracts are then combined for MS preparation and analysis [44]. This method allows a researcher to compare levels of individual protein translation between two cellular conditions, collecting data for around half of all proteins that have detectable mRNA levels (data in relation to HeLa cells [44]). This technique has been found to be extremely useful to determine the short-term effects of different molecules on protein expression, such as the effects that microRNAs have on cells [45].

The drawbacks of pSILAC are similar to those of SILAC, although the restrictions due to cell passage number and the extent of arginine to proline conversion are normally not an issue due to the shorter experimental workflow.

2.3.3 Super-SILAC

Another derivation of the SILAC protocol is called super-SILAC, a method in which a group of labeled cell types is combined to form an internal standard for analyses [46]. By doing so, a more robust

standard is created, exhibiting data more analogous to full tissue, allowing for much higher accuracy for quantitative comparisons across samples, cell lines and experiments [47]. This standard is then mixed with every condition and is termed a "spike-in standard" [48].

This technique is a solution to a limiting factor of SILAC with its inability to be used in more complex subjects, like humans. Instead of working with heterogeneous, collected tissue, individual human cell lines can be grown and labeled. In regular SILAC this is not feasible due to variations in protein expression across cell types, and there is the potential for significant errors between experiments. But when using super-SILAC, one study showed that several thousand proteins could be quantified with an error of only a few percent [46].

In Super-SILAC, the costs of labeling are reduced, while keeping the robustness and reproducibility of SILAC. Suggested applications include biomarker research and quantitation, personalized medicine and proteome studies. The main downside is the long and potentially expensive preparatory process of selecting (or purchasing) multiple cell lines to make the labeled spike-in standard.

2.3.4 SILAM

Despite super-SILAC's solution to heterogeneous samples, the SILAC family of protocols is still not compatible with more complex, intact, living organisms. As such, a protocol named Stable Isotope Labeling of Amino Acids in Mammals (SILAM) can be an alternative to SILAC to be used in this type of biological system [49]. In the original SILAM, an unlabeled or a ^{15}N-enriched spirulina diet was given to two groups of rats, effectively labeling entire animals. When the diet was started immediately after weaning, a 74–92% incorporation of the heavy nitrogen was observed, depending on the protein turnover rate [50].

SILAM can be an invaluable tool for proteomic studies in living organisms and has been successful in mice, rats and squirrels [49, 51, 52], among others. It allows full proteome labeling of a complex living organism without any observed detrimental effects on growth or development after a single generation [49]. It also has potential when studying organism-wide changes from environmental or pharmacological effects, as well as studying animal models of diseases. SILAM can also be especially useful when comparing different tissue types under the same conditions.

The most significant downside to this method of quantitation is the high cost of the reagents. Preparing an entire food source for mammals with ^{15}N enrichment is expensive and requires special care during the protocol, and much of the diet's cost can be wasted if the entire organism is not studied. As with any experiment, multiple replicates are recommended in addition to any required

controls, meaning food, care and sacrifice of several animals, further increasing costs. Additionally, since the identification software has to search for global variables of ^{14}N or ^{15}N [53], the data processing steps are more resource-intensive and time consuming.

In a similar fashion, plants have been shown to be compatible with this type of in vivo labeling in a technique called Stable Isotope Labeling In Planta (SILIP) [54]. A proof of concept study was performed with tomato plants, showing 99% incorporation of the nitrogen-based label.

2.4 LFQ

In contrast with the methods above, label-free quantitation (LFQ), as the name implies, requires no chemical tags or isotopic labels on samples. This makes the following approaches attractive for many experiments, as sample preparation can have a lower reagent cost and fewer experimental steps, and is similar to global standards in the sense that there is no limit to the number of different conditions that can be compared.

At its core, LFQ is based on two different data-collection approaches: (1) spectral counting or (2) ion abundance, the choice of which is in part influenced by the type of data collection used. These are data-dependent acquisition (DDA) or data-independent acquisition (DIA), both of which are described further below.

2.4.1 AQUA

In one LFQ technique, MRM (*see* below) can be combined with the stable isotope dilution (SID) technique. A known quantity of a purified, labeled peptide standard is used with the unlabeled sample, allowing for the absolute quantitation of a protein of interest in a complex mixture in what is referred to as Absolute Quantification (AQUA) [55]. Although different peptides can ionize and fragment differently, using the same peptide as a label allows the user to make a relative comparison with a known quantity.

AQUA is capable of measuring posttranslationally modified proteins, such as by phosphorylation, along with proteins found in full-lysate samples [56] and can be fully optimized in around a week's time [57]. This technique is highly compatible with clinical tests for a specific protein and in biomarker research. However, is not effective in global proteome studies, since the label only accurately grants quantitation to a single peptide. One study found AQUA to have a median coefficient of variation (CV) of about 10% [58]. However this can vary drastically based on the samples and mass spectrometer used.

2.4.2 QconCAT

Due to the high cost of labeled peptide standards used in AQUA, a modified technique was designed to help reduce this expense. A technique able to quantify up to 20–30 proteins with over 50 peptides, QconCAT (for Quantitative concatemers) was developed [59]. A long protein sequence that contains all of the desired peptides can be expressed in *Escherichia coli* grown in stable

isotope-labeled media, providing a single, tryptic protein for the quantitation of a large set of proteins of interest [60]. Since the labeled peptides are not designed, ordered, and created by a third party, both time and money can be saved.

The QconCAT polypeptides are purified after expression, such as via an included histadine tag in the sequence, and a known amount of this protein can be spiked into a sample, allowing for comparative quantitation to the known standard by extracted ion chromatogram (XIC, *see* below) or MRM (*see* below), and thus providing absolute quantitative data [61].

The result is a much cheaper protocol, allowing for the absolute quantitation of a larger number of proteins in unlabeled samples. A major drawback however is the protocol length as this requires around 3–4 weeks with additional lead time to design and order the QconCAT gene [60, 62].

2.4.3 LRP

Due to the financial impact of commercially available AQUA reagents or labeled media for QconCAT when studying multiple proteins of interest, a single, labeled reference peptide (LRP) can be used for higher accuracy of relative quantitation to help account for variations between experiments [58]. While considerably cheaper than SID (AQUA), LRP has a higher median CV at 20–30% due to the variation of ionization, among other factors, but it still has competitive values when compared to immunoblotting (CVs of 20–40%) [58].

LRP is best suited for experiments seeking large-scale quantitative data by MS. Due to a relatively high variance, it is suggested that LRP be used for initial studies, before being investigation by other, more precise methods.

2.4.4 PSAQ

Somewhat an extension to the idea behind AQUA, and in an attempt to further increase accuracy, a method was developed that uses an intact protein standard, called Protein Standard Absolute Quantification (PSAQ) [63]. Since a fully intact, labeled protein is used, the sample can be extensively fractionated before sample preparation and quantitation, additionally providing a control against sites or regions of incomplete digestion.

Owing to the fact that the whole protein is used, high accuracy and precision can be obtained even in extremely diluted samples. One study found a quantification accuracy of 77% and a precision of <5% at concentrations as low as 1 ng/mL [64], and showing comparable sensitivity to enzyme-linked immunosorbent assay (ELISA) [65]. While not the best choice for large-scale operations, this technique has great potential for high-accuracy quantitation of small sets of low-abundance proteins, for example in biomarker and clinical applications.

2.4.5 Other Methods

Additionally, without using any reference peptides or spike-ins, it is possible to use various types of spectral data for quantitation as well. This is done by directly using targeted, DDA, or DIA methods (described below) and performing identification, quantitation and normalization steps on that data with compatible software. Since there is no known protein/peptide quantity added to the samples, these methods are only capable of providing relative quantitation data between samples.

3 Methods of Data Acquisition

Once samples are prepared, they must be injected into a mass spectrometer for analysis. There are several methods for data acquisition, some of which are hardware-based and some which are actually methods selected for a specific function of a spectrometer. Furthermore, some methods of data acquisition and analysis allow for relative quantitation without the need for any labels, standards, or spike-ins (as mentioned earlier).

3.1 Targeted Quantitation

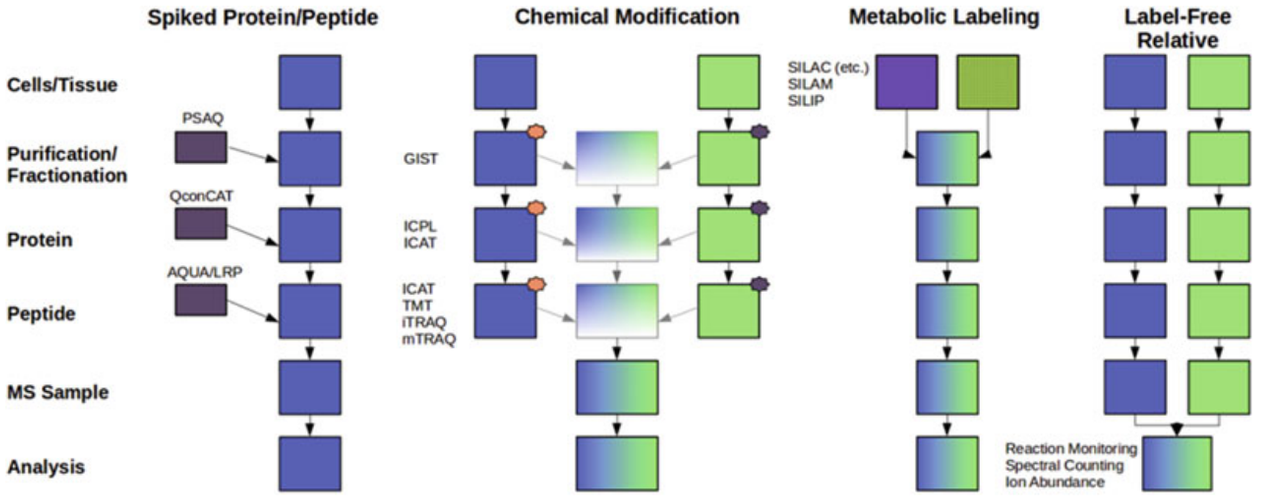
When collecting data, a mass spectrometer first selects for a precursor ion of interest before it is fragmented and those fragment ions are used to determine the sequence of the precursor. It is possible to quantify a protein by measuring the number of fragment ions that reach the final detector, which suggests the amount of the peptides and thus proteins in the original sample.

3.1.1 SRM and MRM

Selective Reaction Monitoring (SRM) is an MS technique that uses a triple quadrupole (QQQ) mass spectrometer to accurately quantify a single protein of interest at a given time [66]. SRM is based on the detection of one or more precursor-fragment ion pairs (or "transitions"). A single precursor ion for a unique peptide of interest is selected by its m/z (and LC elution time) in the first quadrupole (Q1). The ion is then fragmented in the second quadrupole (Q2) or collision cell by CID, and a single, specific fragment is then selected in the third quadrupole (Q3), reaching the detector and generating a signal (Fig. 3b).

The total area under the XIC (also written EIC, a graph that shows peak intensity as a function of time) can be used to estimate the quantity of the peptide precursor ion and therefore the protein. This technique has proven itself capable of quantitation down to femtomole per milligram levels and spanning four orders of magnitude [66]. On average, up to 2000 transitions can be monitored in a single experiment, translating to up to 1000 proteins, although this number can be smaller to read more than one peptide per protein or more than two transitions per peptide [67].

During the acquisition time, the quadrupoles are not scanning other, "unimportant" m/z values, resulting in a 100% duty cycle



Preparation Time	++	++	+++	+
Analysis Time	+(+); number of targets	++	++	+++
Compatible Sources	Any protein/peptide	Any proteins/peptides	Cell colonies, small mammals, plants	Any sample source

Fig. 3 (Top) Experimental flowchart for various methods, detailing the point of convergence of samples. In spiked protocols, the label is added at one of various points in the experiment. In chemical modification, samples are labeled and combined at different points. In metabolic labeling, samples are combined immediately after collection. In label-free analysis, samples are run separately and data analysis is used to compare protein levels. (Bottom) Chart approximating the cost, preparation time, analysis time, and compatible sample types (adapted from a figure by S.-E. Ong and M. Mann [153]). Abbreviations: *PSAQ* protein standard absolute quantification, *QconCAT* quantitative concatemers, *AQUA* absolute quantification, *LRP* labeled reference peptide, *GIST* global internal standard technology, *ICPL* isotope-coded protein labeling, *ICAT* isotope-coded affinity tag, *TMT* tandem mass tags, *iTRAQ* isobaric tag for relative and absolute quantitation, *mTRAQ* mass-differential tags for relative and absolute quantitation, *SILAC* stable isotope labeling of amino acids in cell culture, *SILAM* stable isotope labeling of amino acids in mammals, *SILIP* stable isotope labeling in planta

for a transition of interest, thereby making SRM a sensitive technique. In practice though, normally one or two additional precursor-fragment ion pairs are also monitored to increase specificity and reproducibility. This practice slightly decreases sensitivity, but increases the reliability of the data [67].

MRM is the use of SRM to simultaneously detect more than one precursor/fragment pair. MRM has achieved subnanogram/milliliter quantitation by SID [68, 69] under some conditions. However, it can lose sensitivity in more complex mixtures, making global analyses less feasible at these levels of accuracy [70]. Although specialized software is required for this assay in both experimental preparation and data analysis, there are multiple tools available to perform these experiments, including open-source software like Skyline, created and maintained by the scientific community [71].

Both SRM and MRM techniques are best suited for label-free relative quantitation and standard-assisted absolute quantitation of many, preselected proteins in a simple to relatively complex solution at good to excellent levels of accuracy.

To perform SRM/MRM experiments, a user must complete multiple data preparation and processing steps. These include the

selection of a peptide/ion database, software for peptide and transition selection, software to execute selected methods, and programs to detect peaks, perform quality assessment and statistical analyses. Rather than list each package here, we defer to Colangelo et al., who have already written an extensive review [72] on the software tools for the design, data collection, and analysis steps of MRM proteomics.

3.1.2 PRM

Building upon SRM/MRM technology, to enable high-throughput analyses of multiple product ions at the same time, Parallel Reaction Monitoring (PRM) was developed [73]. Due to the amount of data collected, a high-resolution mass spectrometer is required for this technique. Using PRM, it is possible to both identify and quantify hundreds of proteins in a single sample, in contrast with SRM, where proteins must be preselected and are not identified with the mass spectrometer [74].

Instead of monitoring a single fragment or group of fragments from one precursor, PRM uses a high-resolution mass analyzer to register all generated fragments from the target precursor. This highly robust method of analysis increases the dynamic range while keeping the achievable linearity over that range [73]. For example, one group found it was possible to detect levels of ubiquitin chains at subfemtomole quantities [75].

PRM has a wider dynamic range, although there are still more precise measurements obtained with SRM likely due to the higher sampling rate and SRM was in some cases still able to function at lower limits of quantitation when background interference was not present [70]. The main advantage of PRM over SRM and MRM is that a preselected list of peptide transitions to be monitored is not required and, in complex samples, the identification step is able to remove some background noise of coselected peptides [74]. PRM data collection is compatible with both absolute (e.g., SID or LRP) and relative (using no labeled reference) quantitation.

Proprietary software for mass spectrometers can be used to process PRM data as well as multifunctional packages like Skyline [71] and SpectroDive (Biognosys AG).

3.2 DDA

When collecting data from complex samples, such as in shotgun proteomics, a mass spectrometer is not able to select all of the eluting precursor ions. The machine must then make many rapid choices as it must be determined which of the MS1 peaks should be fragmented for identification and quantitation, and which must be ignored.

One way to select peaks is through DDA, in which the mass spectrometer sequentially selects the most intense peaks recorded on MS1 spectrum. Those peaks are then selected for subsequent, individual fragmentation. After a cycle of MS and several MS/MS spectrum acquisitions, the mass spectrometer performs another MS

reading and the cycle restarts. To collect data from peptides with lower intensities, a process called dynamic exclusion time is usually employed, omitting peptides that have already been read from future selections. Using this concept, several methods have been developed to perform LFQ.

3.2.1 Spectral Counting

One such method is called spectral counting [76], which is a two-step procedure for calculating relative protein abundance. In the first step, the MS/MS stage identifies a peptide through its fragments, repeating this step for every precursor ion. A complete list of all peptides is then compiled and proteins are identified using these peptides.

For a given protein to be quantified, its constituent peptides that were identified must be summed, using the number of times each peptide's MS/MS spectra were registered. This score can then be used to estimate the total amount of that protein in the sample due to its near linear relationship over two orders of magnitude [76]. This method is somewhat controversial since ionization efficiency of different peptides varies with physical properties and chromatographic behavior [77] and, as such, this method requires the acquisition of a large number of spectra for high quality data [78] and is considered to only provide a relative abundance for proteins without the use of SID or LRP.

3.2.2 Spectral Counting Normalization

In attempt to increase accuracy of the data obtained, several free and commercial methods and software applications for standardization have been published. These include APEX [79], Crux [80], emPAI calc [81], PepC [82], QSpec [83], QProt [84], and Spectral Index [85]. These methods perform calculations often based on a database of peptide ionization information to increase accuracy of the conversion between the spectral counts and the protein quantity present in the sample such as by taking the expected number of peptides and peptide length into consideration. ProteoIQ (PREMIER Biosoft, Inc.) and Scaffold (Proteome Software, Inc.) are commercial examples of software that utilize the aforementioned methods, with the incorporation of user interfaces and additional tools.

Each option offers slightly different features, user interfaces, and metrics. Such metrics for standardization include Normalized Spectral Index (SI_N) [86], Exponentially Modified Protein Abundance Index (emPAI) [87], Normalized Spectral Abundance Factor (NSAF) [88], and distributed Normalized Spectral Abundance Factor (dNSAF) [89]. These and other metrics basics are explained below.

3.2.3 Ion Abundance/Ion Counting

Differing from spectral counting, Ion Abundance (also known as Ion Counting) [90] measures the intensities of MS peaks in tandem with LC elution profiles in what are called XIC integrated areas, and which only uses MS/MS data for peptide identification. There are several studies that differ in opinion regarding the accuracy and precision of ion abundance compared to spectral counting [78, 91, 92]. This discrepancy is possibly due to the various operations possible (such as comparing one protein across runs or comparing two proteins to each other within the same run) and due to the availability of multiple formulas for calculations [91].

3.2.4 Ion Abundance Normalization

To obtain the most precise results possible, extensive data processing is required, as LC graphs must be aligned between runs by reference peaks found across samples, and must also be normalized with data from housekeeping genes to allow accurate comparisons between different runs. Several software options are available for peak picking and XIC alignment such as MapQuant [93], MaxQuant [94], OpenMS [95], Peaks Studio [96], Progenesis QI (Nonlinear Dynamics), ProSE (Proteios Software Environment) [97], Serac [78], SpecArray [98], and SuperHirn [99]. For additional information, Välikangas et al. compared performance parameters of five of the most commonly used software suites (MaxQuant, OpenMS, Peaks, Progenesis, and Proteios) [100].

3.2.5 Additional Resources

Once data are collected, there are several ways to quantify the proteins present in the original sample. Using peak intensity data, one such way is Hi-N (or topN) [101], based on the discovery that the three tryptic peptides with the highest intensities can be used with high accuracy to quantify their parent protein. Another method is to sum all of the peptide intensities and divide that by the total number of observable peptides of that protein in intensity-Based Absolute Quantification (iBAQ) [102]. In a paper by Krey et al., Hi-N and iBAQ were found to be equal in data quality [103].

Using spectral counting data, NSAF [88] is a method that takes protein length into consideration, since a longer protein will inherently have more observable peptides than a smaller one. Building on NSAF is dNSAF [89], which additionally takes shared peptides into consideration when quantifying the parent proteins. Yet another method is Statistical Model for Protein Quantification (SCAMPI) [104] which not only takes shared peptides into consideration; but it explicitly incorporates them into data processing. SI_N [86] is a method that combines three features of abundance data: peptide count, spectral count, and MS/MS intensity. The result is a significant and reproducible data set, and allows the quantitation of thousands of proteins in a complex sample. Finally, although not an exhaustive list, emPAI [87] is a method that attempts to standardize data using a number of observable peptides

per protein metric, and is modified to an exponential scale for absolute quantitation.

McIlwain et al. have suggested that, between SI_N, emPAI, NSAF, and dNSAF, it is the NSAF method which has the highest reproducibility in their review using Crux software for spectral counting [105].

3.3 DIA

In DDA, there can be issues with reproducibility, low-abundance peptides, and undersampling [106, 107]. An alternative is DIA [108]. In this methodology, the mass spectrometer does not specifically select precursor ions from MS peaks to fragment, like in DDA mode. Instead, all precursor ions in a specific m/z window are allowed to be fragmented simultaneously, allowing many more peptides to be fragmented, scanned, and identified.

This allows for identification and quantitation of multiple fragments at the same time. Doing so does not affect the method of quantitation (using XIC data). Instead it only modifies the method for which peptides are selected for further fragmentation. This increase in data comes at the price of a more convoluted MS/MS spectrum, thus requiring more data analysis time.

Some sources refer to this method as relatively new. However, it should be noted that publications have referred to this type of protocol since 2003 in a proof of principle [109] and was successfully used in proteomics in 2004 [108]. DIA quantitation approaches fall into two main categories: high/low collision energy alternation and stepwise or randomized windows.

3.3.1 CE Alternation

In methods such as MS^E [110] (in qTOF spectrometers) and All Ion Fragmentation (AIF) [111] (in Orbitrap spectrometers), the collision energy of the full m/z window is alternated, leading to high- and low-energy fragmentation data. This provides complete MS and MS/MS data but generates a more complex and convoluted spectrum. The increase in data processing requirements comes at the benefit of reducing the chance of missing any quickly eluting proteins. The MS/MS data is then deconvoluted and assigned to precursor ions, helped by the fact that precursor and fragment ions must have the same chromatographic elution profile [112]. The deconvoluted MS/MS spectra are searched against a protein databank for identification. The precursor XICs are then used for relative [113] or absolute quantitation [101].

Other workflows have built upon MS^E to increase data quality, such as high- and ultra-definition MS^E, $HDMS^E$ (Waters Corp.) and $UDMS^E$ (Waters Corp.) [114], respectively but the principle of quantitation remains the same [115]. In $HDMS^E$ and $UDMS^E$, more proteins can be identified and with higher confidence when compared to standard MS^E [116]. This is due to the integration of ion mobility separation (IMS), in which ions separate based on their

gas phase mobility in an electric field, increasing peak capacity through the addition of this extra dimension of resolution [115].

Collision energy (CE) levels are what distinguish HDMS^E and UDMS^E. In HDMS^E, the CE increases stepwise during the high-energy scan, as the IMS cycles are run. In UDMS^E, the IMS cycles are instead individually run with their own small CE ramp, which repeats in each cycle. This inclusion of IMS greatly increases the number of proteins identified in complex samples. However quantitation in high-abundance samples can sometimes be reduced due to oversaturation of the detector [117].

3.3.2 Fragmentation Windows

Instead of fragmenting the entire, available set of precursor ions, small windows can be selected of fixed or variable widths. In doing so, spectral data is less complex. However some eluates may be lost during the long cycle times. Several methods exist to select the window sizes and cycle times, such as extended data-independent acquisition (XDIA) [118], PaCIFIC [119], FT-ARM [120], and SWATH [121]. For more information on DIA quantitation methods, an article by Bilbao et al. elaborates on the differences and similarities of each [110].

Generally speaking, a small window of MS ions is allowed to be fragmented at any given time, increasing stepwise before returning and repeating. This provides MS/MS data for many proteins in an elution profile as no individual peak is selected for fragmentation. The MS and stepwise MS/MS spectra can then be compared against a peptide spectral library to identify the proteins present in the sample; and the areas of the XIC peaks are used to calculate protein abundance [122, 123].

Similar to stepwise windows, Multiplexed MS/MS (MSX) [124] chooses small windows for higher-energy fragmentation steps. However it is done at random, instead of in a stepwise manner. When performing experiments with SWATH, the use of variable fragmentation windows [125] to reduce cross fragment ion interference has been proven to aid in the identification of more proteins.

3.3.3 DIA Software

Multiple software packages exist to use complex DIA data, using different methods to obtain quantitative data. Bilbao et al. [110] classified software into three groups: XIC construction from spectral libraries, demultiplexing into pseudo-DDA spectra, and theoretical spectrum comparing.

In XIC construction, a data library is required to assist in the identification of spectra and if data is not present for a peptide, it cannot be identified. Since then, several attempts have been made to solve this shortcoming of DIA by extracting pseudo-DDA spectra. Demultiplexing software individually subtracts MS/MS spectra from MS precursor data, loosely based on research by Purvine et al.

[109]. However, at that time, this process was done by hand and no software was proposed in the original study.

Some software tools capable of pseudo-DDA spectral extraction are DIA-Umpire [126], PeakView (AB Sciex), Skyline [71], Group-DIA [127], MSPLIT-DIA [128] and PECAN [129]. At the time of writing this chapter, no systematic comparison and review of these different methods was available. Other, more specific software packages have become available, for example MaxQuant [111] for AIF, XDIA Processor [118] for XDIA, and Complementary Finder [130] for collision-activated dissociation (CAD).

4 Additional Tools

Beyond the methods and tools for data acquisition and analysis, the following protocols have been considered to be potentially useful for the creation of certain project workflows. These tools are SPS-MS3 for increased sensitivity in multiplexing, the proteomic ruler protocol for cell number normalization, and AP-MS for sample enrichment and depletion.

4.1 SPS-MS3

In high-number multiplexing experiments like iTRAQ and TMT, the sheer number of ions present can cause significant levels of interference during quantitation steps due to unintentionally coselected species during fragmentation [131]. To remedy this, MS3 scans have been used to remove the distortion of the ratio signals [132]. Unfortunately, this increase in data quality originally came at the price of a significantly reduced sensitivity [131].

To regain the lost sensitivity, a method called Synchronous Precursor Selection MS/MS/MS (SPS-MS3) fragments multiple MS/MS peaks at the same time, increasing the number of reporter ion signals and ultimately increases the quality of data obtained [131]. The main drawback to this method is the requirement for a compatible mass spectrometer, capable of performing this type of simultaneous MS3 scanning.

4.2 Proteomic Ruler

In some experiments, it may be desirable to calculate the number of protein copies per cell, achieved through cell counting and absolute quantitation steps. While this technique is good in theory, there are multiple steps in which user errors can drastically affect such a sensitive measurement, such as cell counting steps, varying cell size, protein concentration measurements, and protein reactivity to quantitative assays [133].

A fast, simple way to reduce these sources of error is to sample the MS signals from histone proteins. This can be done because the original number of cells is directly proportional to the quantity of DNA present in a full lysate, and this, in turn, is associated with a direct relation to histone presence at a nearly 1:1 ratio

[134]. Wiśniewski et al. have described a protocol named Proteomic Ruler [133] to use this relation to provide copy numbers per cell and protein concentrations with no additional experimental steps, and with comparable precision compared to standard methods.

4.3 AP-MS

In many experiments, the data from the entire proteome may not be required but rather a small subset of proteins is desired. To enrich the sample and obtain a simpler sample, tandem affinity purification (TAP) was often performed [135, 136]. TAP is a process for enriching a sample for targets of interest, discarding nonspecific molecules.

This enrichment can occur by: immobilizing antibodies [136–138] against a protein, epitope, or posttranslational modification; or immobilizing other ligands like chemicals [139–142], lipids, proteins [143–146], peptides [147], DNA [148], RNA [149], or ions (e.g., anions for histidine tags or cations for phosphorylation) [150], and then eluting the selectively bound protein targets. Other methods ranging from reversible immobilization of phosphopeptides to amine-containing resins [151] have been published.

As the modifications of a protein are sometimes just as important as its quantitation, enrichment by affinity pulldown, ion exchange and reversible immobilization can provide crucial data to an experiment. In combination with shotgun proteomics, individual sites of protein modifications and their stoichiometry can be determined. In addition to the above methods, a different type of affinity pulldown uses pan-antibodies, a type of antibody that recognizes a variety of targets such as post-translational modifications, regardless of the flanking sequences [152, 153].

Once proteins are enriched, they can either be quantified by label-free methods or labeled and quantified by Affinity Purification Mass Spectrometry (AP-MS). Due to the nature of this type of purification and enrichment technique, extra care should be taken when comparing quantitative data from different experiments. For example, antibodies can vary between batches and the presence of other proteins in a lysate can affect which and how much of a protein subset is eluted.

5 Method Comparisons

With the sheer quantity of methods available for use, selecting a single one can be a daunting task. Since many methods have specific benefits and drawbacks for certain applications, this comparative chapter and accompanying chart (Fig. 4) can be used to help select a protocol for a given experiment.

To determine the method that best suits an experiment at hand, one must first look at the type of quantitation desired, such as the precise measurement of a single protein or the global quantitation

Method Name	Type of Quantitation		Target Parameters			Strong Points	Weak Points
	Relative	Absolute	Specificity	Multiplexing	Targeted/Shotgun		
ICAT	X	(X)	Cysteinyl Groups	2	Shotgun	Compatible with low-resolution spectrometers	Many proteins can be left unlabeled due to cysteine labeling
ICPL	X	X	Lysine + N-termini	4	Shotgun	Balanced with lower-resolution spectrometers	Modifying at lysine kills tryptic site of digestion, increasing analysis time
GIST	X	(X)	Varies	2	Shotgun	Works well with PTMs and proteins with difficult sequences	Depending on method, unintended or incomplete labeling possible
iTRAQ	X	X	Primary Amine Groups	2 – 8	Shotgun	Reporter tags help with inter-protein comparisons	Ready-made kits can increase experimental costs
mTRAQ	X	X	Primary Amine Groups	2 + Standard	Shotgun	Global standard assists cross-experiment comparisons	Slightly fewer proteins are quantified than iTRAQ; only two conditions
TMT	X	X	1° Amine, Cysteinyl, or Carbonyl Groups	2 – 11	Shotgun	Highest multiplexing capability	Ready-made kits can increase costs; high multiplexing might reduce accuracy
SILAC	X	(X)	Lysine- or Arginine-Containing Proteins	2 – 3	Shotgun	High accuracy with global labeling; all samples mixed early in experiments	High medium cost; only works with cell lines and after 6+ passages
SILAM/SILIP	X		All Nitrogen Atoms	2	Shotgun	*In vivo* labeling with mammals/plants	High reagent costs; labels the entire organism, regardless of goal
Super-SILAC	X		Lysine- or Arginine-Containing Proteins	2	Shotgun	Imitates complex organisms that can't be otherwise metabolically labeled	Long and potentially expensive process of preparing multiple cell lines
pSILAC	X		Lysine- or Arginine-Containing Proteins	2	Shotgun	Compares protein translation rates in different conditions	High reagent costs; not all cell lines accept changes in medium
AQUA		X	1 Selected Peptide	1	Targeted	High accuracy absolute quantitation; compatible with PTMs	Only quantifies a single peptide
QconCAT		X	20-30 Proteins with 50 Peptides	1	Targeted	Cheaper method for quantifying many proteins	Long preparatory protocol; user makes the labeled peptide
LRP	X		Approximate Global Comparison	1	Targeted	Inter-run comparisons without directly labeling samples	High variance due to variable ionization rates
PSAQ		X	1 Protein with All its Peptides	1	Targeted	Extremely high accuracy; works well with low-abundance proteins	Intact, labeled proteins can incur high costs
Label-Free	X		All Ionizable, Detectable Peptides	1	Shotgun	Very high throughput; no labeling necessary; works with PTMs	Processing is very resource intensive; slightly lowered accuracy

Fig. 4 A concise chart comparing various quantitation methods, detailing the following points: compatible with relative and absolute quantitation [the methods with (X) can be modified to work with absolute quantitation, but one multiplexing condition is lost and a known quantity of protein must be used and labeled]; compatible with the method; how many multiplexing conditions are possible; if the proteins/peptides must be preselected (targeted) or not (shotgun); and the strong/weak points of each method. Abbreviations: *ICAT* isotope-coded affinity tag, *ICPL* isotope-coded protein labeling, *GIST* global internal standard technology, *iTRAQ* isobaric tag for relative and absolute quantitation, *mTRAQ* mass-differential tags for relative and absolute quantitation, *TMT* tandem mass tags, *SILAC* stable isotope labeling of amino acids in cell culture, *SILAM* stable isotope labeling of amino acids in mammals, *SILIP* stable isotope labeling in planta, *AQUA* absolute quantification, *QconCAT* quantitative concatemers, *LRP* labeled reference peptide, *PSAQ* protein standard absolute quantification, *PTM* posttranslational modification

of all proteins in a sample, for example. Then, the analysis equipment available (mass spectrometer setup and analysis systems) must be taken into account to filter for feasible methods. Taking these steps into account will narrow down the available options. The level of data precision desired and the amount of time and money that can be dedicated to the project will then help determine which choice would best fit an experiment. New and improved software is continually being released for various methods, some open-source and some paid, which should also be taken into consideration in addition to any reagents when determining the budget.

Lastly, as a general rule, when selecting a technique, when samples are combined farther up the workflow, the chance of having propagating experimental errors is reduced. Steps for pre-fractionation, digestion, and analysis can all add confounding and perpetuating variables, affecting results. In some LFQ software, XIC alignment via peak picking helps retroactively correct for some of these variables. Workflows and sample combination times are elaborated upon in Fig. 3, inspired by and expanding upon a design originally published by Shao-En Ong and Matthias Mann [154].

5.1 Absolute or Relative Quantitation

When choosing between absolute and relative quantitation, the ultimate goal of the experiment is the leading influential factor. While absolute quantitation can be key in biomarker studies, clinical applications and copy number calculations, not every experiment needs such a high level of accuracy. Since absolute quantitation uses labels or standards, this increases the cost of the experiment. Therefore, in cases where it is not necessary, and determination of fold-changes between samples is sufficient, relative quantitation is recommended.

5.1.1 Absolute Quantitation

Generally speaking, absolute quantitation can be performed using either protein/peptide tags or labeling (ICAT, ICPL, GIST, SILAC, etc.), or reporter ions (iTRAQ, TMT, etc.), or with spike-ins (AQUA, QconCAT, PSAQ, etc.).

When using tags, proteins or peptides are globally labeled, allowing for the identification of peptides belonging to different conditions. If one of those conditions is a known amount of a protein standard, then the intensity of the sample condition could be compared to that standard. However, due to varying rates of ionization of peptides and other confounding factors, this comparison can only be made for the standard proteins' pairs in the sample using this method. As such, these methods are normally not recommended here for absolute quantitation. In a method termed Absolute SILAC [155], protein standards have been used in this way with cell cultures to determine copy numbers.

When using reporter ions, multiplexing capability combined with the consistency of reporter ionization offers better compatibility with absolute quantitation of samples. In general, isobaric tags (quantitation by MS/MS data; e.g., iTRAQ, TMT) provide better data than standard tags (ICAT, ICPL, GIST) because there are no undesired peak overlaps from different tags, the LC elution profile of peptides remains the same, and reporter tags ionize with similar efficiency between peptides. Between iTRAQ and TMT, there is no clear advantage of using either one over the other [24] and in principle they are nearly identical. However, using higher multiplexing, the potential to reduce accuracy is possible [25–28]. mTRAQ has a clear advantage when collecting data over long periods of time, on different machines, or under different conditions but one study found that it may come at the cost of a reduction in identification of proteins with posttranslational modifications and an increase in data variability [20].

Lastly, absolute quantitation is possible with the use of a labeled standard. In QconCAT, AQUA, and PSAQ, a reference protein or peptide is used as a comparison for signal intensity. These can quantify a limited number of peptides/proteins with high accuracy, with some publications claiming that LFQ outperforms iTRAQ and equivalents in identification and coverage [156–158] with a slight decrease in accuracy [159]. One of the most significant

downsides to using label-free based methods is the resource requirement for high-resolution mass spectrometers and large-scale data processing.

Al Feteisi et al. [160] detail the cost–benefit relations between a few different absolute quantitation methods, AQUA, QconCAT, and PSAQ, comparing each method's cost with its sensitivity, reproducibility, and time. They also concluded that LFQ is the most cost-effective method, although depending on the experimental design and data accuracy required, other methods may of course be better suited for a specific protocol.

5.1.2 Relative Quantitation

Within the realm of relative quantitation, a few more possibilities are available to users. Along with the methods listed above, many of them being compatible with both relative and absolute quantitation, some additional LFQ methods are also compatible. As previously mentioned, unless an experiment explicitly requires absolute quantitation, many times the cost of performing such an experiment does not recompense the data obtained. If one sample source with two or more conditions is being compared, fold-change can be more than enough data and all samples can be compared to a control base value.

5.2 SIL or LFQ

Quantitation can be performed by either spectral counting-based methods or by means of XIC data. Using either method, relative quantitation can be SIL and LFQ.

When comparing a label-based and label-free method (iTRAQ and CE alteration for DIA), Patel et al. [161] found that the number of protein identifications is similar and there is high agreement between the two methods; however, when identifications by a single peptide were removed, LFQ performed better. In another study by Li et al. [24], LFQ was found to have a wider dynamic range than TMT, iTRAQ, and metabolic labeling but variation between replicates was higher, meaning that the quantitative data was less precise.

The reduction in experimental complexity and lower cost due to a lack of labels or labeling steps increases the attractiveness of LFQ methods, while higher reproducibility and data accuracy especially with low-abundance targets can be obtained with the use of label-based methods.

5.2.1 SIL

Between SIL methods, many protocols, along with their benefits and drawbacks, are similar; but some advantages have been recorded of one method over others. For example, iTRAQ and TMT show higher precision than metabolic labeling in relative quantitation [24]. However, in experiments that require extensive preparatory steps before MS analysis such as fractionation, abundant protein depletion, co-immunoprecipitation, other enrichments and metabolic labeling still remains a strong candidate as it removes several stages at which user-based errors could occur.

5.2.2 LFQ

When comparing label-free spectral counting and XIC methods, conflicting evidence on superiority has been documented [78, 91, 92]. However, this is thought to be due to differences in protocol and the mass spectrometers in use when these comparisons were made. When these variables were removed, neither spectral counting nor XIC methods inherently carried a specific advantage over the other [162]. However, processing methods of the obtained data can still have a significant effect on inter-replicate reproducibility and accuracy.

It is this difference which carries the most weight for the selection of a label-free method. In a study comparing five different analysis formulas, MaxLFQ and NSAF provided better results and replicability, while SI_N gave the lowest standard quantification error values, and NSAF best identified background proteins from protein targets [162].

5.3 Targeted, DDA or DIA

When collecting both labeled and label-free data, three main methods of mass spectrometer settings can be used: targeted (MRM/PRM), DDA, or DIA. Targeted acquisition is reserved exclusively for small-scale experiments, since whole-proteome "shotgun" analyses are not possible as all peptides to be quantified must be selected before injecting the sample. However, this preselection process increases signal-to-noise ratios and greatly increases quantitation accuracy [66]. Targeted acquisition is also most compatible with older, lower-resolution mass spectrometers.

DDA in comparison is able to identify and quantify proteins due to a peptide selection process during data acquisition. This acquisition method is most often used when performing SIL quantitation. Due to the protocol having existed for more than two decades, there is extensive software available for data processing, and is the default mode on most commercially available mass spectrometers [163].

Lastly, made available due to advances in computational technology, DIA improves upon DDA in some ways, while getting closer to the data quality found in targeted methods. This is the method of choice for LFQ in conjunction with special ion abundance methods. There is a relatively high accuracy in quantitation, with high peptide identification and reproducibility; but this comes at the price of significantly more complex data processing steps, which are still being perfected due to the recent development of this method. Hu et al. have published a much more in-depth comparison between these three methods [163].

5.3.1 Targeted

Between SRM, MRM, and PRM, the latter has the capability to provide quantitative data over a wider dynamic range and confers higher specificity, while having the potential for a simpler assay development stage than SRM [73]. This can be contrasted with a potential decrease in precision of PRM [73]. However, further

studies have suggested that for some applications, PRM can show comparable data quality in linearity and dynamic range, as well as precision [164].

Targeted data acquisition can be extremely useful in specific cases that require high precision and accuracy on a small subset of proteins, such as when quantifying sites of posttranslational modifications [165], measuring enzyme activity by quantification of the protein substrates [166], or when comparing various posttranslational modification sites on a single protein and their stoichiometry [167, 168]. For a more global measurement of this type of analysis, AP-MS can be combined with DDA/DIA methods. However due to inherent variations with antibody-associated analyses, targeted acquisition still confers higher quantitative accuracy.

5.3.2 DDA

DDA takes in a wider scope of data compared with targeted acquisition and is useful for shotgun proteomics, allowing a large number of proteins to be identified and quantified in a single experiment without the need to select peptides before the experiment. When performing large-scale experiments, targeted acquisition methods are not feasible, especially with respect to time required to prepare the experiments and the amount of sample that would be required. As such, DDA has become a standard for whole-proteome quantitation. On the negative side, this large increase in identification and quantitation led to significant drops in reproducibility, in some cases having only around 60% overlap when comparing multiple replicates [169].

One major supporting factor for DDA is its ease of use and broad availability of software for data processing as well as its compatibility with both label-free and stable isotope-based, relative quantitation. However, since peptides are not repeatedly sampled in attempts to obtain the largest amount of identification data possible (and in some cases dynamically excluded to increase coverage of proteins with lower abundance), absolute quantitation is difficult at best.

5.3.3 DIA

One additional step of complexity beyond DDA is DIA, which slightly reduces the number of identifications and number of quantified proteins in exchange for higher reproducibility. This also comes at the cost of more resource-intensive data processing steps, since the spectra obtained are much more convoluted. There is also a vulnerability to data variation due to the presence of other peptides in a sample, since multiple peptides are sent for fragmentation at the same time.

If the resources are available for DIA acquisition and processing, it is the best fit for quantitative shotgun proteomics due to the large amount of data (and higher accuracy) possible with such a small amount of machine and sample preparation.

6 Concluding Remarks

Quantitative proteomics is a continually evolving field that has existed for nearly 20 years. Over time, improvements to mass spectrometers, experimental techniques and computational ability have led to a sprawling array of available methods. These methods allow scientists to identify and quantify a single to a few thousand proteins and, in certain cases, with subfemtomole accuracy. With the increasing importance of proteomics and related fields in both biological and clinical research, mass spectrometry is also growing increasingly more valuable. With its broad applicability, innumerable protocols have been perfected for specific uses in MS-based protein quantitation. Most simply divided, quantitation of proteins can be classified as labeled or label-free quantitation.

In labeled quantitation (stable-isotope and metabolic labeling), multiple samples can be run through the mass spectrometer at once (multiplexing), providing extremely high accuracy for relative (or absolute) abundance measurements. This high accuracy can be vital in clinical applications, where only a small sample is available or when dealing with proteins with extremely low abundance, as is often the case with disease and medication efficacy biomarkers. Several techniques have also been published using labeled standards to produce absolute values for protein quantitation, which provides better reproducibility and accuracy, since data is not relative to other samples and less prone to user error. Labeling proteins has been proven to function with samples consisting of peptides, proteins, cell cultures, and even living mammals and plants. Data processing is relatively light, balancing out the often longer and more costly protocols, due to the requirement for pure, isotopically labeled tags or media.

Comparatively, label-free quantitation uses no chemical tags, reducing the cost and preparation time for individual experiments, and allows large-scale analyses to be performed quickly and robustly, and thousands of proteins in a single, complex sample can be identified and relatively quantified. Due to the lack of tags, multiplexing is not possible and different machine and environmental conditions can change results, although steps are usually taken to correct such issues. Data analysis is relatively lengthier due to additional processing such as an alignment step and signal deconvolution. With the addition of a single labeled reference protein or protein spike from another organism, absolute quantitation is also possible.

There is rarely a single correct answer when designing any experiment, and methods are constantly being developed and improved upon. Each technique has its own niche and strong points, along with its restrictions and drawbacks. We believe that this guide provides a summary of each method with its strong and

weak points and would help the user to determine which method (or methods) would be best for a quantitative proteomic experiment at hand.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Prasad B, Vrana M, Mehrotra A, Johnson K, Bhatt DK (2017) The promises of quantitative proteomics in precision medicine. J Pharm Sci 106:738–744
2. Achour B, Al-Majdoub ZM, Al Feteisi H, Elmorsi Y, Rostami-Hodjegan A (2015) Ten years of QconCATs: application of multiplexed quantification to small medically relevant proteomes. Int J Mass Spectrom 391:93–104
3. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A et al (2015) Tissue-based map of the human proteome. Science 347:1260419. https://doi.org/10.1126/science.1260419
4. Dongré AR, Jones JL, Somogyi Á, Wysocki VH (1996) Influence of peptide composition, gas-phase basicity, and chemical modification on fragmentation efficiency: evidence for the Mobile proton model. J Am Chem Soc 118:8365–8374
5. King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T (2000) Mechanistic investigation of ionization suppression in electrospray ionization. J Am Soc Mass Spectrom 11:942–950
6. Hansen KC, Schmitt-Ulms G, Chalkley RJ, Hirsch J, Baldwin MA, Burlingame AL (2003) Mass spectrometric analysis of protein mixtures at low levels using cleavable 13C-isotope-coded affinity tag and multidimensional chromatography. Mol Cell Proteomics 2:299–314
7. Zhang R, Sioma CS, Wang S, Regnier FE (2001) Fractionation of isotopically labeled peptides in quantitative proteomics. Anal Chem 73:5142–5149
8. Griffiths J (2007) Quantitative proteomics comes of age. Anal Chem:6451–6454
9. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol 17:994–999
10. Yi EC, Li X-J, Cooke K, Lee H, Raught B, Page A et al (2005) Increased quantitative proteome coverage with (13)C/(12)C-based, acid-cleavable isotope-coded affinity tag reagent and modified data acquisition scheme. Proteomics 5:380–387
11. Schmidt A, Kellermann J, Lottspeich F (2005) A novel strategy for quantitative proteomics using isotope-coded protein labels. Proteomics 5:4–15
12. UniProt Consortium T (2018) UniProt: the universal protein knowledgebase. Nucleic Acids Res 46:2699–2699
13. Chakraborty A, Regnier FE (2002) Global internal standard technology for comparative proteomics. J Chromatogr A 949:173–184
14. Ji J, Chakraborty A, Geng M, Zhang X, Amini A, Bina M et al (2000) Strategy for qualitative and quantitative analysis in proteomics based on signature peptides. J Chromatogr B Biomed Sci App 745:197–210
15. Oda Y, Huang K, Cross FR, Cowburn D, Chait BT (1999) Accurate quantitation of protein expression and site-specific phosphorylation. Proc Natl Acad Sci U S A 96:6591–6596
16. Veenstra TD, Martinović S, Anderson GA, Pasa-Tolić L, Smith RD (2000) Proteome analysis using selective incorporation of isotopically labeled amino acids. J Am Soc Mass Spectrom 11:78–82
17. Schnölzer M, Jedrzejewski P, Lehmann WD (1996) Protease-catalyzed incorporation of 18O into peptide fragments and its

application for protein sequencing by electrospray and matrix-assisted laser desorption/ionization mass spectrometry. Electrophoresis 17:945–953

18. Küster B, Mann M (1999) 18O-labeling of N-glycosylation sites to improve the identification of gel-separated glycoproteins using peptide mass mapping and database searching. Anal Chem 71:1431–1440
19. DeSouza LV, Taylor AM, Li W, Minkoff MS, Romaschin AD, Colgan TJ et al (2008) Multiple reaction monitoring of mTRAQ-labeled peptides enables absolute quantification of endogenous levels of a potential cancer marker in cancerous and normal endometrial tissues. J Proteome Res 7:3525–3534
20. Mertins P, Udeshi ND, Clauser KR, Mani DR, Patel J, Ong SE et al (2012) iTRAQ labeling is superior to mTRAQ for quantitative global proteomics and phosphoproteomics. Mol Cell Proteomics 11. https://doi.org/10.1074/mcp.M111.014423
21. Thompson A, Schäfer J, Kuhn K, Kienle S, Schwarz J, Schmidt G et al (2003) Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal Chem 75:1895–1904
22. Hung C-W, Tholey A (2012) Tandem mass tag protein labeling for top-down identification and quantification. Anal Chem 84:161–170
23. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S et al (2004) Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 3:1154–1169
24. Li Z, Adams RM, Chourey K, Hurst GB, Hettich RL, Pan C (2012) Systematic comparison of label-free, metabolic labeling, and isobaric chemical labeling for quantitative proteomics on LTQ Orbitrap Velos. J Proteome Res 11:1582–1590
25. Sandberg A, Branca RMM, Lehtiö J, Forshed J (2014) Quantitative accuracy in mass spectrometry based proteomics of complex samples: the impact of labeling and precursor interference. J Proteome 96:133–144
26. Pottiez G, Wiederin J, Fox HS, Ciborowski P (2012) Comparison of 4-plex to 8-plex iTRAQ quantitative measurements of proteins in human plasma samples. J Proteome Res 11:3774–3781
27. Casey TM, Khan JM, Bringans SD, Koudelka T, Takle PS, Downs RA et al (2017) Analysis of reproducibility of proteome coverage and quantitation using isobaric mass tags (iTRAQ and TMT). J Proteome Res 16:384–392
28. Pichler P, Köcher T, Holzmann J, Mazanek M, Taus T, Ammerer G et al (2010) Peptide Labeling with isobaric tags yields higher identification rates using iTRAQ 4-Plex compared to TMT 6-Plex and iTRAQ 8-Plex on LTQ Orbitrap. Anal Chem 82:6549–6558
29. McAlister GC, Huttlin EL, Haas W, Jedrychowski MP, Rogers JC, Kuhn K et al (2012) Increasing the multiplexing capacity of TMT using reporter ion isotopologues with isobaric masses. Anal Chem 84:7469–7478
30. Jiang H, English AM (2002) Quantitative analysis of the yeast proteome by incorporation of isotopically labeled leucine. J Proteome Res 1:345–350
31. Ong S-E, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A et al (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 1:376–386
32. Gu S, Pan S, Bradbury EM, Chen X (2003) Precise peptide sequencing and protein quantification in the human proteome through in vivo lysine-specific mass tagging. J Am Soc Mass Spectrom 14:1–7
33. Ong S-E, Mittler G, Mann M (2004) Identifying and quantifying in vivo methylation sites by heavy methyl SILAC. Nat Methods 1:119–126
34. Ong S-E, Mann M (2006) A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). Nat Protoc 1:2650–2660
35. Ibarrola N, Kalume DE, Gronborg M, Iwahori A, Pandey A (2003) A proteomic approach for quantitation of phosphorylation using stable isotope labeling in cell culture. Anal Chem 75:6043–6049
36. Zhang R, Regnier FE (2002) Minimizing resolution of isotopically coded peptides in comparative proteomics. J Proteome Res 1:139–147
37. Hwang S-I, Lundgren DH, Mayya V, Rezaul K, Cowan AE, Eng JK et al (2006) Systematic characterization of nuclear proteome during apoptosis: a quantitative proteomic study by differential extraction and stable isotope labeling. Mol Cell Proteomics 5:1131–1145
38. Ong S-E, Kratchmarova I, Mann M (2003) Properties of 13C-substituted arginine in

stable isotope labeling by amino acids in cell culture (SILAC). J Proteome Res 2:173–181

39. Schmidt F, Strozynski M, Salus SS, Nilsen H, Thiede B (2007) Rapid determination of amino acid incorporation by stable isotope labeling with amino acids in cell culture (SILAC). Rapid Commun Mass Spectrom 21:3919–3926
40. Hoof DV, Pinkse MWH, Oostwaard DW-V, Mummery CL, Heck AJ, Krijgsveld J (2007) An experimental correction for arginine-to-proline conversion artifacts in SILAC-based quantitative proteomics. Nat Methods 4:677–678
41. Bendall SC, Hughes C, Stewart MH, Doble B, Bhatia M, Lajoie GA (2008) Prevention of amino acid conversion in SILAC experiments with embryonic stem cells. Mol Cell Proteomics 7:1587–1597
42. Lössner C, Warnken U, Pscherer A, Schnölzer M (2011) Preventing arginine-to-proline conversion in a cell-line-independent manner during cell cultivation under stable isotope labeling by amino acids in cell culture (SILAC) conditions. Anal Biochem 412:123–125
43. Bicho CC, de Lima Alves F, Chen ZA, Rappsilber J, Sawin KE (2010) A genetic engineering solution to the "arginine conversion problem" in stable isotope labeling by amino acids in cell culture (SILAC). Mol Cell Proteomics 9:1567–1577
44. Schwanhäusser B, Gossen M, Dittmar G, Selbach M (2009) Global analysis of cellular protein translation by pulsed SILAC. Proteomics 9:205–209
45. Kaller M, Oeljeklaus S, Warscheid B, Hermeking H (2014) Identification of microRNA targets by pulsed SILAC. Methods Mol Biol 1188:327–349
46. Geiger T, Cox J, Ostasiewicz P, Wisniewski JR, Mann M (2010) Super-SILAC mix for quantitative proteomics of human tumor tissue. Nat Methods 7:383–385
47. Neubert TA, Tempst P (2010) Super-SILAC for tumors and tissues. Nat Methods 7:361–362
48. Geiger T, Wisniewski JR, Cox J, Zanivan S, Kruger M, Ishihama Y et al (2011) Use of stable isotope labeling by amino acids in cell culture as a spike-in standard in quantitative proteomics. Nat Protoc 6:147–157
49. Wu CC, MacCoss MJ, Howell KE, Matthews DE, Yates JR 3rd (2004) Metabolic labeling of mammalian organisms with stable isotopes for quantitative proteomic analysis. Anal Chem 76:4951–4959
50. Rauniyar N, McClatchy DB, Yates JR (2013) Stable isotope labeling of mammals (SILAM) for in vivo quantitative proteomic analysis. Methods 61:260–268
51. Huttlin EL, Chen X, Barrett-Wilt GA, Hegeman AD, Halberg RB, Harms AC et al (2009) Discovery and validation of colonic tumor-associated proteins via metabolic labeling and stable isotopic dilution. Proc Natl Acad Sci U S A 106:17235–17240
52. Rose JC, Epperson LE, Carey HV, Martin SL (2011) Seasonal liver protein differences in a hibernator revealed by quantitative proteomics using whole animal isotopic labeling. Comp Biochem Physiol Part D Genomics Proteomics 6:163–170
53. McClatchy DB, Yates JR (2014) Stable isotope labeling in mammals (SILAM). Methods Mol Biol 1156:133–146
54. Schaff JE, Mbeunkui F, Blackburn K, Bird DM, Goshe MB (2008) SILIP: a novel stable isotope labeling method for in planta quantitative proteomic analysis. Plant J 56:840–854
55. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc Natl Acad Sci U S A 100:6940–6945
56. Kirkpatrick DS, Gerber SA, Gygi SP (2005) The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. Methods 35:265–273
57. Kettenbach AN, Rush J, Gerber SA (2011) Absolute quantification of protein and post-translational modification abundance with stable isotope-labeled synthetic peptides. Nat Protoc 6:175–186
58. Zhang H, Liu Q, Zimmerman LJ, Ham AJ, Slebos RJ, Rahman J et al (2011) Methods for peptide and protein quantitation by liquid chromatography-multiple reaction monitoring mass spectrometry. Mol Cell Proteomics 10. https://doi.org/10.1074/mcp.M110.006593
59. Al-Majdoub ZM, Carroll KM, Gaskell SJ, Barber J (2014) Quantification of the proteins of the bacterial ribosome using QconCAT technology. J Proteome Res 13:1211–1222
60. Pratt JM, Simpson DM, Doherty MK, Rivers J, Gaskell SJ, Beynon RJ (2006) Multiplexed absolute quantification for proteomics using concatenated signature peptides encoded by QconCAT genes. Nat Protoc 1:1029–1043
61. Scott KB, Turko IV, Phinney KW (2016) Chapter eleven—QconCAT: internal

standard for protein quantification. Methods Enzymol 566:289–303

62. Brownridge PJ, Harman VM, Simpson DM, Beynon RJ (2012) Absolute multiplexed protein quantification using QconCAT technology. Methods Mol Biol 893:267–293
63. Dupuis A, Hennekinne J-A, Garin J, Brun V (2008) Protein standard absolute quantification (PSAQ) for improved investigation of staphylococcal food poisoning outbreaks. Proteomics 8:4633–4636
64. Adrait A, Lebert D, Trauchessec M, Dupuis A, Louwagie M, Masselon C et al (2012) Development of a protein standard absolute quantification (PSAQTM) assay for the quantification of Staphylococcus aureus enterotoxin a in serum. J Proteome 75:3041–3049
65. Kaiser SE, Riley BE, Shaler TA, Trevino RS, Becker CH, Schulman H et al (2011) Protein standard absolute quantification (PSAQ) method for the measurement of cellular ubiquitin pools. Nat Methods 8:691–696
66. Liebler DC, Zimmerman LJ (2013) Targeted quantitation of proteins by mass spectrometry. Biochemistry 52:3797–3806
67. Lange V, Picotti P, Domon B, Aebersold R (2008) Selected reaction monitoring for quantitative proteomics: a tutorial. Mol Syst Biol 4:222. https://doi.org/10.1038/msb.2008.61
68. Stahl-Zeng J, Lange V, Ossola R, Eckhardt K, Krek W, Aebersold R et al (2007) High sensitivity detection of plasma proteins by multiple reaction monitoring of N-glycosites. Mol Cell Proteomics 6:1809–1817
69. Keshishian H, Addona T, Burgess M, Kuhn E, Carr SA (2007) Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. Mol Cell Proteomics 6:2212–2229
70. Kitteringham NR, Jenkins RE, Lane CS, Elliott VL, Park BK (2009) Multiple reaction monitoring for quantitative biomarker analysis in proteomics and metabolomics. J Chromatogr B 877:1229–1239
71. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B et al (2010) Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics 26:966–968
72. Colangelo CM, Chung L, Bruce C, Cheung K-H (2013) Review of software tools for design and analysis of large scale MRM proteomic datasets. Methods 61:287–298
73. Peterson AC, Russell JD, Bailey DJ, Westphall MS, Coon JJ (2012) Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. Mol Cell Proteomics 11:1475–1488
74. Rauniyar N (2015) Parallel reaction monitoring: a targeted experiment performed using high resolution and high mass accuracy mass spectrometry. Int J Mol Sci 16:28566–28581
75. Tsuchiya H, Tanaka K, Saeki Y (2013) The parallel reaction monitoring method contributes to a highly sensitive polyubiquitin chain quantification. Biochem Biophys Res Commun 436:223–229
76. Liu H, Sadygov RG, Yates JR (2004) A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem 76:4193–4201
77. Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B (2007) Quantitative mass spectrometry in proteomics: a critical review. Anal Bioanal Chem 389:1017–1031
78. Old WM, Meyer-Arendt K, Aveline-Wolf L, Pierce KG, Mendoza A, Sevinsky JR et al (2005) Comparison of label-free methods for quantifying human proteins by shotgun proteomics. Mol Cell Proteomics 4:1487–1502
79. Braisted JC, Kuntumalla S, Vogel C, Marcotte EM, Rodrigues AR, Wang R et al (2008) The APEX quantitative proteomics tool: generating protein quantitation estimates from LC-MS/MS proteomics results. BMC Bioinformatics 9:529. https://doi.org/10.1186/1471-2105-9-529
80. Park CY, Käll L, Klammer AA, MacCoss MJ, Noble WS (2008) Rapid and accurate peptide identification from tandem mass spectra. J Proteome Res 7:3022–3027
81. Shinoda K, Tomita M, Ishihama Y (2010) emPAI Calc—for the estimation of protein abundance from large-scale identification data by liquid chromatography-tandem mass spectrometry. Bioinformatics 26:576–577
82. Heinecke NL, Pratt BS, Vaisar T, Becker L (2010) PepC: proteomics software for identifying differentially expressed proteins based on spectral counting. Bioinformatics 26:1574–1575
83. Choi H, Fermin D, Nesvizhskii AI (2008) Significance analysis of spectral count data in label-free shotgun proteomics. Mol Cell Proteomics 7:2373–2385
84. Choi H, Kim S, Fermin D, Tsou CC, Nesvizhskii AI (2015) QPROT: statistical method for testing differential expression using protein-level intensity data in label-free

quantitative proteomics. J Proteome 129:121–126

85. Fu X, Gharib SA, Green PS, Aitken ML, Frazer DA, Park DR et al (2008) Spectral index for assessment of differential protein expression in shotgun proteomics. J Proteome Res 7:845–854
86. Griffin NM, Yu J, Long F, Oh P, Shore S, Li Y et al (2010) Label-free, normalized quantification of complex mass spectrometry data for proteomic analysis. Nat Biotechnol 28:83–89
87. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J et al (2005) Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics 4:1265–1272
88. Paoletti AC, Parmely TJ, Tomomori-Sato C, Sato S, Zhu D, Conaway RC et al (2006) Quantitative proteomic analysis of distinct mammalian mediator complexes using normalized spectral abundance factors. Proc Natl Acad Sci U S A 103:18928–18933
89. Zhang Y, Wen Z, Washburn MP, Florens L (2010) Refinements to label free proteome quantitation: how to deal with peptides shared by multiple proteins. Anal Chem 82:2272–2281
90. Bondarenko PV, Chelius D, Shaler TA (2002) Identification and relative quantitation of protein mixtures by enzymatic digestion followed by capillary reversed-phase liquid chromatography-tandem mass spectrometry. Anal Chem 74:4741–4749
91. Milac TI, Randolph TW, Wang P (2012) Analyzing LC-MS/MS data by spectral count and ion abundance: two case studies. Stat Interface 5:75–87
92. Zybailov B, Coleman MK, Florens L, Washburn MP (2005) Correlation of relative abundance ratios derived from peptide ion chromatograms and spectrum counting for quantitative proteomic analysis using stable isotope labeling. Anal Chem 77:6218–6224
93. Leptos KC, Sarracino DA, Jaffe JD, Krastins B, Church GM (2006) MapQuant: open-source software for large-scale protein quantification. Proteomics 6:1770–1782
94. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26:1367–1372
95. Sturm M, Bertsch A, Gröpl C, Hildebrandt A, Hussong R, Lange E et al (2008) OpenMS - an open-source software framework for mass spectrometry. BMC Bioinformatics 9:163. https://doi.org/10.1186/1471-2105-9-163
96. Zhang J, Xin L, Shan B, Chen W, Xie M, Yuen D et al (2012) PEAKS DB: de novo sequencing assisted database search for sensitive and accurate peptide identification. Mol Cell Proteomics 11. https://doi.org/10.1074/mcp.M111.010587
97. Häkkinen J, Vincic G, Månsson O, Wårell K, Levander F (2009) The proteios software environment: an extensible multiuser platform for management and analysis of proteomics data. J Proteome Res 8:3037–3043
98. Li X, Yi EC, Kemp CJ, Zhang H, Aebersold R (2005) A software suite for the generation and comparison of peptide arrays from sets of data collected by liquid chromatography-mass spectrometry. Mol Cell Proteomics 4:1328–1340
99. Mueller LN, Rinner O, Schmidt A, Letarte S, Bodenmiller B, Brusniak MY et al (2007) SuperHirn—a novel tool for high resolution LC-MS-based peptide/protein profiling. Proteomics 7:3470–3480
100. Välikangas T, Suomi T, Elo LL (2017) A comprehensive evaluation of popular proteomics software workflows for label-free proteome quantification and imputation. Brief Bioinform. https://doi.org/10.1093/bib/bbx054
101. Silva JC, Gorenstein MV, Li G-Z, Vissers JP, Geromanos SJ (2006) Absolute quantification of proteins by LCMSE a virtue of parallel ms acquisition. Mol Cell Proteomics 5:144–156
102. Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J et al (2011) Global quantification of mammalian gene expression control. Nature 473:337–342
103. Krey JF, Wilmarth PA, Shin J-B, Klimek J, Sherman NE, Jeffery ED et al (2014) Accurate label-free protein quantitation with high- and low-resolution mass spectrometers. J Proteome Res 13:1034–1044
104. Gerster S, Kwon T, Ludwig C, Matondo M, Vogel C, Marcotte EM et al (2014) Statistical approach to protein quantification. Mol Cell Proteomics 13:666–677
105. McIlwain S, Mathews M, Bereman MS, Rubel EW, MacCoss MJ, Noble WS et al (2012) Estimating relative abundances of proteins from shotgun proteomics data. BMC Bioinformatics 13:308. https://doi.org/10.1186/1471-2105-13-308
106. Michalski A, Cox J, Mann M (2011) More than 100,000 detectable peptide species elute

in single shotgun proteomics runs but the majority is inaccessible to data-dependent LC–MS/MS. J Proteome Res 10:1785–1793

107. Kuster B, Schirle M, Mallick P, Aebersold R (2005) Scoring proteomes with proteotypic peptide probes. Nat Rev Mol Cell Biol 6:577–583
108. Venable JD, Dong M-Q, Wohlschlegel J et al (2004) Automated approach for quantitative analysis of complex peptide mixtures from tandem mass spectra. Nat Methods 1:39–45
109. Purvine S, Eppel JT, Yi EC, Goodlett DR (2003) Shotgun collision-induced dissociation of peptides using a time of flight mass analyzer. Proteomics 3:847–850
110. Aivett B, Emmanuel V, Jeremy L, Strambio-De-Castillia C, Hopfgartner G et al (2015) Processing strategies and software solutions for data-independent acquisition in mass spectrometry. Proteomics 15:964–980
111. Geiger T, Cox J, Mann M (2010) Proteomics on an Orbitrap benchtop mass spectrometer using all-ion fragmentation. Mol Cell Proteomics 9:2252–2261
112. Silva JC, Denny R, Dorschel CA, Gorenstein M, Kass IJ, Li GZ et al (2005) Quantitative proteomic analysis by accurate mass retention time pairs. Anal Chem 77:2187–2200
113. Silva JC, Denny R, Dorschel C, Gorenstein MV, Li GZ, Richardson K et al (2006) Simultaneous qualitative and quantitative analysis of the Escherichia coli proteome a sweet tale. Mol Cell Proteomics 5:589–607
114. Distler U, Kuharev J, Navarro P, Levin Y, Schild H, Tenzer S (2014) Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics. Nat Methods 11:167–170
115. Distler U, Kuharev J, Navarro P, Tenzer S (2016) Label-free quantification in ion mobility-enhanced data-independent acquisition proteomics. Nat Protoc 11:795–812
116. Bond NJ, Shliaha PV, Lilley KS, Gatto L (2013) Improving qualitative and quantitative performance for MSE-based label-free proteomics. J Proteome Res 12:2340–2353
117. Shliaha PV, Bond NJ, Gatto L, Lilley KS (2013) Effects of traveling wave ion mobility separation on data independent acquisition in proteomics studies. J Proteome Res 12:2323–2339
118. Carvalho PC, Han X, Xu T, Carvalho Mda G, Barbosa VC, Yates JR 3rd (2010) XDIA: improving on the label-free data-independent analysis. Bioinformatics 26:847–848
119. Panchaud A, Jung S, Shaffer SA, Aitchison JD, Goodlett DR (2011) Faster, quantitative, and accurate precursor acquisition independent from ion count. Anal Chem 83:2250–2257
120. Weisbrod CR, Eng JK, Hoopmann MR, Baker T, Bruce JE (2012) Accurate peptide fragment mass analysis: multiplexed peptide identification and quantification. J Proteome Res 11:1621–1632
121. Gillet LC, Navarro P, Tate S, Röst H, Selevsek N, Reiter L et al (2012) Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. Mol Cell Proteomics 11. https://doi.org/10.1074/mcp.O111.016717
122. Vowinckel J, Capuano F, Campbell K, Deery MJ, Lilley KS, Ralser M (2014) The beauty of being (label)-free: sample preparation methods for SWATH-MS and next-generation targeted proteomics. F1000Res 2:272. https://doi.org/10.12688/f1000research.2-272.v2
123. Egertson JD, MacLean B, Johnson R, Xuan Y, MacCoss MJ (2015) Multiplexed peptide analysis using data-independent acquisition and skyline. Nat Protoc 10:887–903
124. Egertson JD, Kuehn A, Merrihew GE, Bateman NW, MacLean BX, Ting YS et al (2013) Multiplexed MS/MS for improved data-independent acquisition. Nat Methods 10:744–746
125. Zhang Y, Bilbao A, Bruderer T, Luban J, Strambio-De-Castillia C, Lisacek F et al (2015) The use of variable Q1 isolation windows improves selectivity in LC–SWATH–MS acquisition. J Proteome Res 14:4359–4371
126. Tsou C-C, Avtonomov D, Larsen B, Tucholska M, Choi H, Gingras AC et al (2015) DIA-umpire: comprehensive computational framework for data-independent acquisition proteomics. Nat Methods 12:258–264
127. Li Y, Zhong C-Q, Xu X, Cai S, Wu X, Zhang Y et al (2015) Group-DIA: analyzing multiple data-independent acquisition mass spectrometry data files. Nat Methods 12:1105–1106
128. Wang J, Tucholska M, Knight JDR, Lambert JP, Tate S, Larsen B et al (2015) MSPLIT-DIA: sensitive peptide identification for data independent acquisition. Nat Methods 12:1106–1108
129. Ting YS, Egertson JD, Bollinger JG, Searle BC, Payne SH, Noble WS et al (2017) PECAN: library-free peptide detection for

data-independent acquisition tandem mass spectrometry data. Nat Methods 14:903–908

130. Kryuchkov F, Verano-Braga T, Hansen TA, Sprenger RR, Kjeldsen F (2013) Deconvolution of mixture spectra and increased throughput of peptide identification by utilization of intensified complementary ions formed in tandem mass spectrometry. J Proteome Res 12:3362–3371
131. McAlister GC, Nusinow DP, Jedrychowski MP, Wühr M, Huttlin EL, Erickson BK et al (2014) MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. Anal Chem 86:7150–7158
132. Ting L, Rad R, Gygi SP, Haas W (2011) MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. Nat Methods 8:937–940
133. Wiśniewski JR, Hein MY, Cox J, Mann M (2014) A "proteomic ruler" for protein copy number and concentration estimation without spike-in standards. Mol Cell Proteomics 13:3497–3506
134. van Holde KE (1989) The proteins of chromatin. I. Histones. In: Chromatin, 1st edn. Springer-Verlag, New York, p 70 ISBN 978-1-4612-3490-6
135. Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Séraphin B (1999) A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol 17:1030–1032
136. Puig O, Caspary F, Rigaut G, Rutz B, Bouveret E, Bragado-Nilsson E et al (2001) The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods 24:218–229
137. Dunham WH, Mullin M, Gingras A-C (2012) Affinity-purification coupled to mass spectrometry: basic principles and strategies. Proteomics 12:1576–1590
138. Smith BJ, Cassoli JS, Guest PC, Martins-de-Souza D (2017) Co-immunoprecipitation for deciphering protein Interactomes. Adv Exp Med Biol 974:229–236
139. Bantscheff M, Eberhard D, Abraham Y, Bastuck S, Boesche M, Hobson S et al (2007) Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. Nat Biotechnol 25:1035–1044
140. Kool J, Jonker N, Irth H, Niessen WMA (2011) Studying protein-protein affinity and immobilized ligand-protein affinity interactions using MS-based methods. Anal Bioanal Chem 401:1109–1112
141. Raida M (2011) Drug target deconvolution by chemical proteomics. Curr Opin Chem Biol 15:570–575
142. Müller J, Hemphill A (2011) Identification of a host cell target for the thiazolide class of broad-spectrum anti-parasitic drugs. Exp Parasitol 128:145–150
143. Jansen G, Wu C, Schade B, Thomas DY, Whiteway M (2005) Drag&Drop cloning in yeast. Gene 344:43–51
144. Slobodin B, Gerst JE (2011) RaPID: an aptamer-based mRNA affinity purification technique for the identification of RNA and protein factors present in ribonucleoprotein complexes. Methods Mol Biol 714:387–406
145. Tsai BP, Wang X, Huang L, Waterman ML (2011) Quantitative profiling of in vivo-assembled RNA-protein complexes using a novel integrated proteomic approach. Mol Cell Proteomics 10. https://doi.org/10.1074/mcp.M110.007385
146. Wei X, Herbst A, Ma D, Aiken J, Li L (2011) A quantitative proteomic approach to prion disease biomarker research: delving into the glycoproteome. J Proteome Res 10:2687–2702
147. Schulze WX, Mann M (2004) A novel proteomic screen for peptide-protein interactions. J Biol Chem 279:10756–10764
148. Lambert J-P, Pawson T, Gingras A-C (2012) Mapping physical interactions within chromatin by proteomic approaches. Proteomics 12:1609–1622
149. Oeffinger M (2012) Two steps forward—one step back: advances in affinity purification mass spectrometry of macromolecular complexes. Proteomics 12:1591–1608
150. Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jørgensen TJ (2005) Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. Mol Cell Proteomics 4:873–886
151. Zhou H, Watts JD, Aebersold R (2001) A systematic approach to the analysis of protein phosphorylation. Nat Biotechnol 19:375–378
152. Cheng Z, Tang Y, Chen Y, Roschitzki B, Schlapbach R, Greber UF et al (2009) Molecular characterization of propionyllysines in non-histone proteins. Mol Cell Proteomics 8:45–52
153. Zhang K, Chen Y, Zhang Z, Zhao Y (2009) Identification and verification of lysine propionylation and butyrylation in yeast core histones using PTMap software. J Proteome Res 8:900–906

154. Ong S-E, Mann M (2005) Mass spectrometry–based proteomics turns quantitative. Nat Chem Biol 1:252–262
155. Hanke S, Besir H, Oesterhelt D, Mann M (2008) Absolute SILAC for accurate quantitation of proteins in complex mixtures down to the Attomole level. J Proteome Res 7:1118–1130
156. Trinh HV, Grossmann J, Gehrig P, Roschitzki B, Schlapbach R, Greber UF et al (2013) iTRAQ-based and label-free proteomics approaches for studies of human adenovirus infections. Int J Proteomics 2013:16. https://doi.org/10.1155/2013/581862
157. Wang H, Alvarez S, Hicks LM (2012) Comprehensive comparison of iTRAQ and label-free LC-based quantitative proteomics approaches using two Chlamydomonas reinhardtii strains of interest for biofuels engineering. J Proteome Res 11:487–501
158. Latosinska A, Vougas K, Makridakis M, Klein J, Mullen W, Abbas M et al (2015) Comparative analysis of label-free and 8-Plex iTRAQ approach for quantitative tissue proteomic analysis. PLoS One 10:e0137048. https://doi.org/10.1371/journal.pone.0137048
159. Megger DA, Pott LL, Ahrens M, Padden J, Bracht T, Kuhlmann K et al (2014) Comparison of label-free and label-based strategies for proteome analysis of hepatoma cell lines. Biochim Biophys Acta 1844:967–976
160. Al Feteisi H, Achour B, Barber J, Rostami-Hodjegan A (2015) Choice of LC-MS methods for the absolute quantification of drug-metabolizing enzymes and transporters in human tissue: a comparative cost analysis. AAPS J 17:438–446
161. Patel VJ, Thalassinos K, Slade SE, Connolly JB, Crombie A, Murrell JC et al (2009) A comparison of labeling and label-free mass spectrometry-based proteomics approaches. J Proteome Res 8:3752–3759
162. Bubis JA, Levitsky LI, Ivanov MV, Tarasova IA, Gorshkov MV (2017) Comparative evaluation of label-free quantification methods for shotgun proteomics. Rapid Commun Mass Spectrom 31:606–612
163. Hu A, Noble WS, Wolf-Yadlin A (2016) Technical advances in proteomics: new developments in data-independent acquisition. F1000Res 5. F1000 Faculty Rev-419. https://doi.org/10.12688/f1000research.7042.1
164. Ronsein GE, Pamir N, von Haller PD, Kim DS, Oda MN, Jarvik GP et al (2015) Parallel reaction monitoring (PRM) and selected reaction monitoring (SRM) exhibit comparable linearity, dynamic range and precision for targeted quantitative HDL proteomics. J Proteome 113:388–399
165. Wolf-Yadlin A, Hautaniemi S, Lauffenburger DA, White FM (2007) Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. Proc Natl Acad Sci U S A 104:5860–5865
166. Sheng Z, Zhang S, Bustos D, Kleinheinz T, Le Pichon CE, Dominguez SL et al (2012) Ser1292 autophosphorylation is an indicator of LRRK2 kinase activity and contributes to the cellular effects of PD mutations. Sci Transl Med 4:164ra161. https://doi.org/10.1126/scitranslmed.3004485
167. Phu L, Izrael-Tomasevic A, Matsumoto ML, Bustos D, Dynek JN, Fedorova AV et al (2011) Improved quantitative mass spectrometry methods for characterizing complex ubiquitin signals. Mol Cell Proteomics 10. https://doi.org/10.1074/mcp.M110.003756
168. Kirkpatrick DS, Hathaway NA, Hanna J, Elsasser S, Rush J, Finley D, King RW et al (2006) Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. Nat Cell Biol 8:700–710
169. Tabb DL, Vega-Montoto L, Rudnick PA, Variyath AM, Ham AJ, Bunk DM et al (2010) Repeatability and reproducibility in proteomic identifications by liquid chromatography–tandem mass spectrometry. J Proteome Res 9:761–776

CHAPTER 2

Protein Succinylation and Malonylation in Schizophrenia

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Keywords: Schizophrenia; Post-translational Modification; Mass Spectrometry; Succinylation; Malonylation; Oligodendrocytes; Postmortem

Abstract:

Schizophrenia (SCZ) is a multifactorial mental disorder that affects nearly 1% of the population worldwide. Patients are affected in various ways; and there is no known cure for this disease. Cellular pathways associated with energy metabolism are dysregulated, and metabolic disruption is also one of the side effects of antipsychotics, the principal way to manage the symptoms of SCZ. In 2011, two post-translational protein modifications (PTMs), the succinylation and malonylation of lysine residues, were discovered to be widely present in many domains of life, and furthermore have been observed on many proteins associated with glycolysis and metabolism. The precursors to these PTMs, understood to be succinyl-CoA and malonyl-CoA, are also both a part of central metabolic processes, and their prevalence as a PTM in cells can vary with metabolism-related stimuli, such as hypoxia, also a potential environmental trigger for developing SCZ. In this work, shotgun mass spectrometry-based quantitative proteomics was used to determine what differences in succinyllysine and malonyllysine profiles exist under various conditions. Postmortem brain tissue of SCZ patients was compared with mentally sound controls. Additionally, human oligodendrocyte precursor cell cultures (MO3.13 lineage) were treated with MK-801, a SCZ-mimetic compound and/or 3 antipsychotics before being analyzed. Multiple differences in the acylation profiles were found when comparing these conditions and their associated controls.

Introduction:

Schizophrenia (SCZ) is a complex and multifactorial illness that is estimated to affect between 0.3-0.7% of the population worldwide [1]. It is characterized by changes in an individual's ability to perceive and react to the world around them; and physical, observable changes in brain function and morphology are also visible [2,3]. Its onset is brought on by a culmination of biological and environmental factors [4], not all of which are known. These factors, in combination, lead to an individual experiencing an array of symptoms that cause deficits in social appropriateness and forms of hallucinations or psychosis [5], among other symptoms.

SCZ is known to have a strong genetic influence, as proven by longitudinal studies in twins [6]; but this does not paint the whole picture, as many environmental factors can affect the chances to develop the disorder such as postnatal hypoxia, prenatal vitamin D deficiency, cannabis abuse as a teenager, a stressful childhood, certain viral infections, and diet [7]. However, the etiology of SCZ is unknown and there is currently no cure. Instead, medication allows a patient to manage their symptoms and lead a more fulfilling life.

In general, treatment is based on symptom management with antipsychotics, which are not always effective in all patients [8]. Antipsychotics fall into two overall categories: typical and atypical. Despite the beneficial reduction of positive symptoms, antipsychotic medication also may cause various debilitating side effects, especially typical antipsychotics[9]. The low efficacy of first prescription choices and severe side effects lead to a high percentage of patients eventually abandoning treatment [8].

Typical antipsychotics (TAPs) are antagonists of the dopamine D_2 receptor (D_2R), and were first used in the 1950s [10]. In patients with SCZ, they are administered to reduce positive symptoms by blocking dopamine's effects; however, not every

patient will respond to a particular antipsychotic, and less than half of patients are considered to be good responders to the first antipsychotic prescribed to them [11], requiring more time to find an optimal treatment, frustrating the patient, and wasting resources. Complicating treatment further is the prevalence of tardive dyskinesia, a side effect of TAPs that affects a patient's motor abilities [12].

Alternatives to TAPs have since been discovered, now called atypical antipsychotics (AAPs). This class of medication binds to an array of receptors at different strengths, including the serotonin 5-HT_{2A} receptor [13]; though, how different binding profiles lead to changes in side effects and therapeutic profiles is still undetermined [14].

While AAPs present a slightly lower risk for tardive dyskinesia – 3.9% compared to 5.5% [12] – there is the added chance of developing a blood condition called agranulocytosis, among other side effects. In a study in Iceland on 611 patients with SCZ, they found that a comparable number of patients treated with TAPs developed the condition [15]. Additionally, AAPs can also induce an array of side effects that are jointly called metabolic syndrome, but the incidence rate can vary between AAPs [16]. The main documented symptoms of metabolic syndrome are insulin sensitivity and weight gain [17].

Although overall changes due to SCZ can be seen in samples such as postmortem brain tissue, other models must be used to allow the fine tuning of specific variables and remove confounding factors. One model is cultured oligodendrocytes (OLDs), cells that provide neurons with structure, nutrients, and protection through myelination. OLDs have been recurrently found to be associated with some of the

dysregulations in and symptoms of SCZ [18–22]. This association is also in agreement with the dysfunction of myelin-associated proteins, observed and implicated in SCZ [23].

A human OLD precursor cell line MO3.13 has been shown to stay in an “arrested” immature development state [24]. To simulate SCZ in these cells, they are treated with dizocilpine (MK-801), which has been established as a model via animal behavioral studies. In rats, MK-801 induces negative symptoms [25], cognitive symptoms [26], brings about similar neurochemical changes to those that are seen in first-episode patients [27], and potentially induces positive symptoms, although in a manner unlike what is seen in the PCP model [25,28]. A protocol has been published that elaborates on the use of MK-801-treated cells as a model to study schizophrenia [29].

One form of studying cells under conditions such as these is through proteomics. Due to the known existence of both genetic and environmental factors and the dynamically responsive nature of the proteome, proteomics is an extremely useful tool to study illnesses such as schizophrenia. Beyond the presence and quantity of proteins, mass spectrometry and proteomics can reveal information about post-translational modifications (PTMs) on proteins. PTMs include small organic molecules, polysaccharides, other proteins, and fatty acids, as well as structural changes like cysteine disulfide bridges. New discoveries are constantly being made about additional modifications, such as the 2011 discovery of the widespread addition of the acyl groups succinate and malonate to lysine residues [30,31].

Protein succinylation was discovered less than a decade ago, and confirmed to be a widespread PTM after a study with E. coli, S. cerevisiae, HeLa, and mouse liver cells [31,32]. Succinyl-CoA itself is a key intermediate in the TCA cycle, strengthening its link to metabolism and energy production and a large number of histone proteins as

well as mitochondrial and metabolic proteins are modified with succinyl groups (2,572 sites on 990 proteins) [32]. In addition, the protein succinylation profile has been found to be extremely sensitive to certain external metabolic changes, even after stimuli as short as 20 minutes [33].

Malonyl-CoA was confirmed to be a PTM source after a knockout study of the enzyme that converts malonate to malonyl-CoA [34]. Since malonyl-CoA is an intermediate in fatty acid synthesis, it was also hypothesized to have strong ties with metabolism and energy regulation, a fact that has been confirmed in both analytical and knockout studies [34,35]. One study has linked weight gain from antipsychotics with perturbations in this branch of metabolism, and malonyl-CoA was specifically mentioned [36].

Both modifications are now considered important PTMs and have been observed in various prokaryotic and eukaryotic cells [30,32,37,38]. Sites of modification have been discovered to be highly prevalent on metabolic proteins and other mitochondrial proteins, although the effects of these modifications are still mainly unknown [33,33,34,37,39,40]. Lastly, sites are unlikely to be random or otherwise unregulated, as software has been developed that helps predict which lysine residues are sites of succinylation [41,42] and malonylation [43], hinting at a type of site specificity and stringent regulation for addition and removal such as the theory that SIRT5 is the enzyme responsible for desuccinylation [40] and demalonylation [35]. Tying this in to SCZ, there is substantial proof that metabolic dysregulation and oxidative stress occur in SCZ [44–49]. As such, there is great potential in researching a correlation between SCZ and these recently discovered PTMs to perhaps uncover new medicinal targets or research foci.

In this work, the locations and prevalence of these two PTMS were compared under conditions in relation to SCZ using post-mortem brain tissue and MO3.13 human OLD precursor cells treated with MK-801 and/or haloperidol, chlorpromazine, and quetiapine. Discovering new sites of modification, especially ones present under only certain conditions, will help the currently growing databases for these PTMs. Comparing PTM profiles can reveal dysregulations in pathways that are not otherwise visible through genomics, transcriptomics, or otherwise unfocused proteomics. These pathways could become the focus for further studies of the development, progression, or treatment of SCZ.

Additionally, understanding how these acylation profiles change in response to MK-801 and antipsychotics can provide insight into their mechanisms of action and side effects, potentially assisting in the development of new medications and the reduction of the detrimental side effects of current treatments. Lastly, understanding which pathways, if any, that antipsychotics balance when cells are disturbed with MK-801 could provide valuable understanding about how these medications function, and direct new studies to determine how to manage the cognitive and other symptoms of SCZ that are left unchecked by antipsychotic-based treatment.

Methods:

4.1 Meta-analysis Sample Sources

The meta-analysis was performed using the RAW files from two other members of the laboratory (see Acknowledgements). The data were collected using a method well suited the researcher's experiment at hand and is described in detail below; but conditions stayed constant within an individual experiment. Data used were either from postmortem corpus callosum brain tissue (from schizophrenia patients and controls, collected at and donated from the Institute of Neuropathology, Heidelberg University, Heidelberg, Germany) (see Appendix 1) or the MO3.13 human OLD precursor cell line.

4.1.1 Postmortem Corpus Callosum Tissue

Postmortem brain tissue from the corpus callosum was lysed and cytosolic proteins were extracted according to the protocol as published [50] and is reiterated below. 20mg of tissue was homogenized in 250µL lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 70mM DTT, 2% Halt™ Phosphatase Inhibitor Cocktail, and 2% Halt™ Protease Inhibitor Cocktail EDTA-Free (Thermo Fisher Scientific)) with a manual grinding kit (GE Healthcare Life Sciences). The lysate was centrifuged at 14,000 rpm for 10 minutes at 4°C to pellet membranes, the supernatant was collected, and the protein level was quantified with a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific).

50µg of lysate was prepared by adding 0.2% Rapigest to the samples for 15 minutes of incubation at 80°C. Proteins were reduced by incubating with 100 mM DTT at 60°C for 30 minutes and then alkylated with 200mM IAA for 30 minutes at room temperature in the dark. Cells were digested with 1:50 trypsin:protein (by mass) at 37°C overnight. Trypsin activity was halted with the addition of TFA to 5% for 90 minutes at 37°C. The sample was then centrifuged at 14,000 rpm for 30 minutes at

4°C and the supernatant was collected. The pH was adjusted with 1M NH_4OH and frozen until MS analysis.

1 µg of peptides were injected into a 2D-RP/RP Acquity UPLC M-Class System (Waters Corporation) coupled to a Synapt G2-Si mass spectrometer (Waters Corporation). Samples were fractionated in first dimension chromatography with an XBridge Peptide BEH C18 NanoEase Column (130Å, 3.5 µm, 300 µm X 50 mm, Waters Corporation). Peptide elutions were performed by using discontinuous steps of acetonitrile (11%, 14%, 17%, 20%, and 50% ACN) for 10 minutes at a flow rate of 2,000nL/min. After each step, peptide loads were carried to a second-dimension separation in an ACQUITY UPLC HSS T3 nanoACQUITY Column (100Å, 1.8 µm, 75 µm X 150mm, Waters Corporation). Peptide elutions were achieved using an ACN gradient from 7% to 40% (v/v) for 54 min at a flow rate of 500nL/min directly into a Synapt G2-Si.

For every measurement, the mass spectrometer was operated in resolution mode with an m/z resolving power of about 35,000 FWHM, using ion mobility with a cross-section resolving power of at least 40Ω/ΔΩ. The effective resolution obtained with the conjoined ion mobility was 1,800,000 FWHM. MS/MS analyses were performed by nano-electrospray ionization in positive ion mode with a NanoLock Spray (Waters Corporation) ionization source. The lock mass channel was sampled every 30 seconds. The spectrometer was calibrated with an MS/MS spectrum of [Glu1]-Fibrinopeptide B human (Glu-Fib) solution delivered through the reference sprayer of the NanoLock Spray source.

4.1.2 Oligodendrocyte Precursor Cells

MO3.13 cells were grown in Dulbecco's Modified Eagle Medium (DMEM): 4.5 g/L D-glucose, L-glutamine (+), sodium pyruvate (-), supplemented with BSA. Plates were kept at 37°C at 5% CO_2 until nearly confluent. Cells were treated with either vehicle (HCl or DMSO), MK-801 (50 µM), an antipsychotic (haloperidol, 50µM; chlorpromazine, 10µM; or quetiapine, 50µM), or the antipsychotic plus MK-801. Treatments and MK-801 were combined with cells for 8 hours, with MK-801 being added after 4 hours in joint conditions.

Cells were collected in PBS 1x by manual scraping, centrifuged at 1,200rpm for 5 minutes, and frozen until lysis. Cell lysis was done in 6M urea, 2M thiourea, 10 mM DTT, phosphatase and protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA), and 0.1mM sodium pervanadate. The solution was heated for 2 hours at 37°C before dilution in 9 volumes of 20mM TEAB, pH 7.5 and subsequent sonication on ice. 200mM iodoacetamide (IAA) in triethylammonium bicarbonate (20mM) was added to a final concentration of 20mM IAA and incubated at RT for 20 minutes. 1:50 (trypsin:protein) was added for overnight (12-16 hours) digestion at 37°C. Formic acid was added to a final concentration of 5% to stop digestion. After 5 minutes at RT, the samples were centrifuged at 14,000xg for 45 minutes at 4°C to remove lipids and other debris.

The peptide-containing supernatant was collected and 0.1% TFA was added to dilute the sample to 1mL and the solution was desalted using Oasis HSB cartridges (Waters, Co). The resulting peptides were concentrated via SpeedVac (Thermo Fisher Scientific) and reconstituted in 20mM ammonium formate, pH 10.

4.2 Mass Spectrometry

For postmortem brain tissue, samples were run as published [51] and a copy of this protocol can be found in Appendix 2. For MO3.13 cells, 500ng of peptides were injected into an Acquity UPLC, M-Class (Waters, Co) coupled online to a Synapt G2-Si Mass Spectrometer (Waters, Co). Samples were run through a reverse phase/reverse phase column (7-40% ACN over 90 minutes) and MS/MS spectra were collected in DIA mode. The lock mass compound was [Glu1]-fibrinopeptide B at 100fmol/µL, sampled every 30 seconds.

4.3 Protein Identification

MS and MS/MS data were collected and analyzed by Progenesis QI for Proteomics (version 3.0.6039). Within each experiment, individual samples were automatically aligned by the software to improve comparative quantitation. Protein identification was performed using the following parameters: maximum ion charge of +8, trypsin cleaving sites (up to one missed cleavage), maximum protein mass of 600kDa, fixed carbamidomethyl C, variable oxidation M, variable acylation (succinylation *or* malonylation) K, minimum of 2 fragments per peptide, 5 fragments per protein, and 1 peptide per protein, and using the Uniprot revised *Homo sapiens* database (February, 2018 for post-mortem studies and October, 2018 for MO3.13 studies). An FDR of 4% was selected using the on-the-fly-reversed list automatically created by Progenesis.

4.4 Data Filtering and Collection

CSV tables from Progenesis were exported containing protein/peptide/ion identifications with a mass error of less than 20 ppm. Peptides containing the modification of interest were selected for, and quantitative differences (between SCZ and control samples) with an ANOVA value below 10% were selected for postmortem studies and 5% for MO3.13 studies. The resulting list of peptides was converted into a list of its parent proteins' Uniprot accession numbers and in turn this was input into Reactome.org [52]. With Reactome.org, top pathways were selected based on Entities pValue, using the percentage of identified entities of a specific pathway.

To determine attenuation, first, modifications with average quantitation scores within 10% between DMSO and HCL conditions were selected for to ensure that differences were not due to the vehicles and a change by MK-801 of at least the attenuation value compared to its vehicle, DMSO. Next, an average of the two controls was used as a baseline for ±10, 25, and 50% (indicating high, partial, and low attenuation). A gene was marked with an asterisk (*) if the change by MK-801 was higher than one category away from the return category (and ** for two). Lastly, the MK-801- and antipsychotic-treated scores were tested to determine if treatment attenuated the prevalence of a modification to the degree of any of the three categories.

Results and Discussion:

5.1 Comparison of Brain Tissue Samples

Under less stringent parameters than with cellular experiments (ANOVA ≤ 0.10), in all the brain tissue samples, 105 succinylated proteins and 170 malonylated proteins were found to be differentially expressed. Little overlap was present between patients: only 8 succinylated and 15 malonylated proteins were found differentially expressed in more than one sample (see Appendix 3). These repeated proteins and brief summaries of their functions (Figures 1 & 2) are strongly associated with the cytoskeleton (yellow) and energy metabolism (green).

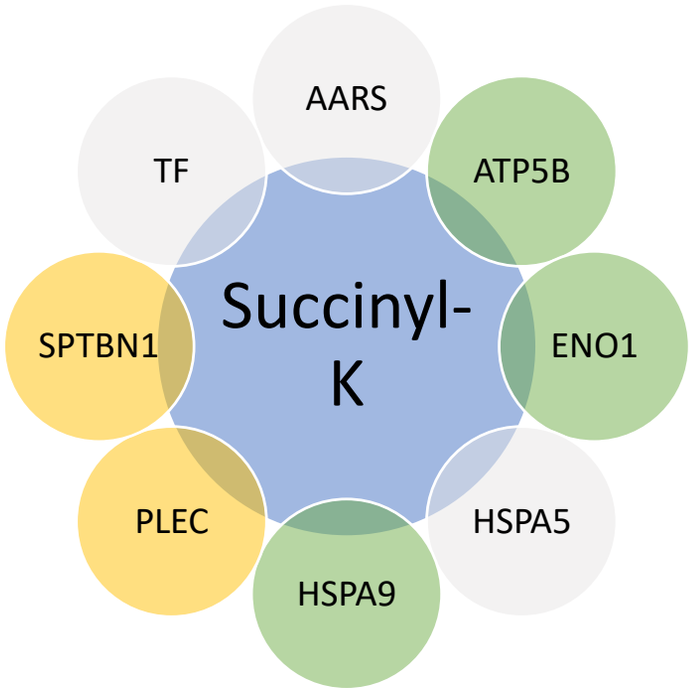

Figure 5-Dysregulated succinylated proteins in SCZ postmortem corpus callosum.

Metabolic proteins are already documented to be associated with these modifications [39], which makes sense due to their inherent relationship with the metabolism. Additionally, previous studies have found that energy, metabolism, and mitochondrial function are dysregulated in schizophrenia [48,53]. Furthermore, demyelination, a process known to be reduced in schizophrenia and suggested to be

a cause of some symptoms of the disorder [20,54], is heavily associated with the cytoskeleton and its modulation. Additionally, Spectrin degradation products have been found in higher levels in the serum of patients with schizophrenia [55].

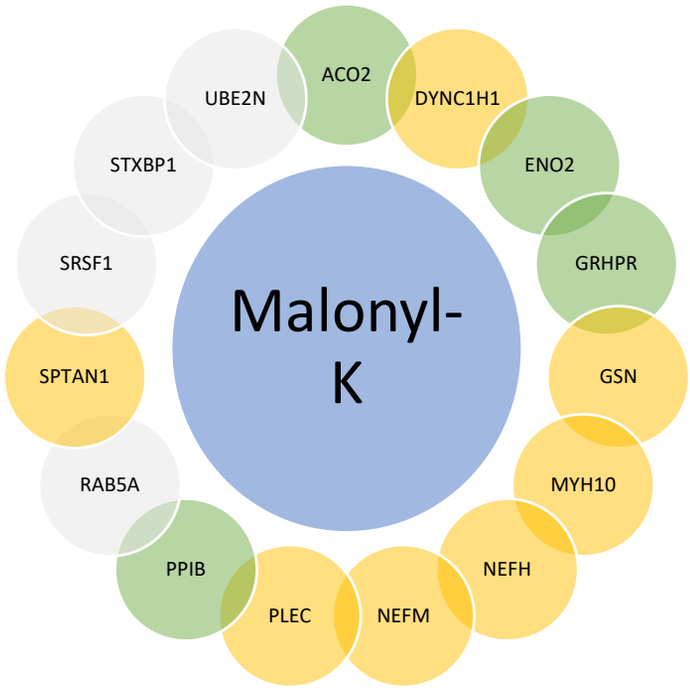

Figure 6-Dysregulated malonylated proteins in SCZ postmortem corpus callosum.

Interestingly, these proteins did not always have a global trend, confirming that there is some level of intentional regulation of these modifications on specific proteins. For example, SPTBN1 had 4 sites (2 succinylated and 2 malonylated) with a lower modification prevalence and 1 site of increased malonylation. In contrast, NEFH was only found to have an increased quantity of malonylated peptides. Overall values of differences follow in Table 1 below, considered unique by Uniprot accession number.

Table 1-Quantity of Differentially Modified Proteins

	Malonylation Upregulated	Malonylation Downregulated	Succinylation Upregulated	Succinylation Downregulated
Unique Proteins	68	112	30	79

Investigating the individual proteins in the list, some others have potentially interesting correlations with schizophrenia. For example, STXBP1 has been hypothesized to be involved with schizophrenia via the known dysfunction in the SNARE complex activity and NMDA reuptake [56]. UBE2N has been previously found to be differentially expressed in postmortem brain tissue from patients with SCZ [57,58].

The mere existence of a difference in expression of this modification hints at a previously unnoticed means of protein regulation that may be directly involved with the morphological and metabolic changes seen in schizophrenia. Further studies could be performed with more samples and brain regions to get a better picture of the changes in individual brain regions as well as the overall profile. Using less invasive sources from living patients also has great potential to reveal differential expression profiles.

It is important to point out that Progenesis quantifies the presence of peptides – or modified peptides – individually between samples, and does not calculate the prevalence to the quantity of the whole protein. Due to this type of processing, it is not possible – without the use of specialized software or extensive manual analysis – to determine if the PTM in question is dysregulated, or if it is the protein itself. Additionally, one study found that conditions such as hypoxia can affect the modification profile in as little as 20 minutes [33], suggesting that tissue may change its expression profile after the patient is deceased. These points should be investigated in future studies.

5.2 Expression Profiles in MO3.13 – MK-801

When analyzing cells treated with MK-801, there were significant (ANOVA ≤ 0.05) changes in their acylation profiles. Many proteins had multiple sites of modification, some of which did not present the same direction of change (see Table

2). When looking solely at proteins with an upregulated or downregulated acylation profile, some trends were observed using Reactome.org.

Table 2-Sites of protein acylation in MK-801-treated cells. Classified by Uniprot Accession Number (AN).

	Malonylation	Succinylation
Total Sites	349	502
Unique ANs	247	322
ANs Upregulated	150	196
ANs Downregulated	140	190
Both Up- and Down-regulated	43	64

One study found that succinate thiokinase (STH) levels in SCZ were reduced[59], an enzyme that converts succinyl-CoA into succinate. Although this would suggest an overall decrease in succinylation, various increases were seen, reinforcing the theory of a regulated and responsive PTM system. Alternatively, this could suggest that MK-801 does not provide a completely accurate representation of SCZ.

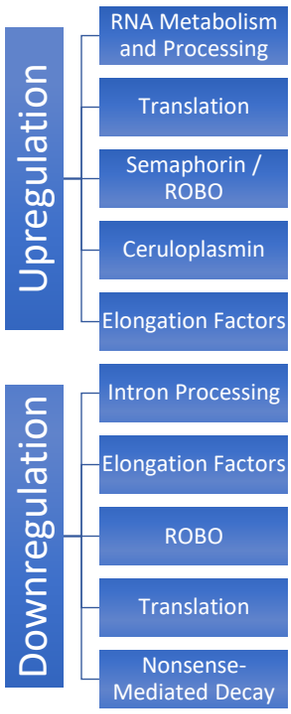

Figure 7-Succinylation dysregulation in MO3.13 by MK-801. ANOVA ≤ 0.05, top 10 pathways selected for each by pValue in Reactome.org.

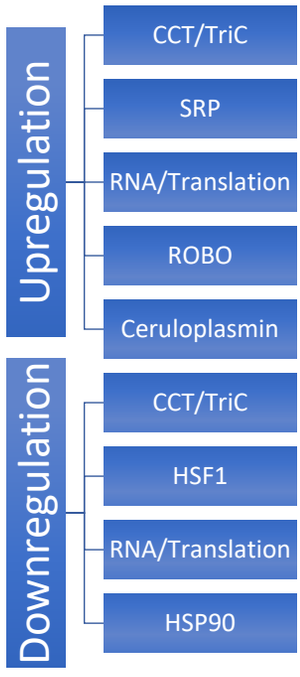

Figure 8-Malonylation dysregulation in MO3.13 by MK-801. ANOVA ≤ 0.05, top 10 pathways selected for each by pValue in Reactome.org.

5.2.1 MK-801 Succinylation

The top 10 pathways by pValue for up- and down-regulated succinylation are summarized in Figure 4 above. In both up- and down-regulated succinylation, pathways involved with RNA processing, splicing, metabolism, and translation were seen to be perturbed. Introns and miRNA specifically have been only rarely suggested to be related to schizophrenia [60], general dysfunctions in mRNA, its processing, spliceosomes, and hnRNPs (heterogeneous ribonucleoproteins) have repeatedly appeared in literature [61–64]. One highlighted subcategory of this is NMD (nonsense-mediated decay), a cellular stress-induced mRNA degradation pathway associated with UPF3B and found to be mutated and/or deregulated in schizophrenia [65,66].

Selecting only the upregulated succinylated proteins, some new pathways were highlighted. Axonal guidance and ceruloplasmin pathways were seen, as in malonylation, along with the added factor of elongation factors, RNA stability, and ribosomal function. AUF1 is implicated in ageing, telomerase activity, and inflammation [67]; though no direct link has been made directly with schizophrenia. Nonetheless, a part of the AUF1 pathways is the YWHAZ protein, which has been associated with schizophrenia in a few studies [68–70].

5.2.2 MK-801 Malonylation

The top 10 pathways by pValue for up- and down-regulated malonylation are listed in Appendix 4 and are summarized in Figure 3 above. In both directions, the CCT/TriC pathway was highlighted, a category of chaperonins that is heavily involved with the cytoskeleton [71]. The neuronal cytoskeleton has many links with schizophrenia, not only due to the known demyelination that occurs, but it also has been mentioned as a potential therapeutic target [72]. One study found that despite the normal expression

of actin, levels of polymerization were decreased [73], something that could be potentially explained by PTMs; and another study suggested that actin dysregulation could be linked to various mental disorders, including schizophrenia [74]. The function of malonylation in these proteins, however, is unknown and more studies would need to be performed to elucidate the effect of this downregulation.

Upregulated-only pathways include ER-targeting for proteins, ROBO-mediated axon guidance, ceruloplasmin expression, and translation. The pathways involved with chaperonins and heat shock proteins are potentially related to the SRP membrane-targeting pathways, since the endoplasmic reticulum is a prime location for protein folding, and stress of this compartment has been implicated in schizophrenia [75]. The Roundabout (ROBO) pathway is considered to be essential for proper neurodevelopment and correct axonal growth [76]. Variations in this gene have been associated with schizophrenia [77] and was found to be a DISC1 interactor [78], but no follow-up studies have been performed to date. Ceruloplasmin expression has been found to be dysregulated in schizophrenia as well [79]. Although, variances in copper levels in patients with schizophrenia, once a considered theory for the cause of schizophrenia [80], have not been studied with much scrutiny since then.

Two downregulated-only pathways that were highlighted are also associated with heat shock and chaperonins, and HSP90 has already been passively referenced in relation to schizophrenia [81,82] and more directly in association with neurite outgrowth [83].

5.2.3 Overall Pathway Changes by MK-801

To see a more overall picture and potentially highlight different affected pathways, both up- and down-regulated proteins for succinylation and malonylation were combined and entered into Reactome.org. An overall summary of the changes follows in Figure 5.

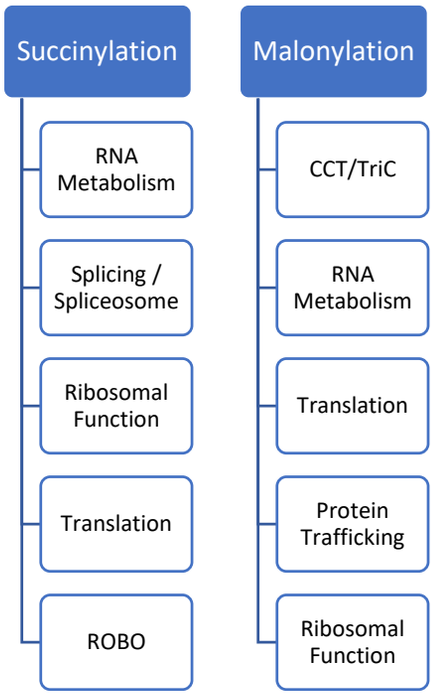

Figure 9-Overall dysregulations in MO3.13 by MK-801.

In summary, various changes were observed in succinylation and malonylation caused by blocking the NMDA receptor in MO3.13 human oligodendrocyte precursor cells. These changes had significant overlap with several processes implicated in SCZ, which reinforces the likelihood that MK-801 is a sufficient schizophrenia-mimetic model. In addition, specifically mentioning the CCT/TriC pathway, it is possible that some of the dysregulations in brain morphology are due to an improperly functioning acylation (and/or deacylation) mechanism.

Comparing these results with the changes seen in the postmortem tissue, there was little overlap with this data and the few proteins found in multiple postmortem

samples. Only 2 of the 8 differentially succinylated proteins (SPTBN1 and HSPA5) and 2 of the 15 malonylated proteins (DYNC1H1 and NEFM) found in the corpus callosum were found in MK-801-perturbed samples. These are 3 cytoskeleton proteins and 1 chaperonin.

5.3 Expression Profiles in MO3.13 – Succinylation

In the MO3.13 cells treated with various antipsychotics, several pathways were affected by changes in succinylation with significance (ANOVA ≤ 0.05) and identified with Reactome.org. The full list of these pathways can be found in the appendices. Overall, there were many similar pathways that were affected by the three antipsychotics; however, some differences were seen.

The first difference observed was the overall number of sites that were changed significantly from the addition of one of the antipsychotics. From haloperidol, 230 sites were upregulated and 169 sites were downregulated, whereas chlorpromazine had 95 upregulated and 70 downregulated sites and quetiapine had 57 upregulated and 4 downregulated sites. This is despite a significant difference in the overall number of identified peptides in the samples. This data is summarized in Table 3 below.

Table 3-Numbers of statistically perturbed succinylation sites in MO3.13 cells due to antipsychotic treatments.

Antipsychotic	Haloperidol	Chlorpromazine	Quetiapine
Overall Number of Sites	538	189	61
Percentage of Modified Peptides	35.8%	20.6%	3.51%
Percentage of Total Peptides	3.16%	1.15%	0.21%

These differences could be due to many factors; however, the main difference between these three compounds is that quetiapine is the only atypical

antipsychotic and has much fewer modified sites. It's possible this is related to the receptors to which the various compounds bind, or could also have some relation to the different side effects found in the two antipsychotic classes like extrapyramidal effects and metabolic syndrome. Which pathways the modified proteins belong to were studied using the Reactome database and analysis tool, and the overall pathways (see Appendices 5-7) were summarized into a few overarching functions based on what components were highlighted in each pathway (see Figure 6). Quetiapine reduced the expression of only 4 modification sites on as many proteins and no pathway with an FDR below 4% was identified.

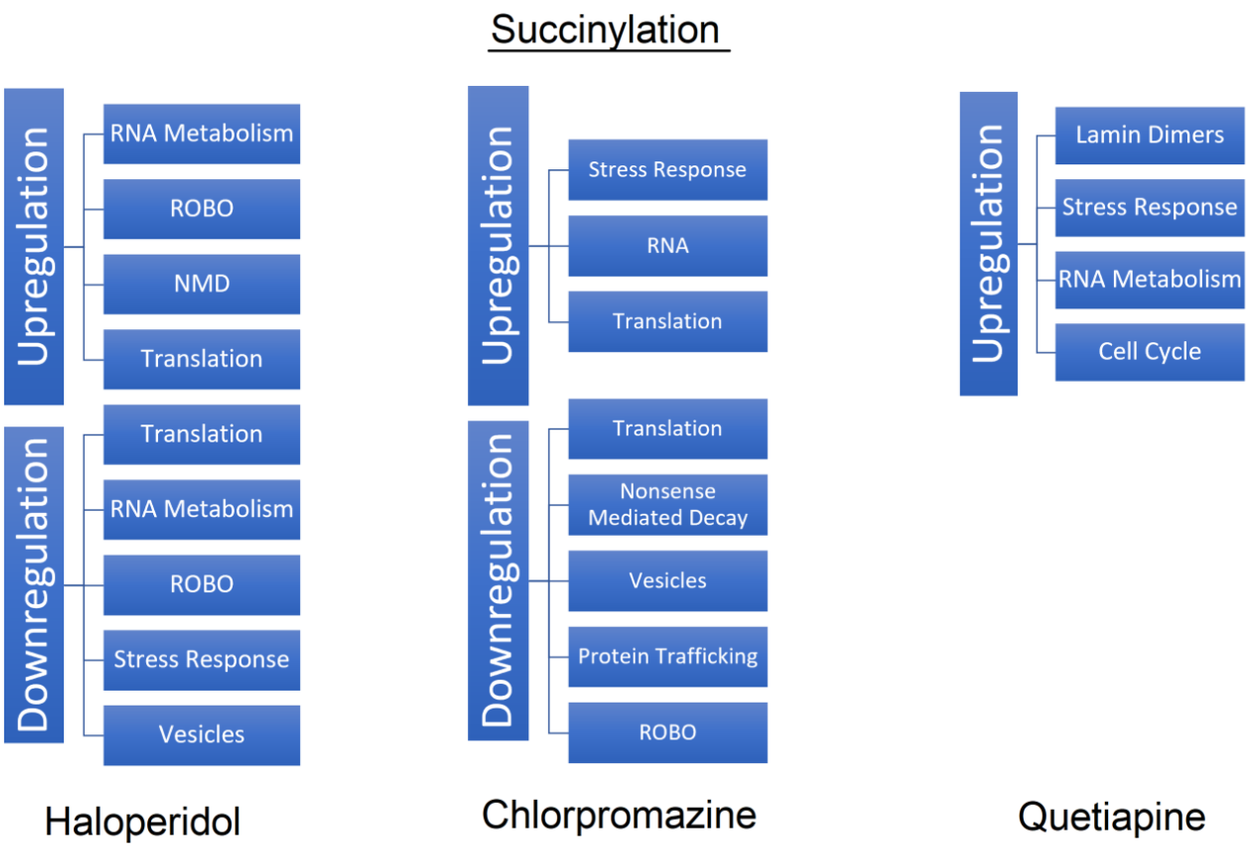

Figure 10-Pathways affected by succinylation dysregulation induced by haloperidol, chlorpromazine, and quetiapine in MO3.13 cells, using Reactome.org database.

To begin, the typical antipsychotic haloperidol induced changes in protein succinylation on proteins associated principally with RNA metabolism and regulation,

nonsense-mediated decay, stress response, and vesicle formation. Some overlap was seen between different pathways such as nonsense-mediated decay (NMD) and RNA metabolism. Several pathways were seen with both increases and decreases of succinylation on its protein members.

Of the proteins differentially succinylated by chlorpromazine, many were involved in pathways associated with stress, metabolism, translation, and vesicles. One study found that chlorpromazine decreased vacuolation and vesicle uptake in gastric epithelial cells [84], a cell type which also contains the 5-HT receptor and is responsive to serotonin [85]. Another study found that vesiculation of red blood cells induced by ATP depletion was inhibited through chlorpromazine, also affecting the phosphorylation levels of a signaling protein PIP_2 [86].

Succinylation changes by quetiapine included lamin dimers and the depolarization of the nuclear lamina, listed as the most dysregulated pathway. Lamin B2 has been found to be dysregulated in SCZ in one shotgun proteomics study [87] and one PTM (phosphorylation) is already known to have an effect on the polymerization readiness of this molecular matrix [88].

All but vesicle transport and lamin dimers could be associated with a metabolic stress-induced response, due to succinylation's known role in the regulation of metabolic enzymes [39] as well as the known effects on metabolism by both typical and atypical antipsychotics [89]. Vesicle transport could be indirectly related to metabolic stress, since vesicles are used for protein transport to the endoplasmic reticulum (ER) and outside the cell. Strong links between ER stress and metabolism/metabolic stress have been observed [90,91].

5.4 Expression Profiles in MO3.13 – Malonylation

The MO3.13 cells treated with various antipsychotics also exhibited several changes in malonylation with significance (ANOVA ≤ 0.05) and the pathways associated to these changes were identified with Reactome.org. The full list of these pathways can be found in the appendices. Again, many pathways appeared in multiple or all three antipsychotics; but some were uniquely present in one.

As in succinylation, the number of significantly changed modification sites varied between antipsychotics. From haloperidol, 280 sites were upregulated and 156 sites were downregulated, chlorpromazine had 114 upregulated and 72 downregulated sites, and quetiapine had 50 upregulated and 5 downregulated sites. This data is summarized in Table 4 below.

Table 4-Numbers of statistically perturbed malonylation sites in MO3.13 cells due to antipsychotic treatments.

Antipsychotic	Haloperidol	Chlorpromazine	Quetiapine
Overall Number of Sites	434	186	55
Percentage of Modified Peptides	34.1%	20.2%	3.51%
Percentage of Total Peptides	2.31%	1.41%	0.21%

Again, the atypical antipsychotic presented a very different profile compared to the typical antipsychotics. The pathways these modified proteins belong to were investigated with the Reactome database and analysis tool, and the overall pathways (see Appendices 5-7) were summarized into a few overarching functions based on what components were highlighted in each pathway (see Figure 7). Quetiapine reduced the expression of only 5 modification sites on as many proteins, though some pathways were still identified with an FDR below 4%.

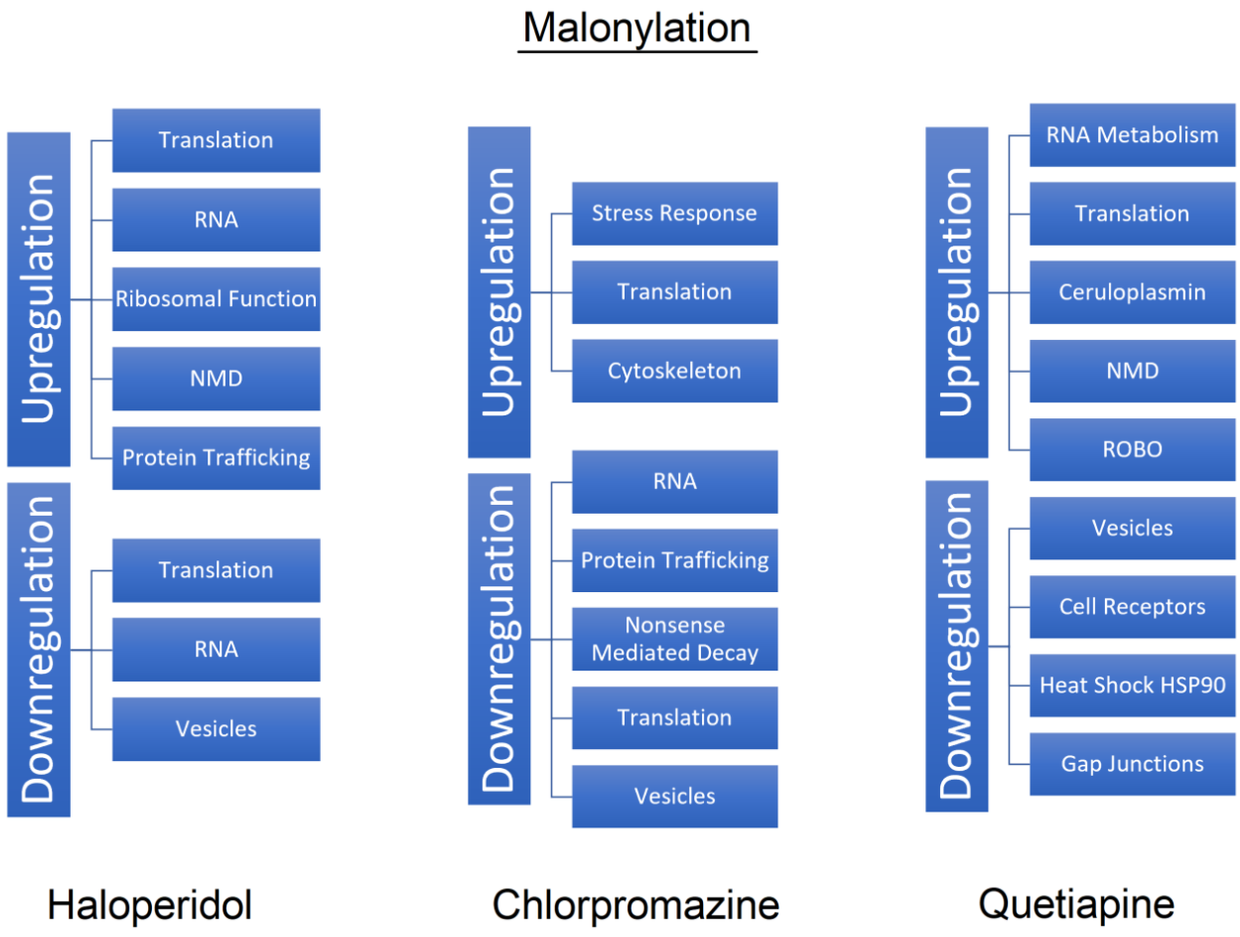

Figure 11-Pathways affected by malonylation dysregulation induced by haloperidol, chlorpromazine, and quetiapine in MO3.13 cells, using Reactome.org database.

Like succinylation, malonylation is also a key player in metabolism [92], and as several metabolic disturbances can result from antipsychotic use [93], it is possible that this PTM is also a form of RNA and translational control or is responding to metabolic stress. In SCZ, one major dysregulation is levels of neurotransmitters [94] and is one hypothesis for certain symptoms [95]. A change in proteins vesicle proteins could be related to a malfunction in the regulation or activity of neurotransmitters or their release.

5.5 Attenuation of MK-801-Induced Changes in MO3.13 by Antipsychotics

Some succinylation sites disturbed by MK-801 were returned to control or near-control levels upon the addition of an antipsychotic and were classified into three groups (see Figure 8). When a site was returned to ±10% of its control value, this was considered high attenuation; ±25%, partial; and ±50%, low. Proteins received an asterisk when they were disturbed to greater levels and attenuated.

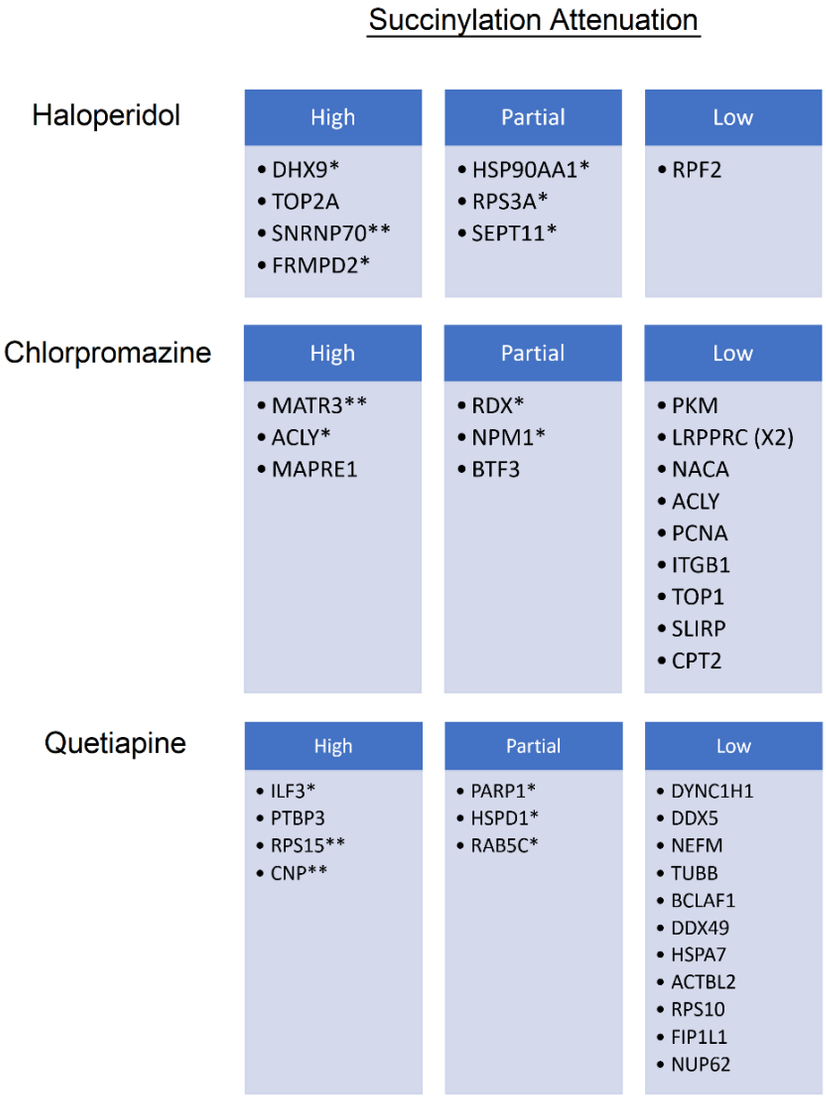

Figure 12-List of proteins with attenuated succinylation disturbances from MK-801 by haloperidol, chlorpromazine, and quetiapine. High ±10%; Partial ±25%; Low ±50%.

The succinylation sites that haloperidol returned closest to their original presence were found on a DNA/RNA helicase (DHX9), a DNA topoisomerase (TOP2A), a snRNP (SNRNP70), and a putative, tight junction-associated protein (FRMPD2). The partially and poorly attenuated sites of modification were found on proteins associated with heat shock, ribosomes, and the cytoskeleton. Chlorpromazine best attenuated a multifunctional nuclear matrix protein (MATR3), a lipid synthesis pathway protein (ACLY), and a microtubule regulating protein (MAPRE1). Quetiapine most closely returned to control levels sites on on ILF3 and PTBP3 (RNA binding proteins), RPS15 (a ribosomal protein), and CNP (a myelin-associated phosphodiesterase).

Many of these proteins could have to do with the symptoms or pathophysiology of SCZ. The sites returned to near-control levels were strongly associated with RNA and translational control, the cytoskeleton, and metabolism, and other partial and low attenuation proteins also often fell into these categories. Looking at the overall attenuation profile, it seems that these modifications are potentially a cellular response to oxidative stress or another similar stimulus. Whether the pathways involved here are due to a direct action on these proteins' function or if they are merely a downstream return to normal activity by the reduction of upstream aggravating stimuli is unknown.

When investigating the levels of malonylation sites that were attenuated with an antipsychotic after perturbations with MK-801, multiple sites were also found and compiled into Figure 10 below.

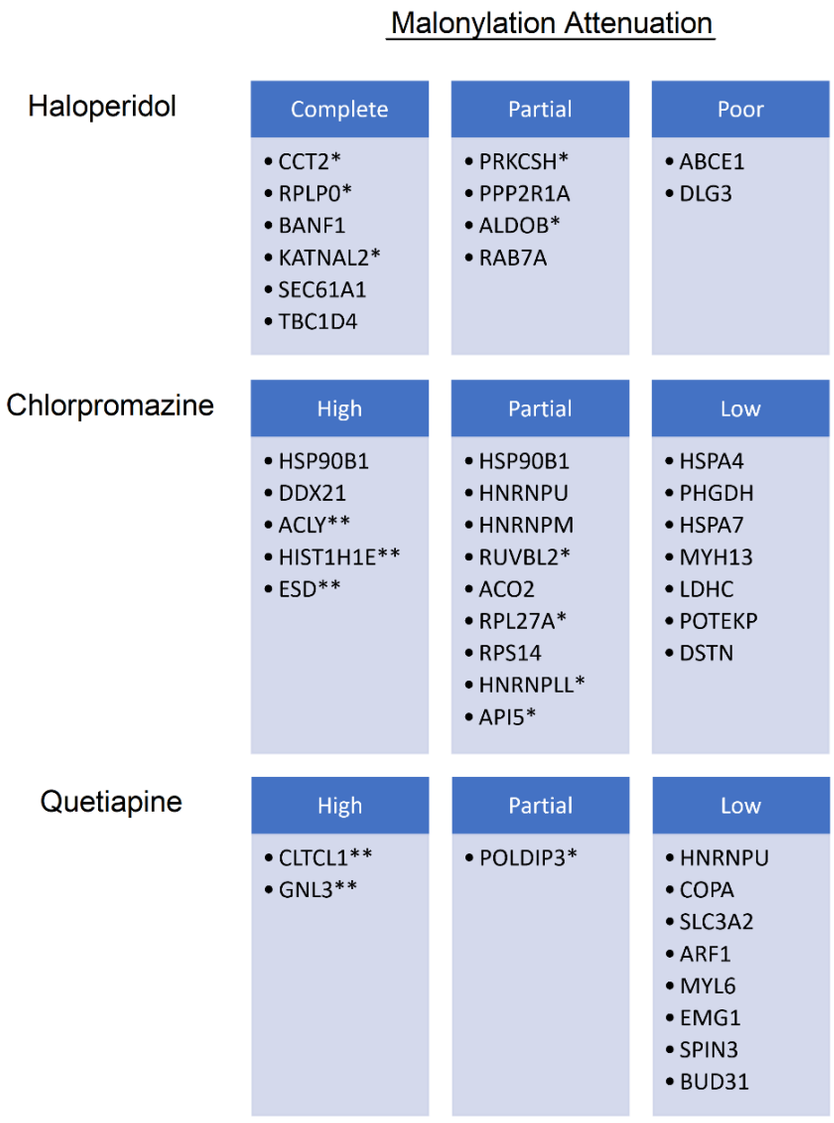

Figure 13-List of proteins with attenuated malonylation disturbances from MK-801 by haloperidol, chlorpromazine, and quetiapine. High ±10%; Partial ±25%; Low ±50%.

The sites of malonylation that haloperidol most closely returned to their original levels were on a chaperonin (CCT2), a ribosomal protein (RPLP0), a chromatin remodeling protein (BANF1), a microtubule-remodeling protein (KATNAL2), an ER protein trafficking protein (SEC61A1), and a RAB-activator/glucose uptake-inducer (TBC1D4). Chlorpromazine closely returned sites on a heat shock protein associated with secretory pathways (HSP90B1), an RNA-binding protein (DDX21), a lipid synthesis pathway protein (ACLY), a histone protein (HIST1H1E), and a protein

involved in carbon metabolism and formaldehyde detoxification (ESD) to their normal malonylation levels. Quetiapine returned malonylation sites on CLTCL1 (a clathrin heavy chain protein) and GNL3 (an MDM2 stabilizer in tumors and stem cells) to more control-like levels.

Multiple proteins seem to be involved with metabolism, protein folding, secretory pathways, and the cytoskeleton. A stem cell proliferation protein was interesting to find as affected, considering the tests were performed on a cell culture; although the purpose of this modification and why it is affected by MK-801 and antipsychotics is not immediately obvious.

Conclusions:

The post-translational modifications succinylation and malonylation seem to be profiles that both respond to various types of environmental stimuli, surpassing the hypoxia response of succinylation previously discovered [33]. The known metabolic disturbances in schizophrenia and from the use of antipsychotics seem to lead to changes in the succinylation and malonylation profiles of postmortem brain tissue and MO3.13 human oligodendrocyte precursor cells, principally in pathways associated with metabolism, the cytoskeleton, RNA processing, and protein translation and folding.

Antipsychotics themselves exhibited an interesting profile difference, with a large difference between the profiles of the two typical and the atypical antipsychotic. However, with only three antipsychotics used, and the fact that atypical antipsychotics bind to a wide array of receptors, more studies with a larger number of compounds would need to be performed to determine if this is what causes the changes, or if there is some other factor involved in these differences.

The schizophrenia-mimetic drug MK-801 also induced many changes in RNA processing, translation, and the cytoskeleton. Despite the general overlap in pathways, very few proteins that were found to be differentially modified in multiple postmortem tissue samples were also found in the MK-801-treated cells, although this could be due to multiple factors, including the differences in cell type and variety, patient-related variables, and state of the tissue before collection.

When the cells were first incubated with MK-801 and subsequently treated with one of three antipsychotics, several but not all pathways were seen to improve and return to their more control-like states. These proteins were highly associated with RNA- and DNA-binding proteins, transcription and translation regulation, the

cytoskeleton, metabolism, and protein transport. Without knowing the effect of these modifications on proteins, it's only possible to speculate on the purpose of the observed changes; however, these results suggest an important mechanism to respond to metabolic (or other) stimuli that goes beyond modifying only metabolism-related proteins and histones.

Future studies can potentially elucidate the purpose of these modifications on the differentially modified proteins and determine if the changes seen here are a result of a direct form of stimulus response to the schizophrenia/MK-801 and the cellular effects they bring on that causes any symptoms of schizophrenia, or if this is a more downstream effect of a larger dysregulation. Additional research can also determine if the metabolic changes and differences between antipsychotics are related to any of the side effects that result from their use and if these changes can be targeted to develop better medication or improve current options.

Conflicts of Interest:

The authors declare no conflicts of interest.

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References:

1. Saha, S., Chant, D., Welham, J. & McGrath, J. A systematic review of the prevalence of schizophrenia. *PLoS Med.* **2**, e141 (2005).

2. Karlsgodt, K. H., Sun, D. & Cannon, T. D. Structural and Functional Brain Abnormalities in Schizophrenia. *Curr Dir Psychol Sci* **19**, 226–231 (2010).

3. McDonald, C. *et al.* Regional volume deviations of brain structure in schizophrenia and psychotic bipolar disorder: Computational morphometry study. *The British Journal of Psychiatry* **186**, 369–377 (2005).

4. Tsuang, M. T., Stone, W. S. & Faraone, S. V. Genes, environment and schizophrenia. *The British Journal of Psychiatry* **178**, s18–s24 (2001).

5. Andreasen, MD, PhD, N. C., Arndt, PhD, S., Alliger, PhD, R., Miller, PharmD, MD, D. & Flaum, MD, M. Symptoms of Schizophrenia: Methods, Meanings, and Mechanisms. *Arch Gen Psychiatry* **52**, 341–351 (1995).

6. Hilker, R. *et al.* Heritability of Schizophrenia and Schizophrenia Spectrum Based on the Nationwide Danish Twin Register. *Biological Psychiatry* **83**, 492–498 (2018).

7. Davis, J. *et al.* A review of vulnerability and risks for schizophrenia: Beyond the two hit hypothesis. *Neurosci Biobehav Rev* **65**, 185–194 (2016).

8. Lieberman, J. A. *et al.* Effectiveness of Antipsychotic Drugs in Patients with Chronic Schizophrenia. *New England Journal of Medicine* **353**, 1209–1223 (2005).

9. Jones, P. B. *et al.* Randomized Controlled Trial of the Effect on Quality of Life of Second- vs First-Generation Antipsychotic Drugs in Schizophrenia: Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia Study (CUtLASS 1). *Arch Gen Psychiatry* **63**, 1079–1087 (2006).

10. Shen, W. W. A history of antipsychotic drug development. *Compr Psychiatry* **40**, 407–414 (1999).

11. Reynolds, G. P. The Pharmacogenetics of Symptom Response to Antipsychotic Drugs. *Psychiatry Investig* **9**, 1–7 (2012).

12. Correll, C. U. & Schenk, E. M. Tardive dyskinesia and new antipsychotics. *Current Opinion in Psychiatry* **21**, 151–156 (2008).

13. Guenette, M. D. *et al.* Atypical antipsychotics and effects of adrenergic and serotonergic receptor binding on insulin secretion in-vivo: An animal model. *Schizophrenia Research* **146**, 162–169 (2013).

14. Sahlholm, K. *et al.* Typical and atypical antipsychotics do not differ markedly in their reversibility of antagonism of the dopamine D2 receptor. *Int J Neuropsychopharmacol* **17**, 149–155 (2014).

15. Ingimarsson, O., MacCabe, J. H., Haraldsson, M., Jónsdóttir, H. & Sigurdsson, E. Neutropenia and agranulocytosis during treatment of schizophrenia with clozapine versus other antipsychotics: an observational study in Iceland. *BMC Psychiatry* **16**, 441 (2016).

16. Association, A. D. Consensus Development Conference on Antipsychotic Drugs and Obesity and Diabetes. *Diabetes Care* **27**, 596–601 (2004).

17. Riordan, H. J., Antonini, P. & Murphy, M. F. Atypical Antipsychotics and Metabolic Syndrome in Patients with Schizophrenia: Risk Factors, Monitoring, and Healthcare Implications. *Am Health Drug Benefits* **4**, 292–302 (2011).

18. Hof, P. R., Haroutunian, V., Copland, C., Davis, K. L. & Buxbaum, J. D. Molecular and Cellular Evidence for an Oligodendrocyte Abnormality in Schizophrenia. *Neurochem Res* **27**, 1193–1200 (2002).

19. Martins-de-Souza, D. Proteome and transcriptome analysis suggests oligodendrocyte dysfunction in schizophrenia. *Journal of Psychiatric Research* **44**, 149–156 (2010).

20. Takahashi, N., Sakurai, T., Davis, K. L. & Buxbaum, J. D. Linking oligodendrocyte and myelin dysfunction to neurocircuitry abnormalities in schizophrenia. *Prog Neurobiol* **93**, 13–24 (2011).

21. Tkachev, D. *et al.* Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. *Lancet* **362**, 798–805 (2003).

22. Uranova, N. A., Vostrikov, V. M., Orlovskaya, D. D. & Rachmanova, V. I. Oligodendroglial density in the prefrontal cortex in schizophrenia and mood disorders: a study from the Stanley Neuropathology Consortium. *Schizophrenia Research* **67**, 269–275 (2004).

23. Karoutzou, G., Emrich, H. M. & Dietrich, D. E. The myelin-pathogenesis puzzle in schizophrenia: a literature review. *Molecular Psychiatry* **13**, 245–260 (2008).

24. Buntinx, M. *et al.* Characterization of three human oligodendroglial cell lines as a model to study oligodendrocyte injury: morphology and oligodendrocyte-specific gene expression. *J. Neurocytol.* **32**, 25–38 (2003).

25. Rung, J. P., Carlsson, A., Rydén Markinhuhta, K. & Carlsson, M. L. (+)-MK-801 induced social withdrawal in rats; a model for negative symptoms of schizophrenia. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **29**, 827–832 (2005).

26. Svoboda, J., Stankova, A., Entlerova, M. & Stuchlik, A. Acute administration of MK-801 in an animal model of psychosis in rats interferes with cognitively demanding forms of behavioral flexibility on a rotating arena. *Front Behav Neurosci* **9**, (2015).

27. Eyjolfsson, E. M., Brenner, E., Kondziella, D. & Sonnewald, U. Repeated injection of MK801: An animal model of schizophrenia? *Neurochemistry International* **48**, 541–546 (2006).

28. Seillier, A. & Giuffrida, A. Evaluation of NMDA receptor models of schizophrenia: Divergences in the behavioral effects of sub-chronic PCP and MK-801. *Behavioural Brain Research* **204**, 410–415 (2009).

29. Brandão-Teles, C., Martins-de-Souza, D., Guest, P. C. & Cassoli, J. S. MK-801-Treated Oligodendrocytes as a Cellular Model to Study Schizophrenia. *Adv. Exp. Med. Biol.* **974**, 269–277 (2017).

30. Peng, C. *et al.* The First Identification of Lysine Malonylation Substrates and Its Regulatory Enzyme. *Mol Cell Proteomics* **10**, M111.012658 (2011).

31. Zhang, Z. *et al.* Identification of lysine succinylation as a new post-translational modification. *Nat Chem Biol* **7**, 58–63 (2011).

32. Weinert, B. T. *et al.* Lysine Succinylation Is a Frequently Occurring Modification in Prokaryotes and Eukaryotes and Extensively Overlaps with Acetylation. *Cell Reports* **4**, 842–851 (2013).

33. Chen, H. *et al.* Mild metabolic perturbations alter succinylation of mitochondrial proteins. *J. Neurosci. Res.* **95**, 2244–2252 (2017).

34. Bowman, C. E. *et al.* The Mammalian Malonyl-CoA Synthetase ACSF3 Is Required for Mitochondrial Protein Malonylation and Metabolic Efficiency. *Cell Chem Biol* **24**, 673-684.e4 (2017).

35. Nishida, Y. *et al.* SIRT5 Regulates both Cytosolic and Mitochondrial Protein Malonylation with Glycolysis as a Major Target. *Mol Cell* **59**, 321–332 (2015).

36. Gonçalves, V. F. *et al.* A Hypothesis-Driven Association Study of 28 Nuclear-Encoded Mitochondrial Genes with Antipsychotic-Induced Weight Gain in Schizophrenia. *Neuropsychopharmacology* **39**, 1347–1354 (2014).

37. Qian, L. *et al.* Global Profiling of Protein Lysine Malonylation in Escherichia coli Reveals Its Role in Energy Metabolism. *J. Proteome Res.* **15**, 2060–2071 (2016).

38. Zhang, Y. *et al.* Global analysis of protein lysine succinylation profiles in common wheat. *BMC Genomics* **18**, 309 (2017).

39. Hirschey, M. D. & Zhao, Y. Metabolic Regulation by Lysine Malonylation, Succinylation, and Glutarylation. *Mol. Cell Proteomics* **14**, 2308–2315 (2015).

40. Rardin, M. J. *et al.* SIRT5 regulates the mitochondrial lysine succinylome and metabolic networks. *Cell Metab.* **18**, 920–933 (2013).

41. Dehzangi, A. *et al.* Improving succinylation prediction accuracy by incorporating the secondary structure via helix, strand and coil, and evolutionary information from profile bigrams. *PLOS ONE* **13**, e0191900 (2018).

42. Ning, Q., Zhao, X., Bao, L., Ma, Z. & Zhao, X. Detecting Succinylation sites from protein sequences using ensemble support vector machine. *BMC Bioinformatics* **19**, 237 (2018).

43. Taherzadeh, G. *et al.* Predicting lysine-malonylation sites of proteins using sequence and predicted structural features. *J Comput Chem* **39**, 1757–1763 (2018).

44. Amorim, M., Moreira, A., Marques, A. & Summavielle, T. Brain metabolic abnormalities in schizophrenia patients. *European Psychiatry* **41**, S802 (2017).

45. Khaitovich, P. *et al.* Metabolic changes in schizophrenia and human brain evolution. *Genome Biol* **9**, R124 (2008).

46. Leonard, B. E., Schwarz, M. & Myint, A. M. The metabolic syndrome in schizophrenia: is inflammation a contributing cause? *J Psychopharmacol* **26**, 33–41 (2012).

47. Marcelis, M., Cavalier, E., Gielen, J., Delespaul, P. & Van Os, J. Abnormal response to metabolic stress in schizophrenia: marker of vulnerability or acquired sensitization? *Psychol Med* **34**, 1103–1111 (2004).

48. Martins-de-Souza, D., Harris, L. W., Guest, P. C. & Bahn, S. The Role of Energy Metabolism Dysfunction and Oxidative Stress in Schizophrenia Revealed by Proteomics. *Antioxidants & Redox Signaling* **15**, 2067–2079 (2010).

49. Nascimento, J. M. & Martins-de-Souza, D. The proteome of schizophrenia. *npj Schizophrenia* **1**, 14003 (2015).

50. Saia-Cereda, V. M. *et al.* Proteomics of the corpus callosum unravel pivotal players in the dysfunction of cell signaling, structure, and myelination in schizophrenia brains. *Eur Arch Psychiatry Clin Neurosci* **265**, 601–612 (2015).

51. Saia-Cereda, V. M., Cassoli, J. S., Schmitt, A., Falkai, P. & Martins-de-Souza, D. Differential proteome and phosphoproteome may impact cell signaling in the corpus callosum of schizophrenia patients. *Schizophr. Res.* **177**, 70–77 (2016).

52. Fabregat, A. *et al.* The Reactome Pathway Knowledgebase. *Nucleic Acids Res.* **46**, D649–D655 (2018).

53. Rezin, G. T., Amboni, G., Zugno, A. I., Quevedo, J. & Streck, E. L. Mitochondrial Dysfunction and Psychiatric Disorders. *Neurochem Res* **34**, 1021 (2009).

54. Mighdoll, M. I., Tao, R., Kleinman, J. E. & Hyde, T. M. Myelin, myelin-related disorders, and psychosis. *Schizophr. Res.* **161**, 85–93 (2015).

55. Cetin, I. & Demirel, O. F. Increased serum levels of spectrin degradation products in patients with schizophrenia. *Turkish Journal of Biochemistry* **43**, 22–29 (2018).

56. English, J. A., Pennington, K., Dunn, M. J. & Cotter, D. R. The Neuroproteomics of Schizophrenia. *Biological Psychiatry* **69**, 163–172 (2011).

57. Martins-De-Souza, D. *et al.* Proteome analysis of schizophrenia brain tissue. *The World Journal of Biological Psychiatry* **11**, 110–120 (2010).

58. Vawter, M. P. *et al.* Microarray analysis of gene expression in the prefrontal cortex in schizophrenia: a preliminary study. *Schizophrenia Research* **58**, 11–20 (2002).

59. Bubber, P., Hartounian, V., Gibson, G. E. & Blass, J. P. Abnormalities in the tricarboxylic acid (TCA) cycle in the brains of schizophrenia patients. *European Neuropsychopharmacology* **21**, 254–260 (2011).

60. Zhu, Y., Kalbfleisch, T., Brennan, M. D. & Li, Y. A MicroRNA gene is hosted in an intron of a schizophrenia-susceptibility gene. *Schizophrenia Research* **109**, 86–89 (2009).

61. Glatt, S. J., Cohen, O. S., Faraone, S. V. & Tsuang, M. T. Dysfunctional Gene Splicing as a Potential Contributor to Neuropsychiatric Disorders. *Am J Med Genet B Neuropsychiatr Genet* **156B**, 382–392 (2011).

62. Ryder, S. P. & Zearfoss, N. R. Regulation of hnRNP A1 Expression by the Schizophrenia Candidate Gene Quaking. *The FASEB Journal* **22**, 603.6-603.6 (2008).

63. Saia-Cereda, V. M., Santana, A. G., Schmitt, A., Falkai, P. & Martins-de-Souza, D. The Nuclear Proteome of White and Gray Matter from Schizophrenia Postmortem Brains. *MNP* **3**, 37–52 (2017).

64. Xu, Y. *et al.* Altered expression of mRNA profiles in blood of early-onset schizophrenia. *Sci Rep* **6**, (2016).

65. Addington, A. M. *et al.* A novel frameshift mutation in UPF3B identified in brothers affected with childhood onset schizophrenia and autism spectrum disorders. *Mol. Psychiatry* **16**, 238–239 (2011).

66. Jolly, L. A., Homan, C. C., Jacob, R., Barry, S. & Gecz, J. The UPF3B gene, implicated in intellectual disability, autism, ADHD and childhood onset schizophrenia regulates neural progenitor cell behaviour and neuronal outgrowth. *Hum. Mol. Genet.* **22**, 4673–4687 (2013).

67. Pont, A. R., Sadri, N., Hsiao, S. J., Smith, S. & Schneider, R. J. mRNA Decay Factor AUF1 Maintains Normal Aging, Telomere Maintenance, and Suppression of Senescence by Activation of Telomerase Transcription. *Molecular Cell* **47**, 5–15 (2012).

68. Foote, M. & Zhou, Y. 14-3-3 proteins in neurological disorders. *Int J Biochem Mol Biol* **3**, 152–164 (2012).

69. Saia-Cereda, V. M., Cassoli, J. S., Martins-de-Souza, D. & Nascimento, J. M. Psychiatric disorders biochemical pathways unraveled by human brain proteomics. *Eur Arch Psychiatry Clin Neurosci* **267**, 3–17 (2017).

70. Wong, A. H. C. *et al.* Genetic and post-mortem mRNA analysis of the 14-3-3 genes that encode phosphoserine/threonine-binding regulatory proteins in schizophrenia and bipolar disorder. *Schizophrenia Research* **78**, 137–146 (2005).

71. Kabir, M. A. *et al.* Functional Subunits of Eukaryotic Chaperonin CCT/TRiC in Protein Folding. *J Amino Acids* **2011**, (2011).

72. Benitez-King, G., Ramírez-Rodríguez, G., Ortíz, L. & Meza, I. The neuronal cytoskeleton as a potential therapeutical target in neurodegenerative diseases and schizophrenia. *Curr Drug Targets CNS Neurol Disord* **3**, 515–533 (2004).

73. Bhambhvani, H. P., Mueller, T. M., Simmons, M. S. & Meador-Woodruff, J. H. Actin polymerization is reduced in the anterior cingulate cortex of elderly patients with schizophrenia. *Transl Psychiatry* **7**, 1278 (2017).

74. Yan, Z., Kim, E., Datta, D., Lewis, D. A. & Soderling, S. H. Synaptic Actin Dysregulation, a Convergent Mechanism of Mental Disorders? *J Neurosci* **36**, 11411–11417 (2016).

75. Patel, S., Sharma, D., Kalia, K. & Tiwari, V. Crosstalk between endoplasmic reticulum stress and oxidative stress in schizophrenia: The dawn of new therapeutic approaches. *Neurosci Biobehav Rev* **83**, 589–603 (2017).

76. Charron, F. Signaling from Within: Endocytic Trafficking of the Robo Receptor Is Required for Midline Axon Repulsion. *PLOS Genetics* **11**, e1005441 (2015).

77. Potkin, S. G. *et al.* A genome-wide association study of schizophrenia using brain activation as a quantitative phenotype. *Schizophr Bull* **35**, 96–108 (2009).

78. Camargo, L. M. *et al.* Disrupted in Schizophrenia 1 Interactome: evidence for the close connectivity of risk genes and a potential synaptic basis for schizophrenia. *Molecular Psychiatry* **12**, 74–86 (2007).

79. Wolf, T. L., Kotun, J. & Meador-Woodruff, J. H. Plasma copper, iron, ceruloplasmin and ferroxidase activity in schizophrenia. *Schizophr. Res.* **86**, 167–171 (2006).

80. Bowman, M. B. & Lewis, M. S. The copper hypothesis of schizophrenia: A review. *Neuroscience & Biobehavioral Reviews* **6**, 321–328 (1982).

81. Bridges, T. M. *et al.* Polymorphisms in human heat shock factor-1 and analysis of potential biological consequences. *Cell Stress Chaperones* **20**, 47–59 (2015).

82. Hashimoto-Torii, K. *et al.* Roles of Heat Shock Factor 1 in neuronal response to fetal environmental risks and its relevance to brain disorders. *Neuron* **82**, 560–572 (2014).

83. Ishima, T., Iyo, M. & Hashimoto, K. Neurite outgrowth mediated by the heat shock protein Hsp90α: a novel target for the antipsychotic drug aripiprazole. *Translational Psychiatry* **2**, e170 (2012).

84. Parker, H., Chitcholtan, K., Hampton, M. & I Keenan, J. Uptake of Helicobacter pylori Outer Membrane Vesicles by Gastric Epithelial Cells. *Infection and immunity* **78**, 5054–61 (2010).

85. Mawe, G. M. & Hoffman, J. M. Serotonin Signaling in the Gastrointestinal Tract: *Nat Rev Gastroenterol Hepatol* **10**, 473–486 (2013).

86. Bütikofer, P. *et al.* Chlorpromazine inhibits vesiculation, alters phosphoinositide turnover and changes deformability of ATP-Depleted RBCs. *Blood* **73**, 1699–704 (1989).

87. Martins-de-Souza, D. *et al.* Prefrontal cortex shotgun proteome analysis reveals altered calcium homeostasis and immune system imbalance in schizophrenia. *Eur Arch Psychiatry Clin Neurosci* **259**, 151–163 (2009).

88. Ottaviano, Y. & Gerace, L. Phosphorylation of the nuclear lamins during interphase and mitosis. *J. Biol. Chem.* **260**, 624–632 (1985).

89. Lieberman, J. A. Metabolic Changes Associated With Antipsychotic Use. *Prim Care Companion J Clin Psychiatry* **6**, 8–13 (2004).

90. Ozcan, L. & Tabas, I. Role of endoplasmic reticulum stress in metabolic disease and other disorders. *Annu. Rev. Med.* **63**, 317–328 (2012).

91. Wang, X. *et al.* ER Stress Modulates Cellular Metabolism. *Biochem J* **435**, 285–296 (2011).

92. Ma, Y. *et al.* Malonylome Analysis Reveals the Involvement of Lysine Malonylation in Metabolism and Photosynthesis in Cyanobacteria. *J. Proteome Res.* **16**, 2030–2043 (2017).

93. Nasrallah, H. A. Atypical antipsychotic-induced metabolic side effects: insights from receptor-binding profiles. *Molecular Psychiatry* **13**, 27–35 (2008).

94. Gill, K. M. & Grace, A. A. *The Role of Neurotransmitters in Schizophrenia*. (Oxford University Press).

95. Schubert, K. O., Föcking, M., Prehn, J. H. M. & Cotter, D. R. Hypothesis review: are clathrin-mediated endocytosis and clathrin-dependent membrane and protein trafficking core pathophysiological processes in schizophrenia and bipolar disorder? *Mol. Psychiatry* **17**, 669–681 (2012).

Appendices:

Appendix 1: Patient information from postmortem donations

Appendix 2: Postmortem Tissue Protein Tables

Appendix 3: MK-801 Pathway Tables

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Appendix 5: Chlorpromazine Pathway Tables

Appendix 6: Quetiapine Pathway Tables

Appendix 1: Patient information from postmortem donations

Case	Age (years)	Gender	PMI (hours)	pH values	Duration of Disease (years)	Duration of Medication (years)	atyptyp	CPE last dose	CPE last ten years	Cause of Death	DSM IV	Age at Onset	Last Medication	Cigarettes	Alcohol	Hosp	ECT
SCZ	73	M	20	6.6	43	40	1	507.4	1.7	heart infarction	295.6	30	Perphenazine 32 mg, Promethazine 150 mg	30/day	no	33	no
SCZ	43	M	18	6.9	22	20	2	464	2.6	heart infarction	295.6	20	Zuclopethixol 40 mg, Valproate 1200 mg, Tiapride 300 mg	0	no	13	no
SCZ	63	F	31	6.8	40	30	3	75	1.8	heart infarction	295.6	24	Olanzapine 15 mg	30/day	no	30	yes
SCZ	71	M	28	6.4	40	35	1	782.4	10	heart infarction	295.6	30	Haloperidol 32 mg, Pipamperone 40 mg	40/day	no	12	no
SCZ	81	M	4	6.7	62	50	1	92.8	1.4	heart insufficiency	295.6	19	Haloperidol 40 mg, Prothypendyl 80 mg	20	no	48	no
Control	41	M	7	6.5						heart infarction				0	no		
Control	57	M	24	6.9						heart infarction				0	no		
Control	53	M	18	7						heart infarction				0	no		
Control	66	M	16	6.8						heart infarction				0	no		
Control	79	M	24	6.4						heart infarction				0	no		

atyptyp: relation between duration of atypical treatment and duration of treatment with typical neuroleptics during lifetime; CPE: medication calculated in chlorpromazine equivalents(mg): CPE last 10 years: the sum of medications during the last 10 years in kg; Hosp: hospitalization time in years; ECT: electro-convulsive therapy; PMI: postmortem interval

Appendix 2: Postmortem Tissue Protein Tables

Table 5-Differentially regulated, succinylated proteins in multiple tissue samples

Gene Name	Protein Name	Brief Functional Summary
AARS	Alanyl-tRNA Synthetase	tRNA synthesis
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	Energy metabolism in mitochondria
ENO1	Enolase 1	Enzyme in glycolysis
HSPA5	Heat shock protein family A (Hsp70) member 5	Protein folding and assembly
HSPA9	Heat shock protein family A (Hsp70) member 9	Cell proliferation; stress response; mitochondrial maintenance
PLEC	Plectin	Cytoskeleton crosslinkers
SPTBN1	Spectrin beta, non-erythrocytic 1	Actin-plasma membrane linker
TF	(sero)Transferrin	Iron homeostasis; high presence in areas of active cell division

Table 6-Differentially regulated, malonylated proteins in multiple tissue samples

Gene Name	Protein Name	Brief Functional Summary
ACO2	Aconitase 2	Metabolism (glycolysis)
DYNC1H1	Dynein cytoplasmic 1 heavy chain 1	Molecular motor; microtubule-activated
ENO2	Enolase 2	Metabolism (glycolysis)
GRHPR	Glyoxylate and hydroxypyruvate reductase	Metabolism
GSN	Gelsolin	Actin plus-end capping
MYH10	Myosin heavy chain 10	Molecular motor; actin-dependent
NEFH	Neurofilament heavy	Brain cytoskeleton
NEFM	Neurofilament medium	Brain cytoskeleton
PLEC	Plectin	Cytoskeleton crosslinkers
PPIB	Peptidylprolyl isomerase B	Protein folding/maturation; role in mitoch. metabolism, apoptosis, redox, inflammation
RAB5A	Member RAS oncogene family 5A	Endosome maturation
SPTAN1	Spectrin alpha, non-erythrocytic 1	Cytoskeleton; plasma membrane stability; DNA repair; cell cycle regulation
SRSF1	Serine and arginine rich splicing factor 1	Splicing regulation (interactors and PTMs can cause activation or repression)
STXBP1	Syntaxin binding protein 1	Regulates syntaxin; causes release of neurotransmitters
UBE2N	Ubiquitin conjugating enzyme E2 N	Ubiquitination mechanism; DNA post-replication repair

Appendix 3: MK-801 Pathway Tables

Table 7-Top 10 upregulated succinylation pathways in MK-801-treated cells from Reactome.org.

Category	Pathway Name
Various	Metabolism of RNA
Various	Translation
Semaphorin / ROBO	Axon guidance
Ceruloplasmin	L13a-mediated translational silencing of Ceruloplasmin expression
Elongation Factors	GTP hydrolysis and joining of the 60S ribosomal subunit
Ceruloplasmin / Elongation Factors	Cap-dependent Translation Initiation
	Eukaryotic Translation Initiation
AUF1 & HuR (ELAVL1 / YWHAZ)	Regulation of mRNA stability by proteins that bind AU-rich elements
AUF1	AUF1 (hnRNP D0) binds and destabilizes mRNA
ROBO	Signaling by ROBO receptors

37 of 196 accession numbers were not found in the Reactome database.

Table 8-Top 10 downregulated succinylation pathways in MK-801-treated cells from Reactome.org.

Category	Pathway Name
Intron Processing / NMD	Metabolism of RNA
Elongation Factors	Eukaryotic Translation Elongation
Spliceosomes / hnRNPs	mRNA Splicing - Major Pathway
	mRNA Splicing
ROBO	Signaling by ROBO receptors
	Regulation of expression of SLITs and ROBOs
Various	Translation
NMD / Stress Response	Nonsense-Mediated Decay (NMD)
	Nonsense Mediated Decay (NMD) enhanced by the Exon Junction Complex (EJC)
hnRNPs	Processing of Capped Intron-Containing Pre-mRNA

37 of 190 accession numbers were not found in the Reactome database.

Table 9-Top 10 upregulated malonylation pathways in MK-801-treated cells from Reactome.org.

Category	Pathway Name
CCT/TriC	Prefoldin mediated transfer of substrate to CCT/TriC
	Folding of actin by CCT/TriC
	Cooperation of Prefoldin and TriC/CCT in actin and tubulin folding
SRP	SRP-dependent cotranslational protein targeting to membrane
Various	Metabolism of RNA
ROBO	Axon guidance
ROBO	Regulation of expression of SLITs and ROBOs
CCT/TriC	Formation of tubulin folding intermediates by CCT/TriC
Ceruloplasmin	L13a-mediated translational silencing of Ceruloplasmin expression
Various	Translation

32 of 150 accession numbers were not found in the Reactome database.

Table 10-Top 10 downregulated malonylation pathways in MK-801-treated cells from Reactome.org.

Category	Pathway Name
CCT/TriC	Folding of actin by CCT/TriC
	Prefoldin mediated transfer of substrate to CCT/TriC
	Cooperation of Prefoldin and TriC/CCT in actin and tubulin folding
	Formation of tubulin folding intermediates by CCT/TriC
	Association of TriC/CCT with target proteins during biosynthesis
HSF1	Cellular response to heat stress
CCT/TriC	Chaperonin-mediated protein folding
	Protein folding
Various	Metabolism of RNA
HSP90	HSP90 chaperone cycle for steroid hormone receptors (SHR)

31 of 140 accession numbers were not found in the Reactome database.

Table 11-Top 10 affected overall succinylation pathways in MK-801-treated cells from Reactome.org.

Category	Pathway Name
Various	Metabolism of RNA
Various	Translation
mRNA Processing	mRNA Splicing - Major Pathway
mRNA Processing	mRNA Splicing
Translation	GTP hydrolysis and joining of the 60S ribosomal subunit
ROBO	Regulation of expression of SLITs and ROBOs
Translation	Eukaryotic Translation Elongation
Translation	Cap-dependent Translation Initiation
Translation	Eukaryotic Translation Initiation
Ceruloplasmin	L13a-mediated translational silencing of Ceruloplasmin expression

65 of 322 accession numbers were not found in the Reactome database.

Table 12-Top 10 affected overall malonylation pathways in MK-801-treated cells from Reactome.org.

Category	Pathway Name
CCT/TriC	Folding of actin by CCT/TriC
Various	Metabolism of RNA
CCT/TriC	Prefoldin mediated transfer of substrate to CCT/TriC
CCT/TriC	Cooperation of Prefoldin and TriC/CCT in actin and tubulin folding
CCT/TriC	Formation of tubulin folding intermediates by CCT/TriC
Translation	Eukaryotic Translation Elongation
Translation	Peptide chain elongation
Ceruloplasmin	L13a-mediated translational silencing of Ceruloplasmin expression
Protein Trafficking	SRP-dependent cotranslational protein targeting to membrane
Translation	GTP hydrolysis and joining of the 60S ribosomal subunit

52 of 247 accession numbers were not found in the Reactome database.

Appendix 4: Haloperidol Pathway Tables

Table 13-Upregulated succinylated pathways by haloperidol in MO3.13 cells from Reactome.org.

Category	Pathway Name
rRNA/mRNA Processing and NMD	Metabolism of RNA
Ceruloplasmin	L13a-mediated translational silencing of Ceruloplasmin expression
ROBO	Regulation of expression of SLITs and ROBOs
NMD	Nonsense-Mediated Decay (NMD)
NMD	Nonsense Mediated Decay (NMD) enhanced by the Exon Junction Complex (EJC)
Translation	Cap-dependent Translation Initiation
Translation	Eukaryotic Translation Initiation
ROBO	Signaling by ROBO receptors
Translation	GTP hydrolysis and joining of the 60S ribosomal subunit
Translation	Formation of a pool of free 40S subunits

46 of 230 accession numbers were not found in the Reactome database.

Table 14-Downregulated succinylated pathways by haloperidol in MO3.13 cells from Reactome.org.

Category	Pathway Name
Various	Translation
Various	RNA Metabolism
ROBO	Axon guidance
Translation	Eukaryotic Translation Elongation
Stress Response	Cellular responses to stress
Translation	Cytosolic tRNA aminoacylation
Translation	rRNA processing
Translation	Major pathway of rRNA processing in the nucleolus and cytosol
Vesicles	Influenza Life Cycle
ROBO	Signaling by ROBO receptors

35 of 169 accession numbers were not found in the Reactome database.

Table 15-Upregulated malonylated pathways by haloperidol in MO3.13 cells from Reactome.org.

Category	Pathway Name
Translation	Peptide chain elongation
Translation	Translation
Translation	Eukaryotic Translation Elongation
Ceruloplasmin	L13a-mediated translational silencing of Ceruloplasmin expression
Ribosomal Function	GTP hydrolysis and joining of the 60S ribosomal subunit
Ribosomal Function	Formation of a pool of free 40S subunits
Translation	Cap-dependent Translation Initiation
Translation	Eukaryotic Translation Initiation
NMD	Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)
Protein Trafficking	SRP-dependent cotranslational protein targeting to membrane

42 of 210 accession numbers were not found in the Reactome database.

Table 16-Downregulated malonylated pathways by haloperidol in MO3.13 cells from Reactome.org.

Category	Pathway Name
Translation	Eukaryotic Translation Elongation
Translation	Peptide chain elongation
RNA Metabolism	Metabolism of RNA
Infection	Influenza Life Cycle
Infection	Influenza Infection
Translation	Translation
Translation	Formation of a pool of free 40S subunits
Vesicles	Infectious disease
RNA Metabolism	rRNA processing
Ceruloplasmin	L13a-mediated translational silencing of Ceruloplasmin expression

23 of 126 accession numbers were not found in the Reactome database.

Appendix 5: Chlorpromazine Pathway Tables

Table 17-Upregulated succinylated pathways by chlorpromazine in MO3.13 cells from Reactome.org.

Category	Pathway Name
Stress Response	Cellular responses to stress
Stress Response	Cellular response to heat stress
Stress Response	Cellular responses to external stimuli
Stress Response	Regulation of HSF1-mediated heat shock response
RNA Metabolism	Metabolism of RNA
Ceruloplasmin	L13a-mediated translational silencing of Ceruloplasmin expression
Translation	GTP hydrolysis and joining of the 60S ribosomal subunit
Translation	Translation
Translation	Cap-dependent Translation Initiation
Translation	Eukaryotic Translation Initiation

15 of 95 accession numbers were not found in the Reactome database.

Table 18-Downregulated succinylated pathways by chlorpromazine in MO3.13 cells from Reactome.org.

Category	Pathway Name
Ceruloplasmin	L13a-mediated translational silencing of Ceruloplasmin expression
Translation	Cap-dependent Translation Initiation
Translation	Eukaryotic Translation Initiation
Translation	Translation
Translation	GTP hydrolysis and joining of the 60S ribosomal subunit
NMD	Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)
Translation	Formation of a pool of free 40S subunits
Vesicles	Influenza Infection
Protein Trafficking	SRP-dependent cotranslational protein targeting to membrane
ROBO	Regulation of expression of SLITs and ROBOs

11 of 70 accession numbers were not found in the Reactome database.

Table 19-Overall dysregulated succinylated pathways by chlorpromazine in MO3.13 cells from Reactome.org.

Category	Pathway Name
Ceruloplasmin	L13a-mediated translational silencing of Ceruloplasmin expression
Translation	Cap-dependent Translation Initiation
Translation	Eukaryotic Translation Initiation
Translation	Translation
Translation	GTP hydrolysis and joining of the 60S ribosomal subunit
ROBO	Regulation of expression of SLITs and ROBOs
RNA Metabolism	Metabolism of RNA
Translation	Peptide chain elongation
Translation	Eukaryotic Translation Elongation
Translation	Formation of a pool of free 40S subunits

25 of 151 accession numbers were not found in the Reactome database.

Table 20-Upregulated malonylated pathways by chlorpromazine in MO3.13 cells from Reactome.org.

Category	Pathway Name
Stress Response	HSP90 chaperone cycle for steroid hormone receptors (SHR)
Translation	L13a-mediated translational silencing of Ceruloplasmin expression
Translation	GTP hydrolysis and joining of the 60S ribosomal subunit
Translation	Cap-dependent Translation Initiation
Translation	Eukaryotic Translation Initiation
Cytoskeleton	Axon guidance
Translation	Translation initiation complex formation
Translation	Ribosomal scanning and start codon recognition
Translation	Activation of the mRNA upon binding of the cap-binding complex and eIFs, and subsequent binding to 43S
Cytoskeleton	Formation of tubulin folding intermediates by CCT/TriC

18 of 98 accession numbers were not found in the Reactome database.

Table 21-Downregulated malonylated pathways by chlorpromazine in MO3.13 cells from Reactome.org.

Category	Pathway Name
RNA Metabolism	Metabolism of RNA
Protein Trafficking	SRP-dependent cotranslational protein targeting to membrane
NMD	Nonsense-Mediated Decay (NMD)
NMD	Nonsense Mediated Decay (NMD) enhanced by the Exon Junction Complex (EJC)
RNA Metabolism	Selenoamino acid metabolism
Translation	Peptide chain elongation
Vesicles	Influenza Infection
Translation	Eukaryotic Translation Termination
RNA Metabolism	Selenocysteine synthesis
Translation	Eukaryotic Translation Elongation

9 of 65 accession numbers were not found in the Reactome database.

Table 22-Overall dysregulated malonylated pathways by chlorpromazine in MO3.13 cells from Reactome.org.

Category	Pathway Name
Translation	L13a-mediated translational silencing of Ceruloplasmin expression
Translation	GTP hydrolysis and joining of the 60S ribosomal subunit
RNA Metabolism	Metabolism of RNA
Translation	Cap-dependent Translation Initiation
Translation	Eukaryotic Translation Initiation
NMD	Nonsense-Mediated Decay (NMD)
NMD	Nonsense Mediated Decay (NMD) enhanced by the Exon Junction Complex (EJC)
Translation	Translation initiation complex formation
Translation	Ribosomal scanning and start codon recognition
Translation	Activation of the mRNA upon binding of the cap-binding complex and eIFs, and subsequent binding to 43S

26 of 154 accession numbers were not found in the Reactome database.

Appendix 6: Quetiapine Pathway Tables

Table 23-Upregulated succinylated pathways by quetiapine in MO3.13 cells from Reactome.org.

Category	Pathway Name
Lamin Dimers	Depolymerisation of the Nuclear Lamina
Stress Response	Unfolded Protein Response (UPR)
RNA Metabolism	Metabolism of RNA
Cell Cycle	Nuclear Envelope Breakdown
RNA Metabolism	Processing of Capped Intron-Containing Pre-mRNA
RNA Metabolism	mRNA Splicing - Major Pathway
RNA Metabolism	Major pathway of rRNA processing in the nucleolus and cytosol
RNA Metabolism	mRNA Splicing
RNA Metabolism	rRNA processing in the nucleus and cytosol
RNA Metabolism	rRNA processing

12 of 54 accession numbers were not found in the Reactome database.

Downregulated succinylated pathways by quetiapine in MO3.13 cells did not exhibit an FDR below 4% and were not included in this analysis.

Table 24-Overall dysregulated succinylated pathways by quetiapine in MO3.13 cells from Reactome.org.

Category	Pathway Name
RNA Metabolism	Metabolism of RNA
RNA Metabolism	Major pathway of rRNA processing in the nucleolus and cytosol
Lamin Dimers	Depolymerisation of the Nuclear Lamina
RNA Metabolism	rRNA processing
Stress Response	Unfolded Protein Response (UPR)
Cell Cycle	Nuclear Envelope Breakdown
RNA Metabolism	Processing of Capped Intron-Containing Pre-mRNA
RNA Splicing	mRNA Splicing - Major Pathway
Protein Metabolism	Metabolism of proteins
RNA Metabolism	mRNA Splicing

12 of 57 accession numbers were not found in the Reactome database.

Table 25-Upregulated malonylated pathways by quetiapine in MO3.13 cells from Reactome.org.

Category	Pathway Name
RNA Metabolism	Metabolism of RNA
Translation	GTP hydrolysis and joining of the 60S ribosomal subunit
Translation	Eukaryotic Translation Initiation
Translation	Cap-dependent Translation Initiation
Ceruloplasmin	L13a-mediated translational silencing of Ceruloplasmin expression
RNA Metabolism	rRNA processing
NMD	Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)
ROBO	Regulation of expression of SLITs and ROBOs
RNA Metabolism	Major pathway of rRNA processing in the nucleolus and cytosol
RNA Metabolism	rRNA processing in the nucleus and cytosol

9 of 47 accession numbers were not found in the Reactome database.

Table 26-Downregulated malonylated pathways by quetiapine in MO3.13 cells from Reactome.org.

Category	Pathway Name
Vesicles	Lysosome Vesicle Biogenesis
Cell Receptors	HSP90 chaperone cycle for steroid hormone receptors (SHR)
Vesicles	Clathrin derived vesicle budding
Vesicles	trans-Golgi Network Vesicle Budding
Clathrin (Vesicles)	MHC class II antigen presentation
Heat Shock HSP90	Aryl hydrocarbon receptor signalling
Heat Shock HSP90	Uptake and function of diphtheria toxin
Gap Junctions	Formation of annular gap junctions
Clathrin (Vesicles)	Entry of Influenza Virion into Host Cell via Endocytosis
Gap Junctions	Gap junction degradation

1 of 5 accession numbers were not found in the Reactome database.

Table 27-Overall dysregulated malonylated pathways by quetiapine in MO3.13 cells from Reactome.org.

Category	Pathway Name
RNA Metabolism	Metabolism of RNA
Translation	GTP hydrolysis and joining of the 60S ribosomal subunit
Translation	Eukaryotic Translation Initiation
Translation	Cap-dependent Translation Initiation
Ceruloplasmin	L13a-mediated translational silencing of Ceruloplasmin expression
ROBO	Axon guidance
RNA Metabolism	rRNA processing
Vesicles	Lysosome Vesicle Biogenesis
Macromolecule Export/Clathrin	Influenza Life Cycle
NMD	Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)

10 of 52 accession numbers were not found in the Reactome database.

OVERALL RESULTS

Schizophrenia is still a complex illness with no cure; but every day, research brings the scientific community just a little bit closer to understanding and eventually curing this devastating condition, even if by just an inch. The research contained in this dissertation has brought various metabolic disturbances in schizophrenia and antipsychotics together with a pair of poorly understood post-translational modifications.

Various proteins and pathways are now seen to be affected by schizophrenia, a mimetic drug MK-801, and antipsychotics. Furthermore, antipsychotics seem to revert some changes induced by MK-801, additionally showing differences among different antipsychotic compounds. The new sites of succinylation and malonylation that were revealed will unlock new doors to research the causes and effects of these modifications, not only in relation to schizophrenia, but also to various other metabolic disorders.

Little overlap was seen between MK-801-treated oligodendrocytes and postmortem brain tissue from patients with schizophrenia; however, this could be due to a number of factors and can be further investigated to determine the similarities and differences between these two conditions, thought to be similar.

The large number of acylation changes induced by antipsychotics has created more questions than it has answered, not only due to the translation, RNA metabolism, and vesicle transport pathways that they affect, but also due to the blunt differences between different antipsychotics. Whether this has to do with the therapeutic potential of the drugs, a result of their function, or their undesired side effects is entirely unknown.

Lastly, the number of proteins that were found to be dysregulated by MK-801 and at least mildly attenuated by an antipsychotic urges further study and investigation to determine what roles succinylation and malonylation may play in schizophrenia’s development, symptoms, progression, and treatment.

CONCLUDING REMARKS

The changes in lysine succinylation and malonylation that were seen in this work suggest an important and seriously under-researched form of post-translational protein modification. The roles of the modification precursors in carbon metabolism and fatty acid synthesis, their high prevalence on metabolism-associated proteins, and the already known response of one to metabolic stress factors suggest some kind of yet-undiscovered mechanism to respond to external stimuli.

A large number of post-translational protein modifications have been already discovered, and studies have been slow to comprehend the role of each in cells. Some modifications are performed for signaling, others for stability, others for activity; these modifications seem to be associated with enzyme activity, which could strongly influence related protein pathways.

As schizophrenia, and many other diseases, have a known dysregulation in energy metabolism, learning how this pathway responds to – and affects – its interactors could provide a great deal of insight into how certain proteins and protein pathways become dysregulated, leading to disease. Post-translational modifications like acetylation and phosphorylation are already well known and studied; and their effects on proteins can lead to various negative side effects in diseases, including a suggested role of phosphorylation in the neurodegenerative condition, Alzheimer's disease.

In addition to disease response, it appears that different antipsychotic classes can also exhibit different modification profiles, suggesting either a visible response to the differing side effects, or even potentially a cause for those same differences. Antipsychotics do possess different side effect profiles, including metabolism-related effects. Learning how to control and suppress these changes may help to create new, more effective medications and reduce the detrimental side effects of current treatment options.

This work serves as a stepping stone and a type of proof of concept, since at this time, no article has yet been published studying changes in the "succinylome" in a mental disease. One study found succinylation changes induced by carbon source (Kosono et al., 2015), another has found succinylation differences in cancer (Song et

al., 2017), and yet another has found a potential change in hypoxia-affected heart tissue (Boylston et al., 2015).

Malonylation has garnered less fame since its discovery; however, its supposed regulatory enzyme (SIRT5) has been implicated in various diseases. Although it is important to mention that SIRT5 is also assumed to be the main regulating enzyme for succinylation. That also makes this work the first to suggest important, regulatory changes in the “malonylome” in disease.

Adding to the metabolic regulation aforementioned, succinylation and malonylation seemed to affect the cytoskeleton, protein translation, and vesicle transport, which strengthens the probability of a form of cellular regulation in response to metabolic stimuli. As an additional point, if this modification is found to be as highly regulated as what this study suggests possible, succinylation and malonylation profiles may even be possible to use as biomarker candidates to differentiate between different diseases.

In summary, investigating further these two poorly understood protein modifications in relation to disease, specifically schizophrenia, may reveal previously unthought of methods to identify or treat them. Learning how to manipulate these two modifications can also help to reduce the side effects of certain medications like antipsychotics and help develop new ones.

REFERENCES

Alleyn, M., Breitzig, M., Lockey, R., and Kolliputi, N. (2017). The dawn of succinylation: a posttranslational modification. American Journal of Physiology-Cell Physiology *314*, C228–C232.

Amorim, M., Moreira, A., Marques, A., and Summavielle, T. (2017). Brain metabolic abnormalities in schizophrenia patients. European Psychiatry *41*, S802.

Andreasen, MD, PhD, N.C., Arndt, PhD, S., Alliger, PhD, R., Miller, PharmD, MD, D., and Flaum, MD, M. (1995). Symptoms of Schizophrenia: Methods, Meanings, and Mechanisms. Arch Gen Psychiatry *52*, 341–351.

Arnedo, J., Svrakic, D.M., del Val, C., Romero-Zaliz, R., Hernández-Cuervo, H., Fanous, A.H., Pato, M.T., Pato, C.N., de Erausquin, G.A., Cloninger, C.R., et al. (2014). Uncovering the Hidden Risk Architecture of the Schizophrenias: Confirmation in Three Independent Genome-Wide Association Studies. AJP *172*, 139–153.

Association, A.D. (2004). Consensus Development Conference on Antipsychotic Drugs and Obesity and Diabetes. Diabetes Care *27*, 596–601.

Boison, D., Singer, P., Shen, H.-Y., Feldon, J., and Yee, B.K. (2012). Adenosine hypothesis of schizophrenia – Opportunities for pharmacotherapy. Neuropharmacology *62*, 1527–1543.

Bond, N.J., Shliaha, P.V., Lilley, K.S., and Gatto, L. (2013). Improving Qualitative and Quantitative Performance for MSE-based Label-free Proteomics. J. Proteome Res. *12*, 2340–2353.

Bourassa, S., Fournier, F., Nehmé, B., Kelly, I., Tremblay, A., Lemelin, V., Lamarche, B., Couture, P., and Droit, A. (2015). Evaluation of iTRAQ and SWATH-MS for the Quantification of Proteins Associated with Insulin Resistance in Human Duodenal Biopsy Samples. PLOS ONE *10*, e0125934.

Bowman, C.E., Rodriguez, S., Selen Alpergin, E.S., Acoba, M.G., Zhao, L., Hartung, T., Claypool, S.M., Watkins, P.A., and Wolfgang, M.J. (2017). The Mammalian Malonyl-CoA Synthetase ACSF3 Is Required for Mitochondrial Protein Malonylation and Metabolic Efficiency. Cell Chem Biol *24*, 673-684.e4.

Boylston, J.A., Sun, J., Chen, Y., Gucek, M., Sack, M.N., and Murphy, E. (2015). Characterization of the cardiac succinylome and its role in ischemia-reperfusion injury. J Mol Cell Cardiol *88*, 73–81.

Brandão-Teles, C., Martins-de-Souza, D., Guest, P.C., and Cassoli, J.S. (2017). MK-801-Treated Oligodendrocytes as a Cellular Model to Study Schizophrenia. Adv. Exp. Med. Biol. *974*, 269–277.

Buntinx, M., Vanderlocht, J., Hellings, N., Vandenabeele, F., Lambrichts, I., Raus, J., Ameloot, M., Stinissen, P., and Steels, P. (2003). Characterization of three human oligodendroglial cell lines as a model to study oligodendrocyte injury: morphology and oligodendrocyte-specific gene expression. J. Neurocytol. *32*, 25–38.

Chan, M.K., Tsang, T.M., Harris, L.W., Guest, P.C., Holmes, E., and Bahn, S. (2011). Evidence for disease and antipsychotic medication effects in post-mortem brain from schizophrenia patients. Molecular Psychiatry *16*, 1189–1202.

Chen, H., Xu, H., Potash, S., Starkov, A., Belousov, V.V., Bilan, D.S., Denton, T.T., and Gibson, G.E. (2017). Mild metabolic perturbations alter succinylation of mitochondrial proteins. J. Neurosci. Res. *95*, 2244–2252.

Chua, S.E., and Murray, R.M. (1996). The Neurodevelopmental Theory of Schizophrenia: Evidence Concerning Structure and Neuropsychology. Annals of Medicine *28*, 547–555.

Correll, C.U., and Schenk, E.M. (2008). Tardive dyskinesia and new antipsychotics. Current Opinion in Psychiatry *21*, 151–156.

Davis, J., Eyre, H., Jacka, F.N., Dodd, S., Dean, O., McEwen, S., Debnath, M., McGrath, J., Maes, M., Amminger, P., et al. (2016). A review of vulnerability and risks for schizophrenia: Beyond the two hit hypothesis. Neurosci Biobehav Rev *65*, 185–194.

Dehzangi, A., López, Y., Lal, S.P., Taherzadeh, G., Sattar, A., Tsunoda, T., and Sharma, A. (2018). Improving succinylation prediction accuracy by incorporating the secondary structure via helix, strand and coil, and evolutionary information from profile bigrams. PLOS ONE *13*, e0191900.

Eyjolfsson, E.M., Brenner, E., Kondziella, D., and Sonnewald, U. (2006). Repeated injection of MK801: An animal model of schizophrenia? Neurochemistry International *48*, 541–546.

Fatemi, S.H., and Folsom, T.D. (2009). The Neurodevelopmental Hypothesis of Schizophrenia, Revisited. Schizophr Bull *35*, 528–548.

Gerber, S.A., Rush, J., Stemman, O., Kirschner, M.W., and Gygi, S.P. (2003). Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. PNAS *100*, 6940–6945.

Gonçalves, V.F., Zai, C.C., Tiwari, A.K., Brandl, E.J., Derkach, A., Meltzer, H.Y., Lieberman, J.A., Müller, D.J., Sun, L., and Kennedy, J.L. (2014). A Hypothesis-Driven Association Study of 28 Nuclear-Encoded Mitochondrial Genes with Antipsychotic-Induced Weight Gain in Schizophrenia. Neuropsychopharmacology *39*, 1347–1354.

Guenette, M.D., Giacca, A., Hahn, M., Teo, C., Lam, L., Chintoh, A., Arenovich, T., and Remington, G. (2013). Atypical antipsychotics and effects of adrenergic and serotonergic receptor binding on insulin secretion in-vivo: An animal model. Schizophrenia Research *146*, 162–169.

Gupta, S., and Kulhara, P. (2010). What is schizophrenia: A neurodevelopmental or neurodegenerative disorder or a combination of both? A critical analysis. Indian J Psychiatry *52*, 21–27.

Harrison, P.J. (2011). Using Our Brains: The Findings, Flaws, and Future of Postmortem Studies of Psychiatric Disorders. Biological Psychiatry *69*, 102–103.

Hasan, A., Falkai, P., Wobrock, T., Lieberman, J., Glenthøj, B., Gattaz, W.F., Thibaut, F., Möller, H.-J., and Schizophrenia, on behalf of the W.T.F. on T.G. for (2015). World Federation of Societies of Biological Psychiatry (WFSBP) Guidelines for Biological Treatment of Schizophrenia Part 3: Update 2015 Management of special circumstances: Depression, Suicidality, substance use disorders and pregnancy and lactation. The World Journal of Biological Psychiatry *16*, 142–170.

Higgs, R.E., Knierman, M.D., Gelfanova, V., Butler, J.P., and Hale, J.E. (2005). Comprehensive Label-Free Method for the Relative Quantification of Proteins from Biological Samples. J. Proteome Res. *4*, 1442–1450.

Hilker, R., Helenius, D., Fagerlund, B., Skytthe, A., Christensen, K., Werge, T.M., Nordentoft, M., and Glenthøj, B. (2018). Heritability of Schizophrenia and Schizophrenia Spectrum Based on the Nationwide Danish Twin Register. Biological Psychiatry *83*, 492–498.

Hirschey, M.D., and Zhao, Y. (2015). Metabolic Regulation by Lysine Malonylation, Succinylation, and Glutarylation. Mol. Cell Proteomics *14*, 2308–2315.

Hof, P.R., Haroutunian, V., Copland, C., Davis, K.L., and Buxbaum, J.D. (2002). Molecular and Cellular Evidence for an Oligodendrocyte Abnormality in Schizophrenia. Neurochem Res *27*, 1193–1200.

Howes, O.D., and Kapur, S. (2009). The Dopamine Hypothesis of Schizophrenia: Version III—The Final Common Pathway. Schizophr Bull *35*, 549–562.

Hu, A., Noble, W.S., and Wolf-Yadlin, A. (2016). Technical advances in proteomics: new developments in data-independent acquisition. F1000Res *5*.

Hu, W., MacDonald, M.L., Elswick, D.E., and Sweet, R.A. (2015). The glutamate hypothesis of schizophrenia: evidence from human brain tissue studies. Annals of the New York Academy of Sciences *1338*, 38–57.

Huang, J.T.-J., Wang, L., Prabakaran, S., Wengenroth, M., Lockstone, H.E., Koethe, D., Gerth, C.W., Gross, S., Schreiber, D., Lilley, K., et al. (2008). Independent protein-profiling studies show a decrease in apolipoprotein A1 levels in schizophrenia CSF, brain and peripheral tissues. Molecular Psychiatry *13*, 1118–1128.

Ingimarsson, O., MacCabe, J.H., Haraldsson, M., Jónsdóttir, H., and Sigurdsson, E. (2016). Neutropenia and agranulocytosis during treatment of schizophrenia with clozapine versus other antipsychotics: an observational study in Iceland. BMC Psychiatry *16*, 441.

Jones, P.B., Barnes, T.R.E., Davies, L., Dunn, G., Lloyd, H., Hayhurst, K.P., Murray, R.M., Markwick, A., and Lewis, S.W. (2006). Randomized Controlled Trial of the Effect on Quality of Life of Second- vs First-Generation Antipsychotic Drugs in Schizophrenia: Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia Study (CUtLASS 1). Arch Gen Psychiatry *63*, 1079–1087.

Karlsgodt, K.H., Sun, D., and Cannon, T.D. (2010). Structural and Functional Brain Abnormalities in Schizophrenia. Curr Dir Psychol Sci *19*, 226–231.

Karoutzou, G., Emrich, H.M., and Dietrich, D.E. (2008). The myelin-pathogenesis puzzle in schizophrenia: a literature review. Molecular Psychiatry *13*, 245–260.

Khaitovich, P., Lockstone, H.E., Wayland, M.T., Tsang, T.M., Jayatilaka, S.D., Guo, A.J., Zhou, J., Somel, M., Harris, L.W., Holmes, E., et al. (2008). Metabolic changes in schizophrenia and human brain evolution. Genome Biol *9*, R124.

Klose, J. (1975). Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik *26*, 231–243.

Kosono, S., Tamura, M., Suzuki, S., Kawamura, Y., Yoshida, A., Nishiyama, M., and Yoshida, M. (2015). Changes in the Acetylome and Succinylome of Bacillus subtilis in Response to Carbon Source. PLOS ONE *10*, e0131169.

Koulman, A., Woffendin, G., Narayana, V.K., Welchman, H., Crone, C., and Volmer, D.A. (2009). High-resolution extracted ion chromatography, a new tool for metabolomics and lipidomics using a second-generation orbitrap mass spectrometer. Rapid Commun Mass Spectrom *23*, 1411–1418.

Leonard, B.E., Schwarz, M., and Myint, A.M. (2012). The metabolic syndrome in schizophrenia: is inflammation a contributing cause? J Psychopharmacol *26*, 33–41.

Lieberman, J.A., Stroup, T.S., McEvoy, J.P., Swartz, M.S., Rosenheck, R.A., Perkins, D.O., Keefe, R.S.E., Davis, S.M., Davis, C.E., Lebowitz, B.D., et al. (2005). Effectiveness of Antipsychotic Drugs in Patients with Chronic Schizophrenia. New England Journal of Medicine *353*, 1209–1223.

Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D., and Darnell, J. (2000). The Molecules of Life. Molecular Cell Biology. 4th Edition.

Marcelis, M., Cavalier, E., Gielen, J., Delespaul, P., and Van Os, J. (2004). Abnormal response to metabolic stress in schizophrenia: marker of vulnerability or acquired sensitization? Psychol Med *34*, 1103–1111.

Martins-de-Souza, D. (2010). Proteome and transcriptome analysis suggests oligodendrocyte dysfunction in schizophrenia. Journal of Psychiatric Research *44*, 149–156.

Martins-de-Souza, D., Harris, L.W., Guest, P.C., and Bahn, S. (2010). The Role of Energy Metabolism Dysfunction and Oxidative Stress in Schizophrenia Revealed by Proteomics. Antioxidants & Redox Signaling *15*, 2067–2079.

Martins-de-Souza, D., Guest, P.C., Harris, L.W., Vanattou-Saifoudine, N., Webster, M.J., Rahmoune, H., and Bahn, S. (2012). Identification of proteomic signatures associated with depression and psychotic depression in post-mortem brains from major depression patients. Translational Psychiatry *2*, e87.

McDonald, C., Bullmore, E., Sham, P., Chitnis, X., Suckling, J., Maccabe, J., Walshe, M., and Murray, R.M. (2005). Regional volume deviations of brain structure in schizophrenia and psychotic bipolar disorder: Computational morphometry study. The British Journal of Psychiatry *186*, 369–377.

McLean, J.A., Ruotolo, B.T., Gillig, K.J., and Russell, D.H. (2005). Ion mobility–mass spectrometry: a new paradigm for proteomics. International Journal of Mass Spectrometry *240*, 301–315.

Mighdoll, M.I., Tao, R., Kleinman, J.E., and Hyde, T.M. (2015). Myelin, myelin-related disorders, and psychosis. Schizophr. Res. *161*, 85–93.

Murray, R.M., and Lewis, S.W. (1987). Is schizophrenia a neurodevelopmental disorder? Br Med J (Clin Res Ed) *295*, 681–682.

Nascimento, J.M., and Martins-de-Souza, D. (2015). The proteome of schizophrenia. Npj Schizophrenia *1*, 14003.

Newman, J.C., He, W., and Verdin, E. (2012). Mitochondrial Protein Acylation and Intermediary Metabolism: Regulation by Sirtuins and Implications for Metabolic Disease. J. Biol. Chem. *287*, 42436–42443.

Ning, Q., Zhao, X., Bao, L., Ma, Z., and Zhao, X. (2018). Detecting Succinylation sites from protein sequences using ensemble support vector machine. BMC Bioinformatics *19*, 237.

Nishida, Y., Rardin, M.J., Carrico, C., He, W., Sahu, A.K., Gut, P., Najjar, R., Fitch, M., Hellerstein, M., Gibson, B.W., et al. (2015). SIRT5 Regulates both Cytosolic and Mitochondrial Protein Malonylation with Glycolysis as a Major Target. Mol Cell *59*, 321–332.

O'Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. *250*, 4007–4021.

Ong, S.-E., and Mann, M. (2005). Mass spectrometry–based proteomics turns quantitative. Nat Chem Biol *1*, 252–262.

Owen, M.J., O'Donovan, M.C., Thapar, A., and Craddock, N. (2011). Neurodevelopmental hypothesis of schizophrenia. The British Journal of Psychiatry *198*, 173–175.

Peng, C., Lu, Z., Xie, Z., Cheng, Z., Chen, Y., Tan, M., Luo, H., Zhang, Y., He, W., Yang, K., et al. (2011). The First Identification of Lysine Malonylation Substrates and Its Regulatory Enzyme. Mol Cell Proteomics *10*, M111.012658.

Qian, L., Nie, L., Chen, M., Liu, P., Zhu, J., Zhai, L., Tao, S., Cheng, Z., Zhao, Y., and Tan, M. (2016). Global Profiling of Protein Lysine Malonylation in Escherichia coli Reveals Its Role in Energy Metabolism. J. Proteome Res. *15*, 2060–2071.

Rardin, M.J., He, W., Nishida, Y., Newman, J.C., Carrico, C., Danielson, S.R., Guo, A., Gut, P., Sahu, A.K., Li, B., et al. (2013). SIRT5 regulates the mitochondrial lysine succinylome and metabolic networks. Cell Metab. *18*, 920–933.

Resing, K.A., and Ahn, N.G. (2005). Proteomics strategies for protein identification. FEBS Letters *579*, 885–889.

Reynolds, G.P. (2012). The Pharmacogenetics of Symptom Response to Antipsychotic Drugs. Psychiatry Investig *9*, 1–7.

Riordan, H.J., Antonini, P., and Murphy, M.F. (2011). Atypical Antipsychotics and Metabolic Syndrome in Patients with Schizophrenia: Risk Factors, Monitoring, and Healthcare Implications. Am Health Drug Benefits *4*, 292–302.

Rung, J.P., Carlsson, A., Rydén Markinhuhta, K., and Carlsson, M.L. (2005). (+)-MK-801 induced social withdrawal in rats; a model for negative symptoms of schizophrenia. Prog. Neuropsychopharmacol. Biol. Psychiatry *29*, 827–832.

Saha, S., Chant, D., Welham, J., and McGrath, J. (2005). A systematic review of the prevalence of schizophrenia. PLoS Med. *2*, e141.

Sahlholm, K., Marcellino, D., Nilsson, J., Ögren, S.O., Fuxe, K., and Århem, P. (2014). Typical and atypical antipsychotics do not differ markedly in their reversibility of antagonism of the dopamine D2 receptor. Int J Neuropsychopharmacol *17*, 149–155.

Seillier, A., and Giuffrida, A. (2009). Evaluation of NMDA receptor models of schizophrenia: Divergences in the behavioral effects of sub-chronic PCP and MK-801. Behavioural Brain Research *204*, 410–415.

Selten, J.-P., van der Ven, E., Rutten, B.P.F., and Cantor-Graae, E. (2013). The Social Defeat Hypothesis of Schizophrenia: An Update. Schizophr Bull *39*, 1180–1186.

Shen, W.W. (1999). A history of antipsychotic drug development. Compr Psychiatry *40*, 407–414.

Silva, J.C., Gorenstein, M.V., Li, G.-Z., Vissers, J.P.C., and Geromanos, S.J. (2006). Absolute Quantification of Proteins by LCMSE A Virtue of Parallel ms Acquisition. Mol Cell Proteomics *5*, 144–156.

Song, Y., Wang, J., Cheng, Z., Gao, P., Sun, J., Chen, X., Chen, C., Wang, Y., and Wang, Z. (2017). Quantitative global proteome and lysine succinylome analyses provide insights into metabolic regulation and lymph node metastasis in gastric cancer. Scientific Reports *7*, 42053.

Sullivan, E.M., and O'Donnell, P. (2012). Inhibitory interneurons, oxidative stress, and schizophrenia. Schizophr Bull *38*, 373–376.

Svoboda, J., Stankova, A., Entlerova, M., and Stuchlik, A. (2015). Acute administration of MK-801 in an animal model of psychosis in rats interferes with cognitively demanding forms of behavioral flexibility on a rotating arena. Front Behav Neurosci *9*.

Taherzadeh, G., Yang, Y., Xu, H., Xue, Y., Liew, A.W.-C., and Zhou, Y. (2018). Predicting lysine-malonylation sites of proteins using sequence and predicted structural features. J Comput Chem *39*, 1757–1763.

Takahashi, N., Sakurai, T., Davis, K.L., and Buxbaum, J.D. (2011). Linking oligodendrocyte and myelin dysfunction to neurocircuitry abnormalities in schizophrenia. Prog Neurobiol *93*, 13–24.

Timms, A.E., Dorschner, M.O., Wechsler, J., Choi, K.Y., Kirkwood, R., Girirajan, S., Baker, C., Eichler, E.E., Korvatska, O., Roche, K.W., et al. (2013). Support for the N - Methyl-D-Aspartate Receptor Hypofunction Hypothesis of Schizophrenia From Exome Sequencing in Multiplex Families. JAMA Psychiatry *70*, 582–590.

Tkachev, D., Mimmack, M.L., Ryan, M.M., Wayland, M., Freeman, T., Jones, P.B., Starkey, M., Webster, M.J., Yolken, R.H., and Bahn, S. (2003). Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. Lancet *362*, 798–805.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A *76*, 4350–4354.

Tsuang, M.T., Stone, W.S., and Faraone, S.V. (2001). Genes, environment and schizophrenia. The British Journal of Psychiatry *178*, s18–s24.

Tuominen, H.J., Tiihonen, J., and Wahlbeck, K. (2005). Glutamatergic drugs for schizophrenia: a systematic review and meta-analysis. Schizophrenia Research *72*, 225–234.

Uranova, N.A., Vostrikov, V.M., Orlovskaya, D.D., and Rachmanova, V.I. (2004). Oligodendroglial density in the prefrontal cortex in schizophrenia and mood disorders: a study from the Stanley Neuropathology Consortium. Schizophrenia Research *67*, 269–275.

Wandy, J., Daly, R., Breitling, R., and Rogers, S. (2015). Incorporating peak grouping information for alignment of multiple liquid chromatography-mass spectrometry datasets. Bioinformatics *31*, 1999–2006.

Wang, M., You, J., Bemis, K.G., Tegeler, T.J., and Brown, D.P.G. (2008). Label-free mass spectrometry-based protein quantification technologies in proteomic analysis. Brief Funct Genomics *7*, 329–339.

Washburn, M.P., Wolters, D., and Yates Iii, J.R. (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nature Biotechnology *19*, 242–247.

Watanabe, Y., Someya, T., and Nawa, H. (2010). Cytokine hypothesis of schizophrenia pathogenesis: Evidence from human studies and animal models. Psychiatry and Clinical Neurosciences *64*, 217–230.

Weinert, B.T., Schölz, C., Wagner, S.A., Iesmantavicius, V., Su, D., Daniel, J.A., and Choudhary, C. (2013). Lysine Succinylation Is a Frequently Occurring Modification in Prokaryotes and Eukaryotes and Extensively Overlaps with Acetylation. Cell Reports *4*, 842–851.

Xie, Z., Dai, J., Dai, L., Tan, M., Cheng, Z., Wu, Y., Boeke, J.D., and Zhao, Y. (2012). Lysine succinylation and lysine malonylation in histones. Mol. Cell Proteomics *11*, 100–107.

Zhang, Y., Wang, G., Song, L., Mu, P., Wang, S., Liang, W., and Lin, Q. (2017). Global analysis of protein lysine succinylation profiles in common wheat. BMC Genomics *18*, 309.

Zhang, Z., Tan, M., Xie, Z., Dai, L., Chen, Y., and Zhao, Y. (2011). Identification of lysine succinylation as a new post-translational modification. Nat Chem Biol *7*, 58–63.

Appendix 1: Co-immunoprecipitation for Deciphering Protein Interactomes

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Smith, B.J., Cassoli, J.S., Guest, P.C., and Martins-de-Souza, D. (2017). Co-immunoprecipitation for Deciphering Protein Interactomes. In Proteomic Methods in Neuropsychiatric Research, P.C. Guest, ed. (Cham: Springer International Publishing), pp. 229–236.

Abstract:

A single protein is often capable of binding with many partners, enabling potential effects on either protein, such as modifying its expression or activity. However, due to the complex nature of *in vivo* systems, it is often difficult to perform nontargeted assays with a protein of interest. Methods in discovery proteomics must be used to find potential interactors to pave the way for additional, more focused studies. This protocol describes the biological steps needed to create an interactome focused on a single protein target through co-immunoprecipitation.

Appendix 2: Using co-immunoprecipitation and shotgun mass spectrometry for protein-protein interaction identification in cultured human oligodendrocytes.

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Abstract:

Protein complexes are responsible for major biological processes in cells. Identifying a protein's interaction partners reveals many different aspects related to the target such as its function, regulation, and mechanisms of action. Co-immunoprecipitation (co-IP) followed by shotgun mass spectrometry (MS) is a powerful technique for capturing and identifying endogenous protein complexes. Although it is a simple concept, performing the experiment can be challenging due to the number of steps and the diversity of available protocols. Here we present a certified and detailed protocol to perform co-IP experiments in cultured human oligodendrocytes.

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