THE UNIVERSITY OF CHICAGO

THE ONCOGENIC REGULATION OF HISTONE LYSINE LACTYLATION

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES

AND THE PRITZKER SCHOOL OF MEDICINE

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

COMMITTEE ON CANCER BIOLOGY

BY

MATHEW PEREZ-NEUT

CHICAGO, ILLINOIS

DECEMBER 2021

Table of Contents

LIST OF FIGURES v

LIST OF TABLES vi

ACKNOWLEDGEMENTS vii

ABBREVIATIONS xi

CHAPTER 1: Introduction 1

1.1 Protein posttranslational modifications 1

1.1.1 Properties and functions of protein posttranslational modifications 1

1.1.2 Histone proteins and histone marks 2

1.1.3 Discovery of chemically diverse histone marks 3

1.2 Biological regulation of histone marks 8

1.2.1 Metabolic regulation of histone marks 8

1.2.2 Regualtory elements of histone marks: the readers, writers, erasers 10

1.2.3 Acetyltransferases 11

1.2.4 MYST family of KATs 12

1.3 The MAPK signaling pathway 15

1.3.1 Protein phosphorylation 15

1.3.2 The RAS-RAF-MEK-ERK/MAPK pathway 16

1.3.3 MAPK and cancer 19

1.4 Cancer metabolism 21

1.4.1 The Warburg effect 21

1.4.2 The lactate molecule 23

1.4.3 Unanswered questions 25

CHAPTER 2: LYSINE LACTYLATION IS AN ONCOGENIC HISTONE MARK 27
2.1 Introduction 27
2.2 Results............28
2.2.1 Histone Kla is regulated by EGFR signaling 28
2.2.2 Histone Kla is regulated by KRAS and BRAF signaling 31
2.2.3 Histone Kla is regulated by MEK signaling 34
2.2.4 Comprehensive characterization of MAPK regulated histone Kla 36
2.3 Discussion 40
2.4 Methods 41
CHAPTER 3: LACTATE IS A KEY SOURCE FOR ONCOGENIC HISTONE LYSINE LACTYLATION 44
3.1 Introduction 44
3.2 Results............45
3.2.1 Oncogenic signaling induces the Warburg effect and generates lactate............45
3.2.2 Histone lysine lactylation is coupled to glycolysis............51
3.2.3 Lactate is sufficent for oncogenic histone Kla 56
3.3 Discussion 60
3.4 Methods 60
CHAPTER 4: MOF IS A NOVEL LACTYLTRANSFERASE 63
4.1 Introduction 63
4.2 Results............65
4.2.1 MOF is a lactyltransferase in vitro and in vivo 65
4.2.2 Comprehensive characterization of MOF regulated histone Kla 68

4.2.3 Biochemical modeling of MOF acyl-CoA interaction 70

4.3 Discussion 77

4.4 Methods 78

CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS 82

REFERENCES: 85

APPENDIX: 111

LIST OF FIGURES

Figure 1. 1 Structures of acyllysines discovered Zhao and colleagues. 7

Figure 2. 1 Histone lysine lactylation is regulated by growth factor signaling. 30
Figure 2. 2 The MAPK pathway and oncogenic signaling regulate histone lysine lactylation.....33
Figure 2. 3 MEK is a universal regulator of histone lysine lactylation in cancer cells................35
Figure 2. 4 Proteomic characterization MAPK-regulated histone lysine lactylation. 38
Figure 2. 5 Heatmap comparison of the differential contributions observed in MAPK-regulated histone lysine lactylation. 39

Figure 3. 1 Warburg effect regulatory proteins are modulated by BRAF...................................49
Figure 3. 2 The level of intracellular lactate is controlled by the MAPK pathway. 50
Figure 3. 3 Upstream glycolysis and glycolytic substrates are required for oncogenic histone lysine lactylation. 54
Figure 3. 4 Downstream glycolysis is required for oncogenic histone lysine lactylation............54
Figure 3. 5 Lactate-generating machinery is required for oncogenic histone lysine lactylation...55
Figure 3. 6 Lactate drives histone lysine lactylation independently of active MAPK signaling. .58
Figure 3. 7 Lactate drives histone lysine lactylation independently of active glycolysis. 59

Figure 4. 1 MOF can enzymatically lactylate histone proteins in vitro. 72
Figure 4. 2 MOF regulates histone lysine lactylation levels in cells. 73
Figure 4. 3 MOF Proteomic characterization of MOF-regulated histone lysine lactylation.........74
Figure 4. 4 Molecular modeling of MOF in complex with acetyl-CoA and lactyl-CoA. 75

LIST OF TABLES

Table 1. 1 MYST family KATs, their roles, and the lysine acylation's they catalyze…................14

ACKNOWLEDGEMENTS

I would like to acknowledge first my cat Sylvia, who sits beside me at current, purring gleefully. I would like to thank those who have contributed to the knowledge in which provides the foundation of the work presented herein. I would like to thank my advisor, Dr. Yingming Zhao, who has been a continual source of inspiration to me. My understanding of success is defined by your immense contribution to our understanding of protein post translational modifications and the roles they may play in biology. As your student, I have had the honor of being exposed to tremendous resources and knowledge. I am grateful for your careful and professional philosophy toward science, which has imbued in me an deep respect for science, biology, and the significance that our work can have on ending human diseases Thank you for everything you have done to support me both professionally, and personally.

I would also like to thank my committee members: Dr. Lev Becker, Dr. Peter Savage, and Dr. Xin He. Dr. Lev Becker has been helpful to me in defining key experimental strategies, and in maturing my conceptual perspective. I will forever appreciate and look for 'lighthouses' in my logic because of you. I would like to thank Dr. Peter Savage, who has supported my scientific ambitions since our introduction. Dr. Savage has continually emphasized the importance of hypothesis driven research and the rigor that is required to support a claim. I want to thank Dr. Xin He for your availability and for the critical feedback you have provided me with during our committee meetings.

I want to thank Dr. Nancy Schwartz for guiding me through the NIH Initiative for Maximizing Student Development (IMSD) program. I would like to thank you further, for inspiring my desire for change, and for supporting my initiatives. Dr. Schwartz has been an avid supporter of my social views. Thank you for always shielding me from the dangers inherent in

demanding change. Thank you, Dr. Laurie Risner, for your help in building programs and planning conferences.

Thank you Dr. Kay Mcleod for giving me a chance, and for being my ally when I needed you. I want to thank Dr. Alex Muir for the incredible scientific conversations that we had over lunch, and the fact that you made the time for me. Although you are a new faculty member here, you have already demonstrated your immense value to our academic community.

I would like to thank the Chair of the Cancer Biology program, Dr. Barbara Kee, for the support you provided me toward the end of my graduate education. I want to thank Lisa Abston-Leftridge for guiding me the cancer biology program over the years. Likewise, I want to thank Laura Negrete for guiding me through the thesis defense and dissertation process.

The data collected and presented in this original research work would not have been possible without many people. I want to thank Wenchao Liu and Dr. Jinjun Gao for providing technical assistance with LC-MS/MS experiments. I want to thank Dr. Ruilong Liu and Dr. He Huang for performing the initial screen that identified that MOF could possess lactyltransferase activity. I want to thank Victor Han for performing a number of western blots in the lab under my mentorship.

I want to thank all the members of my lab for teaching me the technical elements of performing experiments and for being role models on how to be a successful and effective research scientist. Thank you, Dr. Di Zhang, Dr. He Haung, Dr. Sunjoo Kim, Dr. Jinjun Gao, Dr. Delaney, Dr. Honggang Ye, and Dr. Jianfeng Du.

I want to thank my lab mate, Dr. Kyle Delaney. Kyle was one year above me in the CCB program. Kyle has been a role model and big brother figure to me during my time as a graduate

student in Dr. Zhao's lab. It was through watching Kyle conquer his own challenges that attributes like steady, meticulous, and collected, can be fierce.

I want to thank my other lab mate, Wenchao Liu. Wenchao is a year below me in the CCB program. The conversations that Wenchao and I have had together have directly made me a much smarter person and scientist. I will forever cherish the moments where I felt that you were the only person in the world that could understand the weird, niche, and hyper specific science that we sometimes engage in. I still remember the moment that made me realize that you're stronger than me.

I want to thank a particular friend that I made in graduate school. Thank you, Cody Hernandez. Cody has been a significant source of inspiration and advice for me during my tenure as a graduate student. Together, Cody and I reformed the culture of the Biological Sciences Division (BSD) to become a more inclusive space for individuals from marginalized backgrounds. We revitalized the University of Chicago SACNAS chapter, and we co-found the Graduate Recruitment Initiative Team (GRIT). We expanded GRIT nationally by helping GRIT chapters develop at other institutions like The University of Virginia (UVA) and the University of North Carolina Chapel Hill (UNC). Together, we acquired the support and signatures for the formulation of a letter that we drafted that demanded the removal of the GRE as an admissions requirement – upon which its release resulted in the removal of the GRE as a required component for admissions in the BSD graduate programs. The change we made will remain stenciled on the mental sheets of unfolding generations, and I am honored to share this legacy with you.

Finally, I would like to thank my family for their continued support of me. I want to thank my brother, Felipe Perez-Neut, for his unyielding love and support. You have always

championed my ambition, even in the absence of evidence that could demonstrate my capabilities. I try and model my inner strength to be akin to yours. I am so grateful and glad you are here. Let it be known that the planet will break before Felipe does. I would like to thank my mother, Nelly Neut, for her unconditional support and love. Thank you for driving me to night school when I was a child. Thank you for teaching me that loving someone is good enough to be fulfilled and to be happy. I know that regardless of this accomplishment, or the next one, you will love me all the same. That is because it is not what I do that makes you proud. It is who I am that makes you proud. I am everything you dreamed for me because I am your son.

ABBREVIATIONS

RNA	ribonucleic acid
PTM	post translational modification
DNA	deoxyribonucleic acid
Kac	acetyllysine
HAT	histone acetyltransferase
KAT	lysine acetyltransferase
HDAC	histone deacetylase
KDAC	lysine deacetylase
Kac	acetyllysine
Kbhb	β-hydroxybutyryllysine
Kbu	butyryllysine
Kbz	benzoyllysine
Kcr	crotonyllysine
Kglu	glutaryllysine
Khib	2-hyrdroxyisobutyryllysine
Khmg	3-hydroxy-3-methylglutaryllysine
Kla	lactyllysine
Kibu	isobutyryllysine
Kmal	malonyllysine
Kpr	propionyllysine
Ksu	succinyllysine
HPLC	high performance liquid chromatography
CoA	coenzyme A
ING	inhibitor of growth
SDH	succinate Dehydrogenase
AML	acute myeloid leukemia
ORC	origin of replication complex
TIP60	tat interaction protein 60
MOF	male absent on first
ATP	adenosine triphosphate
AMP	adenosine monophosphate
MAPK	mitogen activated protein kinase
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
GTP	guanosine triphosphate
GDP	guanosine diphosphate
TCA	tricarboxylic acid cycle

CHAPTER 1

INTRODUCTION

1.1 Protein posttranslational modifications

1.1.1 Properties and functions of protein posttranslational modifications

The cell is composed of four major macromolecules: nucleic acid, lipid, metabolite, and protein[1]. Of these four components, proteins are the most complex biomolecule in cells – an emergent end product resulting from the evolution of nucleic acid. The regulatory activity of proteins can be influenced both prior to and after their complete synthesis in cells through various mechanisms, such as RNA splicing, missense mutations, and protein post-translational modifications. Any unique protein molecules that can be made from permutations of these processes are referred to as proteoforms[2]. While there are approximately 19,000 protein coding genes in the human genome, the number of theoretical proteoforms that can arise from the histone H4 gene is >10^10, with 98,304 H4 proteoforms known to be reported[3,4]. Thus, the number of proteoforms that can be generated from a single histone H4 gene product is several magnitudes greater than the total number of protein coding genes within the human genome (>520K times more). Proteoforms can significantly increase the regulatory versatility of a protein molecule generated from a single protein coding gene[5].

The majority of regulation that controls protein function in cells occurs after their synthesis via a highly conserved and broadly defined biochemical process known as protein post-translational modification (PTM). PTMs can be broadly defined as modifications to the protein structure that occur after protein synthesis, which includes processes like protein folding[6]. Protein folding is largely considered a stochastic process that is governed by thermodynamic principles that drive the assembly of protein molecules into their native secondary and

sometimes tertiary structures[7]. However, the activity of many proteins can be dynamically controlled by another form of PTM that occurs after protein folding. This form of PTM is described by covalent chemical modification of select amino acid residues[8,9]. The chemical modification of select amino acids on proteins, herein referred to as PTMs, defines the major source of known and potential proteoforms.

PTMs can possess diverse biochemical and biological attributes which include: the PTM chemical structure, the co-factor derivative, the mechanism of chemical reaction, the proteins and position on proteins that may be modified, and most importantly, the impact that a PTM can have on cellular processes. PTMs are essential for life processes, and their essentiality is exemplified in disease driven phenotypes that are associated with observed aberrations that occur in PTM regulatory elements[10,11].

One of the most essential cellular processes that defines eukaryotes is the dynamic regulation of chromatin. Central to the dynamic regulation of chromatin are the posttranslational modification of histone proteins, which are critically important for diverse DNA based regulatory processes including epigenetics, genome replication, and DNA repair[12].

1.1.2 Histone proteins and histone marks

Histone proteins are a highly conserved family of proteins found in the nucleus of eukaryotic cells – and have been proposed by select scholars as one of the critical milestones in the evolution of complex multicellular organisms[13]. The core histone protein family is comprised of four key members: histone H3, H2A, H2B, and H4. Histone H4 is the only histone protein that does not have genetic isoforms[14].

Two copies of each core histone protein are assembled into an octameric protein complex that can then be wrapped by approximately 146 base pairs of DNA, or 1.7 turns, to generate a

DNA-protein complex known as the nucleosome. The nucleosome is the principal component to the biomolecular composition of the eukaryotic chromatin.

Nucleosomes are considered to have enabled the evolution of complex multicellular life[15]. This theory posits that nucleosomes can compact significantly larger amounts of genetic material. The compaction of genetic material by histone proteins enables two features that were required for the evolution of eukaryotes: the compartmentalization of genetic material and subsequent emergence of nucleus (2) increase genome size and thereby the capacity to store information[15–17].

However, histone proteins were later discovered to serve many essential roles within chromatin that were independent of chromosome compaction. In the last 35 years, histone proteins have been discovered to be dynamic molecules capable of regulating countless chromatin-based processes, such as DNA replication, recombination, and DNA damage repair[18–20]. Central to histone protein's ability to dynamically regulate chromatin are the posttranslational modifications (PTM) that are known to occur on them[18].

Histone PTMs regulate various chromatin-based processes, including transcription[21]. Histone PTMs are often referred to as histone 'marks.' While histone marks play an essential role in the activation or repression of gene transcription, they are well established in governing several other chromatin-based processes as well. Unique arrangements of histone marks in nucleosomes have been shown to modulate the activity of specified events. Allis and colleagues were the first to propose that these unique arranges of histone marks may constitute a type of code, which they refer to as the 'histone code.'[22]

1.1.3 Discovery of a novel chemical class of histone marks

One of the first discovered and most intensely studied types of histone marks is lysine acetylation (Kac)[23]. Lysine acetylation is the product of the addition of an acetyl moiety to the side chain amine of lysine[24]. While lysine acetylation can technically be classified as an acylation due to the carbonyl that is present in the acetyl moiety, acetylation was known to exist 70 years before the discovery of most other types of lysine acylations[24]. Therefore, it is unsurprising that much what is known about epigenetics stems directly from our understanding of histone Kac. Despite the critical importance of acetylation in epigenetics, recent advances in mass spectrometry-based proteomics, and advances in immunoaffinity enrichment strategies, have led to the discovery dozens of chemically unique lysine acylation's that can occur on histone proteins (**Figure 1.1**)[25–32].

The initially discovery of these novel lysine acylations was made on histone proteins[24]. Histone proteins serve as a valuable system for the discovery of new types of lysine acylation's: histone proteins are abundant in lysine residues, they contain unstructured tails that can readily be mimicked in vitro, and they are highly abundant in cells[33]. Lastly, histone proteins can be extracted from cells by various techniques, including acid extraction and high salt dialysis[34]. These strategies provide alternative approaches to purify histones that harbor PTMs that can be less stable[35].

With histone proteins as a suitable biochemical platform for the discovery of novel types of lysine PTMs, Zhao and colleagues leveraged two essential technologies to make their discoveries: antibody-based immunoprecipitation and mass spectrometric proteomics technologies. These two technologies offered 1) the ability to enrich low abundant PTM-bearing peptides, and 2) the ability to analyze and subsequently identify novel mass shifts that occur as a result of a new chemical modification.

Interestingly, Zhao and colleagues discovered their initial lysine acylation's, propionyllysine (Kpu) and butyryllsyine (Kbu), by using an antibody specific toward Kac. The Kac antibody that was used to enrich Kac bearing peptides demonstrated cross reactivity toward Kpu and Kbu bearing peptides[24]. The cross reactivity is likely enabled by the shared structural similarities between the three modifications. Indeed, this same strategy was later employed by Zhao and colleagues to discovery malonyllysine (Kmal) and glutaryllysine (Kglu), given their similarity to succinyllysine (Ksu)[27,36–38].

With this feature of cross reactivity available, Zhao and colleagues had purified sufficient sample for mass spectrometric detection of novel mass shifts on proteins. The spectra captured from Kac immunopreciptates were subjected to an unrestricted sequence alignment search that resulted in the detection of mass shifts between -100 and +200, with 1 Da increments. This software was called PTMap[39,40]. Using anti-Kac antibody enrichment and PTMap, the initial discovery of Kpu and Kbu were made. In the decade that followed this initial discovery, several more novel lysine acylation's were discovered using this strategy, including the recently identified *iso*-butyryllysine (Kibu)[41]. Zheng and colleagues could isolate and purify *iso*-butyryllysine bearing peptides using a Kbu specific peptide. However, since Kbu and Kibu are isomers, they could only be differentiated via differences found in their retention times when they were subjected to high-performance liquid chromatography (HPLC)[41]. In parallel to the discovery of novel lysine acylation's, significant research efforts dedicated to determining the biochemical regulators and biological significance of these new histone marks emerged[42]. These collective research efforts have allowed us to establish critical similarities and differences that exist between Kac and other histone acylation's, which will be discussed next.

Before we move on, let us revisit proteoform diversity. Protein PTMs significantly increase the diversity of unique proteoforms that can exist. To put this into perspective, earlier we stated that there are 98,304 theoretical H4 proteoforms[4]. These 98,304 theoretical H4 proteoforms do not include any of the novel acylation's that have been discovered. Considering just one of the novel acylation's results in 6,193,152 new theoretical H4 proteoforms for a total of 6,292,566 – approximately 63 times more proteoforms than initially determined.

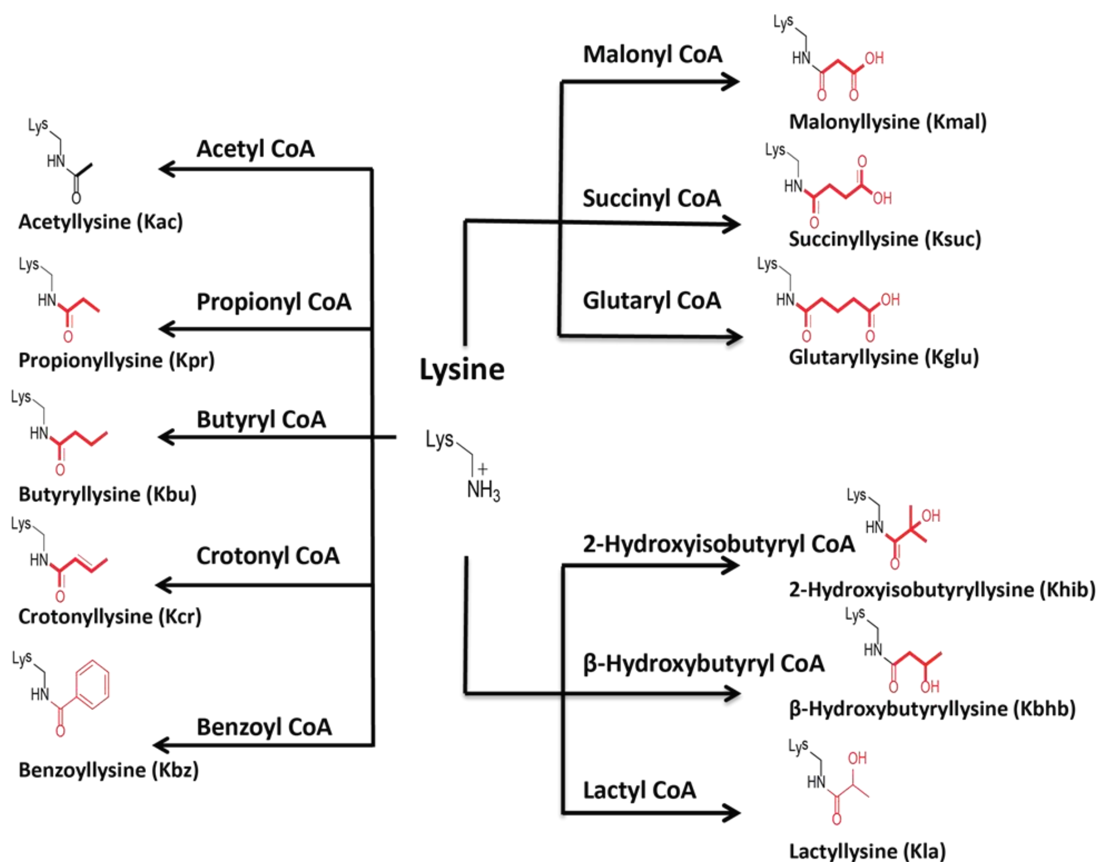


Figure 1.1 Structures of acyllysines discovered Zhao and colleagues.
Chemical structures of the lysine modifications discovered by Zhao and colleagues.
The 10 published lysine acylations discovered by the laboratory of Dr. Yingming Zhao highlighted in red. The structure of acetyllysine is included for reference in black. All relevant acyl-CoA species that give rise to the corresponding lysine acylation are listed beside the structures above. Figure adapted from seminar illustrations. The abbreviations for each acyllysine name are included below each structure.

1.2 Biological regulation of histone marks

1.2.1 Metabolic regulation of histone marks

An intriguing property that is shared between Kac and the other novel lysine acylation's is their proximity to cellular metabolism. More specifically, lysine acetylation and lysine acylation's are primarily derived via a high energy co-factor[43]. Prior to the discovery of lysine acetylation, the co-factor that is required for its generation was determined to result from the ligation of acetate and coenzyme A (CoA), which results in the genesis of acetyl-CoA[44]. The discovery of acetyl-CoA and of protein acetylation were award the Nobel prize[45]. While acetyl-CoA can be derived from the direct ligation of acetate and CoA, several other biosynthetic routes for its generation have since been discovered[46]. Nevertheless, the relationship between the levels of acetyl-CoA and lysine acetylation have been well documented[47]. Changes in acetyl-CoA can be caused by changes in its precursor metabolites, which ultimately impact the levels of Kac in cells[48–50]. The precise mechanisms that govern which Kac sites are preserve during times of limited resource availability are not known.

Appreciating this relationship, Zhao and colleagues demonstrated that this phenomenon is a conserved property of several non-acetyl acylation's that they discovered[42]. Similar to Kac, these novel lysine acylation's were shown to originate from the ligation of a precursor metabolite to CoA, thereby generating acyl-CoA species capable of forming a lysine acylation. One of the best in cell examples was shown by Zhao and colleagues in their report that described the discovery benzoyllysine (Kbz)[25]. In this report, human liver cells that were exposed to increasing doses of deuterated sodium benzoate had observably higher levels of deuterated benzoyl-CoA. The increased levels of intracellular benzoyl-CoA were associated with in increased levels of Kbz[25]. While significant work is required to determine the enzymes responsible for the

generation of these acyl-CoA species, it seems that the paradigms governing lysine acetylation are conserved with respect to the novel acylation's recently discovered.

Many of the novel histone acylation's discovered by Zhao and colleagues have been associated with active transcription[30,31,42]. At first glance, the function of novel histone acylation's may seem redundant with respect to functions that histone acetylation serves. However, mounting evidence suggests that novel acylation's may function to sense the energy status within a cell by altering protein function[50,51]. Evidence of this paradigm was demonstrated in the initial report that was made by Zhao and colleagues of a novel lysine acylation called lysine lactylation (Kla)[30]. Using macrophages as a model, Zhao and colleagues demonstrate that Kla is induced on histone proteins during pro-inflammatory activation. The Kla induced on macrophage histones was shown to be intimately linked to elevated lactate levels. During pro-inflammatory activation, macrophages undergo metabolic reprogramming that includes increased glycolytic flux and subsequently increased lactate production. Using CRISPR to knock out lactate dehydrogenase, Zhao and colleagues determine that lactate generation is necessary to achieve Kla in macrophages. Fascinatingly, the histone Kla levels that were found to occur in macrophage chromatin was shown to occur on genes that are involved in the resolution, or dampening, of inflammation. Dr. Becker posits that the reason for this is that lactate accumulation via glycolysis serves as a 'metabolic clock', that upon reaching a certain level, lactate via Kla can polarize macrophages to terminate pro-inflammatory processes by activating processes involved in resolution and wound repair[52]. While the relationship between lactate and Kla during pro-inflammatory macrophage polaorization is clear, less is known about histone Kla dynamics during processes, including in diseases like cancer. Furthermore, lactyl-CoA was recently detected in cells, albeit before the discovery of Kla[53].

While these lysine acylation's can overlap considerably in their regulatory functions, the metabolic pathways from which they originate are distinct. For example, modulation of intracellular lactate via changes in glycolysis has a direct impact on the abundance of Kla[30,54]. Additionally, dysfunctions found in certain metabolic proteins have been found to result in the generation of excess precursor metabolites that subsequently result in lysine acylation's[55]. Further evidence for this phenomena has been observed in cells with dysfunctions in the tricarboxylic acid cycle (TCA) that cause an accumulation in succinyl-CoA, such as SUCLA2 loss of functions mutations[56,57].

The metabolic similarities and difference that exist between Kac and amongst the newly discovered lysine acylation's represents only a mere fraction of the comparisons that are available to make. Indeed, several comparisons in how these novel acylation's are biochemically governed can already be made, with many more likely to emerge as this research area continues to mature. I will next focus on the biochemical regulatory elements that govern these novel acylation's.

However, it is critical to note that under certain conditions, acetyl-CoA and other types of acyl-CoA species can spontaneously react with proteins to form a variety of different products, including the lysine acylation's discussed herein. In the interest of brevity, I will focus on the regulatory components of lysine acylation's and refer to the reader to the following reviews for more information on non-enzymatic protein modification[58–62].

1.2.2 Regulators of novel histone marks: writers, readers, and erasers

Histone lysine acetylation is thought to be principally governed by the action of three classes of regulatory proteins that are often referred to as writers, readers, and erasers. Writers of histone Kac are technically termed acetyltransferases and are often interchangeably referred to as

(**K**)ATs (**lysine** acetyltransferase) or (**H**)ATs (**Histone** acetyltransferase)[63]. These enzymes catalyze a chemical reaction between acetyl-CoA and lysine to generate Kac and SH-CoA. Reader proteins are described as proteins that contain specialized domains capable of specifically recognizing unique types of histone marks[64,65]. Reader domains can be found in diverse proteins throughout the proteome, including several writers and erasers[63,66]. For example, the acetyltransferase p300 contains a bromodomain, which is a type of reader domain known to bind to acetylated lysine[67,68]. Erasers are enzymes that catalyze the removal of histone marks, returning the modified substrate to native unmodified state. Erasers of histone Kac are technically termed histone deacetylases and are interchangeably referred to as (**K**)DACs (**lysine** de-acetylase) or (**H**)DACs (**Histone** de-acetylase)[69]. The collective action of writers, readers, and erasers are central to the regulation of histone marks. This process of writing, reading, and erasing, can form a dynamically cyclical and thereby sustainable form of chromatin regulation (**Figure 1.4**).

1.2.3 Acetyltransferases

To date, there have been more than a dozen KATs identified in human cells. These specific KATs can classified into 4 distinct families: HAT1, GCN5/PCAF, P300/CBP, and the MYST family[63]. Rigorous research has provided essential knowledge regarding the biochemical and the regulatory characteristics of KATs. Central to their function, the majority of these KATs have been shown to activate transcription from chromatin due to their ability to acetylate chromatin[70]. The major research focuses surrounding KATs have provided a foundational understand of their biochemistry and roles in biology. This research has focused on: the protein complexes these enzymes can exist in, their protein substrates, the mechanisms governing substrate selection, and their biological functions. However, in the last decade, there has been an

increased interest in determining the types of acyltransferase activity that previously annotated KATs may possess[42]. Of particular interest to his work, we will continue by focusing on the MYST family.

1.2.4 MYST family of KATs

The MYST family is comprised of 5 distinct KATs: TIP60, MOZ, MORF, HBO1, and MOF[71]. The MYST family of KATs share a MYST domain that is composed of an acetyl-CoA binding motif and a zinc finger[72]. While the majority of MYST substrates reside on histone proteins, accumulating evidence suggests that this family of KATs can acetylate a variety of non-histone substrates as well[73]. MYST KATs are thought to be exclusively active only when in multiprotein complexes. The majority of the MYST KATs share protein complex subunits from the inhibitor of growth (ING) family of tumor suppressors, with the single exception being MOF[74].

TIP60 was first identified in 1996 by G. Chinnadurai and colleagues through a yeast two-hybrid screen to identify interacting modules of HIV-1 Tat protein, and therefore named Tat interactive protein, 60kDa (TIP60)[75]. In this initial report, it was postulated that TIP60 was a co-factor that interacts with Tat to activate HIV gene expression[75]. It was in 1999 that Robson and colleagues demonstrated that TIP60 is a nuclear hormone receptor coactivator. They determined that the coactivator function of TIP60 is the result of its acetyltransferase activity[76]. TIP60 was later discovered to be critically involved in the acetylation and activation of tumor suppressor gene, p53[77–79]. Beyond acetylation, several reports have demonstrated that TIP60 possesses acyltransferase activity. TIP60 was shown to propionylate and 2-hydroxyisobutryate lysine residues[24,80].

MOZ was initially discovered by Housman and colleagues in 1996 as a resultant fusion partner of CREB-binding protein via a common chromosomal translocation that occurs in acute myeloid leukaemia (AML)[81]. Three years later, MORF was discovered based on its shared structural and functional homology to MOZ[82–84]. MOZ and MORF share a variety of biochemical and biological features, including the subunits that exist within their protein complexes[71]. Both KATs form a complex that contains ING5 and bromodomain and PHD finger containing (BRPF) proteins. MOZ and MORF are largely known for their ability to acetylate histone H3, and are involved in a number of processes, including DNA replication[73]. Both MOZ and MORF have been reported to possess acyltransferase activity[85,86]. Curiously, the double PHD finger (DPF) domain found in MOZ is a reader of lysine crotonylation (Kcr)[87]. Similarly, MORF contains a DPF domain that was shown to bind to several of different types of chemically distinct histone acylation's[85].

HBO1 was first identified by Iizuka and colleagues in 1999 when they identified a protein bound to the origin of replication complex (ORC) 1 protein[88]. Consistent with its initial discovery in the ORC complex, it was later determined that HBO1 plays a critical role DNA replication[89,90]. Much remains to be discovered regarding the role of HBO1 in DNA replication – however the prevailing theory posits that HBO1 regulates DNA replication by acetylating the necessary components of the pre-replication machinery, as well as acetylating histones to facilitate more accessible chromatin[73]. A recent report suggests that HBO1 possess significant propionylase, butyrylase, and crotonylase activity, with a propensity for acylation at on lysine 14 of histone H3 (H3K14)[91]. Another recent report from a different group found that HBO1 possess some *iso*-butyryllysine (Kibu) activity, albeit much weaker than HAT1. HBO1 was the only MYST family KAT that was observed able to generate Kibu marks[41].

Alas, Male Absent on First (MOF), identified in 1997 by Lucchesi and colleagues, was named as such for its identification in *Drosophila* as a putative acetyltransferase involved in dosage compensation[92]. Dosage compensation is the regulatory process that ensures that male and female organisms can control the amount of X-chromosome gene products that are expressed[93]. MOF is a member of the dosage compensation complex and is known for its ability to acetylate histone H4 on position K14 (H4K16ac)[94]. H4K16ac alone has been shown to directly inhibit the formation of condensed chromatin, which likely underscores this histone marks prevalence in dosage compensated X-chromosomes[95]. MOF has been shown to possess crotonyl- and propionyl-transferase activity[96,97]. MOF was shown to propionylate several lysine residues on histone and non-histone proteins, expanding the potential enzymatic impact of MOF far beyond its canonical H4K16ac function[97]. Whether other types of acylation's can be driven by MOF remains unknown. To our knowledge, this will be the first report that demonstrates that MOF can possess lactyltransferase activity.

In the following text, we will learn about novel functions that MOF acyltransferase possess. Indeed, the MYST family of KATs all possess acyltransferase activity. However, as we shall see later, their acyltransferase activity is not precisely conserved to a specific acylation lysine modification. The following table summarizes the transferase activity that the MYST family of KATs possess **(Table 1.1)**.

MYST KAT	Function	Acylations
TIP60	HIV-1 gene rexpression, hormone receptor co-activator	Kac, Kpr, Kibu, Kbhb, Khib
MOZ/MORF	DNA replication	Kac, Kpr, Kibu, Khib
HBO1	Pre-replication liscensing, DNA replication	Kac, Kpr, Kbu, Kcr
MOF	Dosage compensation, gene transcription	Kac, Kpr, Kcr, K(la)

Table 1.1 MYST family KATs, their roles, and the lysine acylation's they catalyze.

1.3 The MAPK signaling pathway

1.3.1 Protein phosphorylation

One of the most studied types of protein posttranslational modifications (PTM) is protein phosphorylation[98]. The discovery of protein phosphorylation was inspired by the 1947 Nobel prize winning work of Carl and Gerti Cori, who discovered an enzyme in muscle that could convert glycogen deposits into readily available glucose via glycogen phosphorylation[99,100]. Throughout the 1950s, Edmond Fischer and Edwin Krebs found that the activity of the enzyme responsible for converting glycogen to glucose could be toggled between an active/inactive (on/off) state[101,102] Fischer and Krebs ultimately revealed that, similar to glycogen phosphorylation, the on/off 'switch' of this given enzyme was mediated by its phosphorylation.

Their discovery of protein phosphorylation awarded them the 1992 Nobel prize in Physiology or Medicine[103].

Protein phosphorylation is a property of all life on earth. In humans, protein phosphorylation can be observed to play a vital role in diverse physiological and homeostatic processes[104,105]. Indeed, these processes include but are not limited to: cellular metabolism, hormone release, muscle contraction, development, and of particular interest to this text, the regulation of cellular signaling through the RAS-RAF-MAPK pathway[106].

Phosphorylation is a reversible protein modification that occur via the enzymatic transfer of the γ-phosphoryl group of adenosine triphosphate (ATP) onto a protein residue[107]. This reaction consumes ATP to generate a phosphorylated protein and adenosine diphosphate (AMP)[105]. While many residues on protein can be phosphorylated, the canonical residues that bear this modification are serine, threonine, and tyrosine[108]. The enzymes responsible for protein phosphorylation are called kinases, while the enzymes responsible for removing the phosphoryl group are called phosphatases[109].

Protein phosphorylation has a central role in the regulation of diverse cell signaling pathways. Briefly, cell signaling can be described as the multilateral flow of information within a living system, which includes the ability to sense, integrate, and response to 'cues'[110]. In this context, cues can come in variety of different forms, including chemical cues such as growth factors, or insulin, which bind to cell surface receptors and mediate a downstream signaling cascade[111]. Mechanical cues exist as well, well exemplified by the hippo signaling pathway and the beta-catenin signaling pathway[112–114]. As briefly mentioned earlier in this section, protein phosphorylation plays a central role in regulating RAS-RAF-MAPK signaling cascade.

1.3.2 The RAS-RAF-MEK-ERK/MAPK pathway

The RAS-RAF-MEK-ERK/MAPK signaling pathway is one of the most well studied signal transduction pathways, which is ironic given the pathways immense complexity[115]. The complexity of this pathway stems largely from three key features. First, there are a number of cues that can activate the RAS-RAF-MEK-ERK/MAPK pathway[116]. Second, the activation of this pathway can be toggled via a poorly understood mechanism, which has been described as the 'amplitude' of response[117]. Third, the RAS-RAF-MEK-ERK/MAPK is a multistep signaling pathway where each step of the pathway can produce signals that emanate lateral to the central MAPK pathway – thus RAF can induce a phenotype independent of MEK activation.[118] Beyond these core features that describe the immense complexity of the RAS-RAF-MEK-ERK/MAPK, the core proteins within the pathway have a number of homologues[119]. Therefore, for the sake of brevity, I will be referring to the RAS-RAF-MEK-ERK/MAPK pathway simply as the MAPK pathway, and I will focus on the broader principals of the MAPK pathway in biology.

The RAS gene was initially discovered in the mid-1960s through research that was being performed on transforming retrovirus by Dr. Jennifer Harvey[120]. Dr. Jennifer Harvey, the sole author on a 1964 article in Nature, reported that an unidentified virus isolated from leukaemic rats could induce rapidly forming tumors in newborn rodents[121]. When the sequences for the tumor promoting properties were found, it was discovered that these viral sequences were in fact derived from the host rat genome[122]. Several decades of research came to pass and the molecular and biochemical regulatory mechanisms that define RAS became clearer. RAS proteins are crucial initiators of growth signal transduction in cells and are often activated down stream of mitogen induced stimulus[123]. Mitogens are broadly defined as extracellular signals that can bind to growth factor receptors. An example of a mitogen and its associated receptor would be epidermal growth factor (EGF) and EGF receptor (EGFR)[124,125]. RAS is activated by its binding

to guanosine triphosphate (GTP), which can be induced via the action of activated RAS-GEF protein modules that release guanosine diphosphate (GDP) in order to form GTP. RAS bound to GTP initiates RAS binding to RAF, the next downstream member of the MAPK pathway[126]. Upon RAS-RAF binding, RAS can phosphorylate the RAF kinase domain on the activating site, which induces the activation of RAF[127]. RAS activation results in a lateral phosphorylation that initiates the activation of a number of other types of signal transduction programs[116,126].

In 1983, Stephenson and colleagues cloned the first of three isoforms that compose the RAF family, which we now refer to as C-RAF[128]. Not long thereafter, A-RAF and B-RAF would be discovered[129,130]. The RAF family are protein kinases that have an important role in the regulation of cell growth, differentiation, and survival[131]. The activity of RAF kinases are modulated by the binding of small G proteins of the RAS family to the N-terminal region of RAF proteins[132,133]. Upon activation of RAS by GTP binding, RAS will bind RAF to form a scaffolding complex[134,135,135]. Within this complex, RAS will phosphorylate the RAF kinase domain on the activating site, which will result in the activation of RAF kinase activity[136]. A-RAF, C-RAF, and B-RAF have been shown to differ slightly in their activation requirements by RAS. However, this slight difference has a profound impact on their proto-oncogenic potential. C-RAF and A-RAF activation is dependent on the phosphorylation of 4 keys residues, where three of these sites are the targets of distinct kinases[137]. Conversely, B-RAF only requires the phosphorylation of 2 sites, thus requiring less regulatory steps to become activated[138,139].

Similar to RAS activation, the activation of RAF results in the downstream phosphorylation of a number of substrates[140–144]. One key substrate that is phosphorylated by RAF are the MEK protein kinases. The MEK protein kinases are activated by phosphorylation on two serine residues: S217 and S221[139,145–147]. Upon activation via phosphorylation, the MEK

protein kinases, similar to their upstream counterparts, can phosphorylate a number of substrates. However, what makes the MEK protein kinases unique, specifically MEK1/2 in this case, is that MEK1/2 protein kinases are the sole activators of ERK1/2[148]. This is why MEK1/2 has been referred to by some scientists as ‘gatekeepers’ for the activation of ERK1/2[148]. Therefore, signaling that emanates from the MAPK that does not result in MEK1/2 activation will therefore also result in inactivated ERK1/2.

ERK1/2 is activated by MEK1/2 via phosphorylation. Upon activation, ERK1/2 will translocate into the nucleus where it can form a variety of different complexes that are involved in the repression or activation of gene transcription[149]. ERK1/2 is a kinase and has been shown to phosphorylate and subsequently activate a number of nuclear coactivators and transcription factors[119]. ERK1/2 has also been shown to activate genes involved in cell growth, including the associated metabolic and cell division genes that are required for cell growth[150].

1.3.3 MAPK and cancer

The role of MAPKs in initiating cancer has been well established[150–152]. Indeed, several of the MAPK proteins that we have discussed have been known to acquire oncogenic mutations that result in their constitutive activation[153]. The constitutive activation of a MAPK protein bypasses the need for mitogen induced activation, therefore locking the cell into a perpetual state of chronic and dysregulated growth[151]. Through decades of scientific research in the field of MAPK signaling, several small molecule inhibitors that target these oncogenic MAPK proteins have been developed and deployed clinically.

Growth factor receptors, which sit directly upstream of RAS activation, are frequently mutated in cancer. Epidermal growth factor receptor (EGFR) and HER2 mutations have been observed to occur predominately in lung and breast cancer, respectively. However, EGFR

mutations and amplifications have been seen in a variety of other cancers. Remarkably, EGFR mutation is observed in 31.6% of all non-small cell lung cancer patients, and 36.5% in adenocarcinoma[154]. EGFR mutations can occur on a number of positions within the protein, via a number of different mutational classes. These mutations include amplifications, point mutations, and deletions[155,156]. HER2 amplification is observed in about 20% of breast cancers and is associated with aggressive disease[154]. Several small molecule inhibitors for EGFR have been developed, such as clinically approved agents, gefitinib and lapatinib (which will be used later in this study)[157,158]. Beyond small molecule inhibitors, biologic inhibitors have been FDA approved that target amplified HER2. This includes the monoclonal antibody, trastuzumab[159].

The discovery of RAS was made by its ability to transform cells[121]. Therefore, it is unsurprising that RAS is mutationally activated in 30% of all known cancers[160]. Despite its ubiquitous role in diverse cancer types, RAS mutations are most prevalent in pancreas (90%), and colon (50%) cancer[161]. RAS has a notorious reputation for being 'undruggable'[162]. However, in the last 5 years, considerable progress has been made toward developing inhibitors that target mutated KRAS[163,164]. These novel small molecule inhibitors leverage the G12C mutation that can occur in KRAS. While the G12C mutation is known to lead to the constitutive activation of KRAS, the mutationally incorporated cysteine residue can be targeted by electrophilic compounds to form a covalent bond via a chemical reaction known as Michael's addition[165–167]. This is precisely what lead to the development of sotorasib, a small molecule covalent inhibitor of KRAS G12C. Sotorasib was shown to be remarkably specific toward KRAS G12C[168]. It was also observed to be a potent inhibitor of this oncogenic form of KRAS. Sotorasib was recently approved by the FDA for the treatment of KRAS G12C harboring NSCLC patients[169].

RAF mutations are seen in approximately 7% of all human cancers[153]. As briefly mentioned earlier, the activation requirements for B-RAF are simpler than A-RAF or C-RAF, which has been suggested as a reason why B-RAF mutations are observed more frequently in cancer[139]. B-RAF mutations are frequently observed in melanoma (60%) and thyroid (50%) cancer[170]. The most prevalent mutation that occurs in B-RAF is the V600E mutation. This mutation occurs in the kinase activating region of B-RAF, which renders B-RAF constitutively active. The development of small molecule inhibitors for BRAF V600E had major clinical success[171]. Prior to the development of vemurafenib, melanoma was one of the most lethal forms of cancer[172].

While it has been reported to occur, MEK is less likely to acquire oncogenic mutations in transformed cells[173–176]. However, given MEKs pivotal position as a gatekeeper to ERK1/2, MEK inhibitors could be leveraged to circumvent acquired resistance that is known to occur during long term use of RAF and EGFR inhibitors[148]. While the road toward MEK inhibitors has been long and arduous, Trametinib has been FDA approved for concurrent use with first-generation B-RAF inhibitors to treat melanoma. At this time, there are no known inhibitors of ERK1/2[177–179].

1.4 Cancer metabolism

1.4.1 The Warburg effect

Cancer has long been associated with changes in cellular metabolism[151,180]. The illustrious Noble prize scientist, Dr. Otto Warburg, observed that cancer cells utilized what at the time he referred to as ‘fermentation’ (we now realize this is glycolysis) despite the availability of oxygen[181]. He concluded that this metabolic phenotype, and cancer itself, was the result of defective mitochondria[181]. Roughly a century after this initial report, we now understand that

cancer is the result of a genetic dysfunction that results in unchecked cellular death and growth programs[182]. Nevertheless, Dr. Warburg's discovery is still the focus of intense research, as altered cellular metabolism is known to occur frequently across diverse types of cancers[183]. The metabolic phenotype Dr. Warburg described is now referred to as the 'Warburg effect'[184].

The Warburg effect describes a cellular metabolic phenotype characterized by excessive lactate generation in the presence of adequate oxygen[185]. In the presence of adequate oxygen, glucose proceeds through glycolysis to eventually generate pyruvate. Pyruvate can be used to generate a number of other products, but the most well-known products of pyruvate are acetyl-CoA or lactate[185]. The widely accepted dogma in central carbon metabolism posits that in the presence of oxygen, pyruvate enters the mitochondria to form acetyl-CoA for further processing in the tricarboxylic acid cycle (TCA)[186]. When oxygen is unavailable, pyruvate is reduced to lactate by the enzyme lactate dehydrogenase (LDH). The Warburg effect violates this dogmatic view of central carbon metabolism. Furthermore, the Warburg effect is not exclusively confined to pathological states[187,188]. It is now widely accepted that the Warburg effect can occur during several homeostatic processes, such as immune cell signaling or stem cell differentiation[189–191]. Nevertheless, the Warburg effect is most widely appreciated for its prevalence in cancer.

In cancer, the Warburg effect can be achieved by two distinct and often overlapping oncogenic mechanisms that result in metabolic reprogramming of the cancer cells. The first oncogenic mechanism that can enable the Warburg effect has been shown to occur via direct transcriptional activation of glycolytic machinery[192]. The transcriptional activation of glycolytic machinery can be achieved by oncogenic transcription factors, HIF1a and c-Myc[192–194]i. HIF1a and c-Myc can induce the Warburg effect by increasing the expression of genes involved in

glucose import and glycolysis, inducing the overexpression of genes such as glucose transporters (GLUTs), hexokinase 2, and LDH[195].

Another mechanism to achieve the Warburg effect in cancer is via oncogenic signaling that arises from activating mutations in the RAS-RAF-MEK-ERK/MAPK (MAPK) pathway. In fact, multiple signaling modalities that emanate from the MAPK pathway have been reported to promote the Warburg effect in cancer[196]. Cantley and Lu demonstrated that EGFR stimulation can induce an ERK dependent phosphorylation of pyruvate kinase M2 (PKM2), resulting in PKM2 co-activating the expression of known oncogenic transcription factors, beta-catenin and c-Myc[118]. Later, Lu and colleagues would later describe a novel ERK-mediated mechanism that induces the Warburg effect via inhibitory phosphorylation of mitochondrial oxidative phosphorylation proteins, thus inhibiting the TCA cycle[197].

1.4.2 The lactate molecule

The lactate molecule is the end product of glycolysis that occurs in hypoxic conditions and is also the end product of glycolysis during the Warburg effect. Lactate has often been referred to as a waste product of metabolism. However, there has been a recent resurgence in research that focuses both on lactates function as a metabolite and as a signaling molecule[198].

The lactate molecule can exist in cells as an enantiomeric anion in either the L form (L-lactate) or the D form (D-lactate)[199]. L-lactate is the molecule generated as an end product of glycolysis and therefore the major physiologically relevant form. D-lactate is the end product of the glyoxalase pathway, which functions to clear a toxic molecule called methylglyoxal that forms spontaneously from the glycolytic intermediate, dihydroxyacetone phosphate (DHAP)[200]. The physiological range of lactate in tissue is approximately 0.5 to 20 mM, with levels that have been reported to reach upward of 40 mM in certain tumor samples[201]. By comparison, the

physiological range for D-lactate is maintained at about 0.01 mM[202–204]. L-lactate that is generated in hypoxic tissues has long been considered to be secreted from the cells into the blood. L-lactate will journey to the liver, where it will enter specialized liver cells that convert L-lactate into pyruvate via LDH. The lactate-derived pyruvate molecule is used as starting material for gluconeogenesis. This process is collectively referred to as the Cori cycle[205–207]. However, emerging evidence suggests that L-lactate can function as a primary fuel source in certain types of cancer, and even in normal physiology[208,209]. Beyond its role in energy metabolism, L-lactate, herein referred to as lactate, has been shown to function as a signaling molecule[210,211].

Several studies have implicated the signaling capability that lactate possesses. Medzhitov and colleagues demonstrate that pro-inflammatory macrophages could be polarized to an anti-inflammatory macrophage through signaling that occurs via tumor-derived lactate[212]. They reason that the lactate can stabilize HIF1a, which then activates the transcription anti-inflammatory associated genes[212]. However, the precise molecular mechanism that underscores lactate's ability to polarize macrophages was not determined. Other groups have reported similar observations regarding the impact that lactate can have on diverse immune cell processes[213,214]. Zhao and colleagues reported a critical finding that may be the smoking gun that describes the mechanism in which lactate may function as a signaling molecule.

In 2019, Zhao and colleagues reported a 72.021 mass shift that occurred on a peptide derived from Hela core histone extracts[30,52]. Using a series of synthetic peptides that each harbored an isomeric lysine modification equal to the mass of 72.021, they determined which synthetic peptide had the same retention time as the *in vivo* peptide[30]. The synthetic peptide whose retention matched the *in vivo* peptide harbored a lactyllysine (Kla) modification. Antibodies that can specifically recognize the Kla modification were developed, and the team

subsequently demonstrated that Kla is novel type of PTM that can occur on histone proteins and is sensitive to intracellular lactate levels. Revisiting the work of earlier work of Medzhitov and colleagues, Zhao and Becker discovered that histone Kla is enriched in bone marrow derived macrophages (BMDMs) that were stimulated to be pro-inflammatory (M1). Using Kla specific antibodies for chromatin immunoprecipitation sequencing (ChIP-Seq), they determined that the genes marked in M1 BMDMs were genes associated with resolution and wound repair – an observation that was consistent with those made by Medzhitov and colleagues. Beyond its role in innate immune cell function, alternative functions for histone Kla remain to be determined.

1.4.3 Unanswered questions

The significance of certain PTMs in the context of cancer has been discussed at length in this text – especially protein prephosphorylation. However, given the cross talk between the MAPK pathway and the Warburg effect in cancer, it is intriguing to speculate that this particular PTM driven pathway can support the establishment of another type of PTM: histone lysine lactylation. Furthermore, discovering the biochemical regulatory elements that govern protein phosphorylation had significant impacts on human life via the development of clinically valuable medicines. Therefore, it stands to reason that identifying the regulatory elements for lysine lactylation could provide a similar advantage to cancer patients. Here, we will show evidence that suggest that the MAPK signaling pathway is a universal regulator of histone lysine lactylation in cancer cells. We will quantitative and map the degree in which the MAPK pathway can establish histone Kla levels. We determine the mechanism in which the MAPK controls histone Kla in cancer cells. Finally, we will report that MOF can possess lactyltransferase activity. We characterize the lactyltransferase activity of MOF using quantitative proteomics and

use molecular simulations to determine the possible mechanism of molecular recognition that MOF utilizes to engage with its putative co-factor, lactyl-CoA.

CHAPTER 2

LYSINE LACTYLATION IS AN ONCOGENIC HISTONE MARK

2.1 Introduction

As introduced in Chapter 1, lysine lactylation (Kla) is a novel PTM found to occur on histone proteins and to be induced by increasing the intracellular concentration of its precursor metabolite: lactate[32]. Lactate is an established end product of the Warburg effect, a process that is frequently observed to occur in cancer cells[183]. Several lines of evidence suggest that the Warburg effect can be realized in cells via oncogenic activating mutations that occur in RAS-RAF-MEK-ERK/MAPK (MAPK) pathway[215,216]. Targeted therapies for oncogenic MAPK proteins have had a significantly positive impact on human health. However, refractory disease remains a major medical burden on patients and society. Therefore, determining novel targets downstream of the MAPK pathway could result in novel therapies akin to those that were initially developed for members of MAPK pathway. However, whether the MAPK can modulate histone Kla levels in cancer cells is poorly understood. We therefore hypothesized that histone Kla could be dynamically established by the MAPK pathway.

In the following chapter, we report a novel regulatory link between the MAPK pathway and histone Kla. We comprehensively dissect the contributions that each MAPK member exerts on the establishment of histone Kla levels in cancer. We demonstrate that EGFR signaling can positively contribute to histone Kla. We next determine that RAS can establish histone Kla levels in cancer cells. Working our way downstream the MAPK pathway, we next interrogate RAF and determine that RAF contributes to histone Kla levels. Alas, we determine that MEK is a universal regulator of histone Kla across all tested cancer cell lines. Lastly, we perform quantitative proteomics to characterize the MAPK regulated histone lysine lactylome. Taken

together, the MAPK pathway can establish histone Kla levels in cancer, and inhibiting oncogenic activating mutations found in RAS and RAF results in perturbed histone Kla levels. These findings support our hypothesis that histone Kla can be controlled by the MAPK signaling pathway.

2.2 Results

2.2.1 Histone Kla is regulated by EGFR signaling

Oncogenic activating mutations in proteins belonging to the MAPK pathway can result in a metabolic switch toward the Warburg effect[118,197,215,217]. It was reported that intracellular lactate levels are reduced in cancer cells exposed to clinically relevant targeted inhibitors of the MAPK[218]. Given that histone lysine lactylation (Kla) was shown to be sensitive to the levels of intracellular L-lactate, we hypothesized that oncogenic signaling resulting from the Warburg effect can promote elevated histone Kla levels.

We first examined impact of EGFR signaling on histone Kla levels. The epidermoid carcinoma cell line A431 was selected for this experiment because these cells contain only wildtype alleles of the MAPK pathway proteins, and because they express high levels of EGFR and are therefore sensitive to EGF stimulation[219]. The EGF pathway is chronically stimulated by normal cell culture media, as serum typically contains complex mixture of diverse mitogens. Nevertheless, treatment of an additional 200 ng/mL of human EGF slightly elevated Kla levels (**Figure 2.1a**). Inhibition of EGFR by two FDA approved EGFR inhibitors, Lapatinib or Gefetinib, resulted in decreased histone Kla levels (**Figure 2.1a**). As a control, the activation and inhibition of the MAPK pathway are represented by the phosphorylation status of ERK1/2 (**Figure 2.1a**).

To corroborate the regulatory function of EGFR inhibitor, we exposed A431 cells to escalating doses of another FDA approved EGFR inhibitor, erlotinib[220]. We observed a dose-dependent decrease in histone Kla levels (**Figure 2.1b**). Intriguingly, adding back increasing concentrations of FBS to serum-starved A431 cells resulted in a dose-dependent fashion results in increased in histone Kla levels, demonstrating that ligand deprivation or inhibitor-mediated inhibition of EGFR can decrease histone Kla levels (**Figure 2.1c**). Taken together, our results indicate that EGFR activity can positively modulate the levels of histone Kla in cancer cells. Furthermore, these preliminary results suggest that oncogenic activating mutations found in the MAPK signaling pathway are likely to positively regulate histone Kla levels.

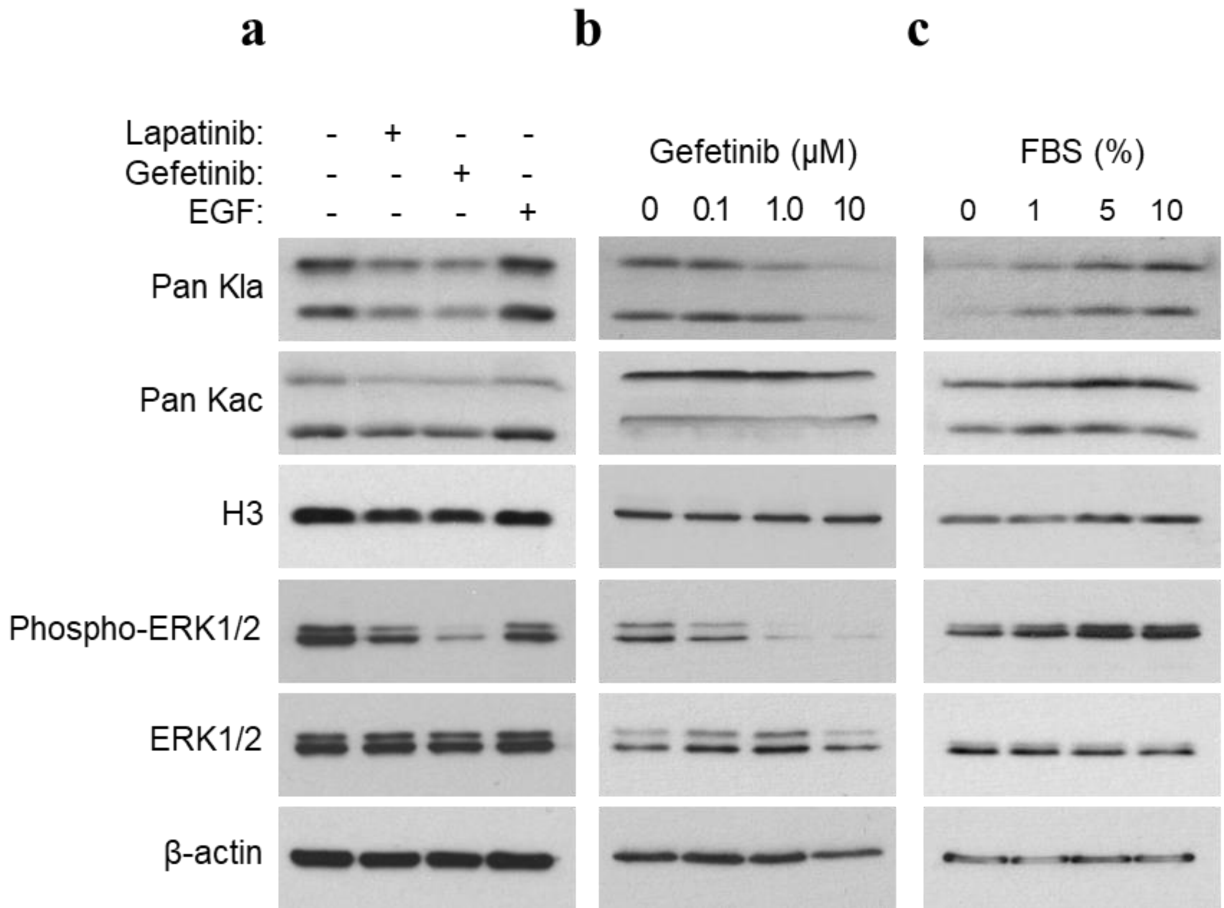


Figure 2.1 Histone lysine lactylation is regulated by growth factor signaling.
Positive or negative modulation of EGFR has direct impacts histone Kla levels (a) A431 cells (BRAF wildtype) were exposed to 10 μM of either gefetinib or lapatinib, or 200 ng of EGF for 24 hours. (b) A431 Cells were exposed to the indicated concentrations of gefetinib for 24 hours. (c) A431 cells were serum starved for 24 hours before the indicated amount of serum containing media (as a percent) was introduced and the cells were cultured for an additional 24 hours. (a, b, c) Western blot results generated from the use of the indicated antibody (left of gel panel). Primary antibody incubated overnight at a working concentration of 1 μg/ μL.

2.2.2 Histone Kla is regulated by KRAS and BRAF signaling

We hypothesized that RAS could establish histone Kla in cancer cells. To test this hypothesis, we took advantage of two tools. The first tool is the newly developed KRAS G12C inhibitor, sotorasib. Sotorasib is small molecule inhibitor that covalently binds to the mutant cysteine found in the G12C mutant KRAS. The other tool we leveraged were KRAS cells lines that were either homozygous for wild type KRAS or harbored at least one allele of the KRAS G12C mutation.

We next investigated if RAS activity could modulate histone Kla levels. To this end, we selected three pancreatic cancer cells lines that contain either an allele for the KRAS G12C mutation (MiaPaca-2, H385), or harbor non-mutated KRAS (PANC-1). We leveraged the use of sotorasib, a novel covalent small molecule inhibitor specific for KRAS G12C that was recently FDA approved for use in non-small cell lung cancer (NSCLC). Remarkably, exposing PANC-1 cells to increasing concentrations of sotorasib did not result in any changes in histone Kla levels (**Figure 2.2a**). By contrast, exposing MiaPaca-2 cells to increasing doses of sotorasib resulted in a dose dependent decrease in histone Kla levels (**Figure 2.2a**). This observation was consistent with H385 cells that were exposed to sotorasib (**Figure 2.2a**). Therefore, sotorasib reduces histone Kla levels in KRAS G12C harboring cells, but not in wildtype KRAS cells. These data demonstrate that histone Kla can be positively regulated by KRAS activity.

Given that we observed that the inhibition of EGFR and the inhibition of KRAS could impact the levels of histone Kla in cancer cells, we hypothesized that BRAF would likely play a role in establishing histone Kla in cancer cells. To test this hypothesis, we leveraged the use of BRAF V600E small molecule inhibitor, vemurafenib, on wildtype and BRAF V600E harboring cancer cells.

To interrogate RAF, we selected three melanoma cancer cells lines that contained either an allele for the BRAF V600E mutation (A375, SKMEL-28), or non-mutated BRAF (A431). We exposed these cells to increasing concentrations of vemurafinib, an FDA-approved small molecule inhibitor specific for BRAF V600E. Exposing A431 cells to increasing concentrations of vemurafinib did not significantly change histone Kla levels in the wild type BRAF cell line (**Figure 2.2b**). However, exposing either A375 or SKMEL-28 cells to increasing concentrations of vemurafenib resulted in a dose-dependent reduction of histone Kla levels (**Figure 2.2b**). Therefore, vemurafenib reduces histone Kla levels in BRAF V600E mutated cell lines, but not in BRAF wildtype cells. These data suggest that histone Kla can be positively regulated by BRAF activity.

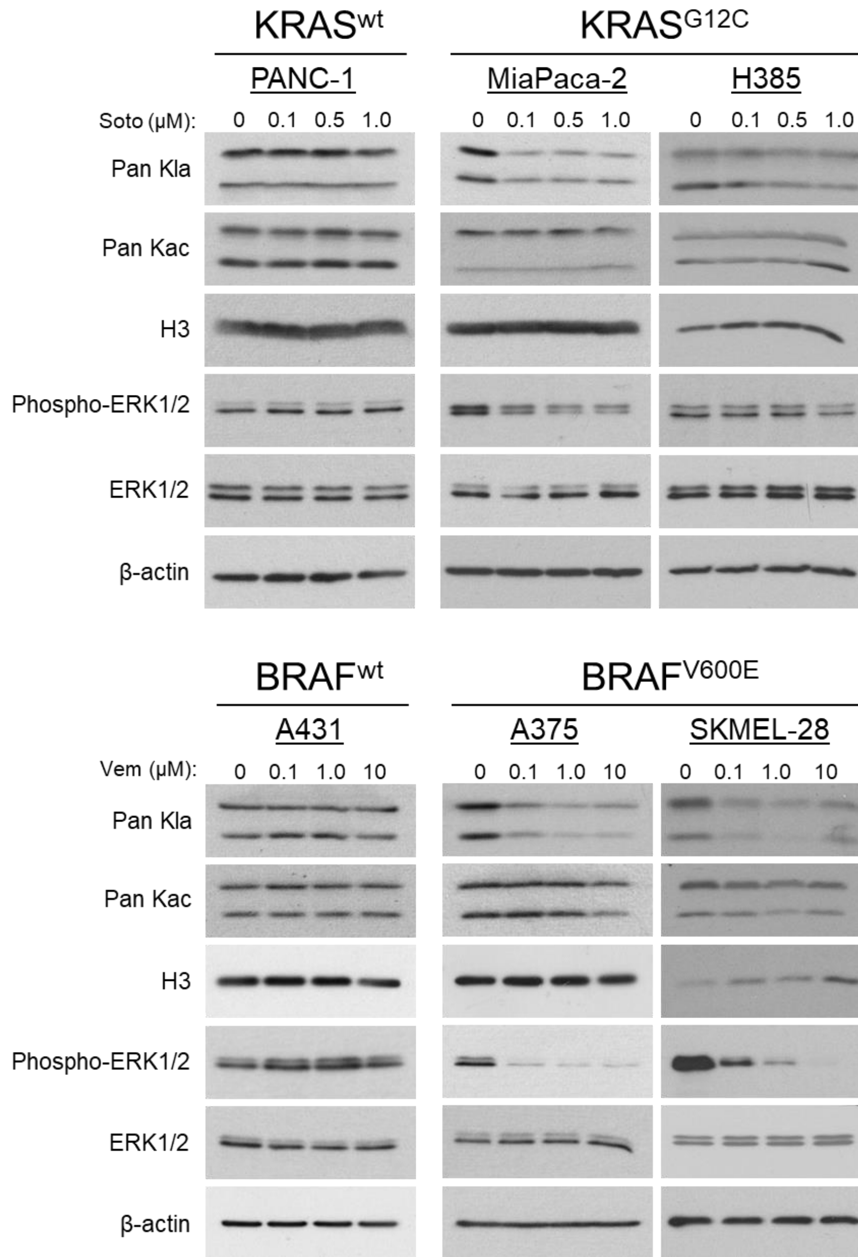


Figure 2.2 The MAPK pathway and oncogenic signaling regulate histone lysine lactylation. Oncogenic signaling from KRAS and BRAF can promote histone lysine lactylation in cancer cells. (a) Pancreatic cancer cell lines PANC-1 (KRAS wildtype), MiaPaca-2 (homozygous KRAS G12C) and H385 (heterozygous KRAS G12C) were exposed to the indicated doses of sotorasib for 24 hours. (b) Melanoma cancer cell lines A431 (BRAF wildtype), A375 (homozygous BRAF V600E) and SKMEL-28 (homozygous BRAF V600E) were exposed to the indicated doses of vemurafenib for 24 hours. (a, b) Western blot results generated from the use of the indicated antibody (left of gel panel). Primary antibody incubated overnight at a working concentration of 1 μg/ μL.

2.2.3 Histone Kla is regulated by MEK signaling

Alas, we assessed how MEK activity could modulate histone Kla levels in cancer. For this experiment, we selected cell lines originating from 5 distinct primary sites that correspond to breast, colon, gastric, lung, prostate, and skin tumors. We leveraged the use trametinib: an FDA approved small molecule inhibitor of MEK. Notably, MEK is the only known activator of ERK1/2 and is often referred to as a 'gatekeeper' that can activate ERKs nuclear translocation and nuclear coactivator function[148]. Accordingly, trametinib exposure resulted in decreased ERK1/2 activity in all tested cell lines, as measured by phospho-ERK1/2 signal (**Figure 2.3**). Remarkably, exposure to trametinib resulted in an almost unambiguous decrease in histone Kla levels in all cell lines that were tested (**Figure 2.3**). These data demonstrate that histone Kla levels are positively regulated by MEK. Moreover, given the specificity between the MEK-ERK interaction, these results suggest that histone Kla can be universally governed by ERK activation.

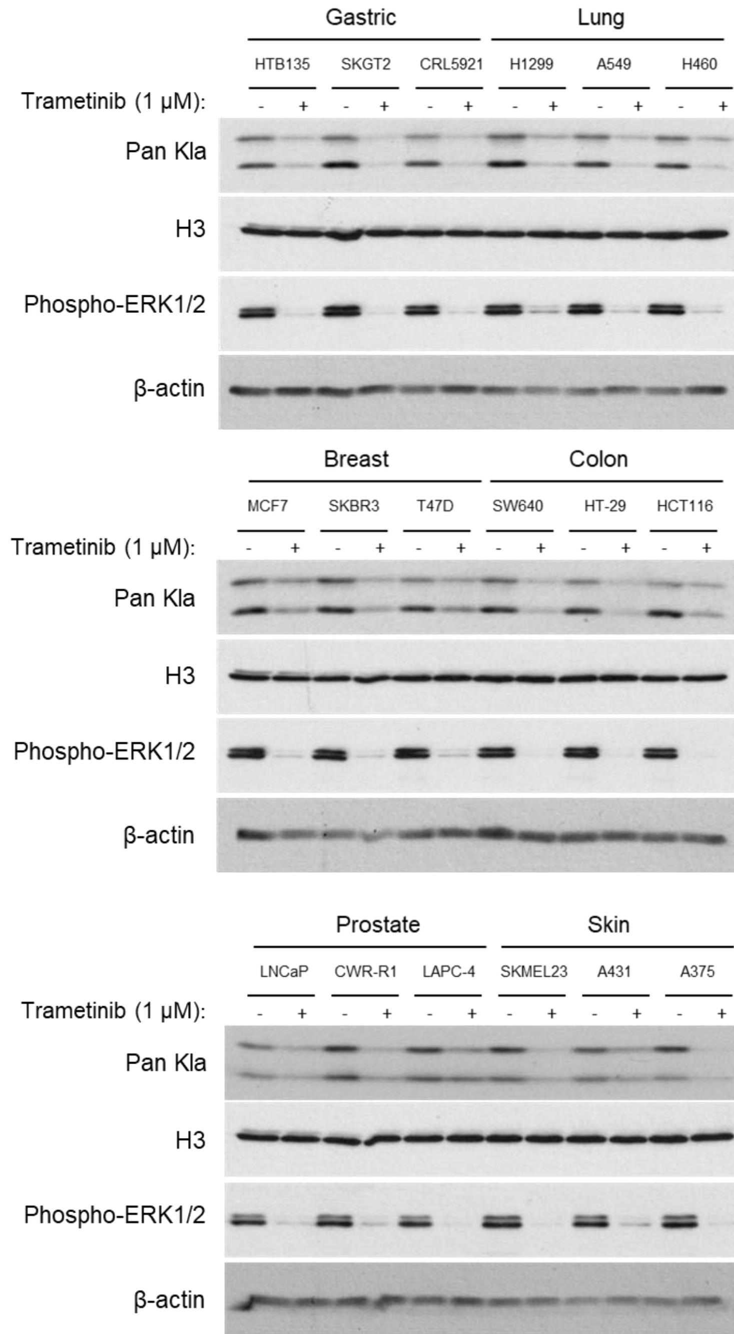


Figure 2.3 MEK is a universal regulator of histone lysine lactylation in cancer cells.
MEK signaling regulates histone lysine lactylation in diverse human cell lines. (a) Three cancer cells lines originating from 5 distinct tissues were exposed to 1 μM of the MEK inhibitor, trametinib. Western blot results generated from the use of the indicated antibody (left of gel panel). Primary antibody incubated overnight at a working concentration of 1 μg/ μL.

2.2.4 Comprehensive characterization of MAPK regulated histone Kla

We previously observed that histone lysine lactylation can be globally driven by oncogenic signaling that emanates from the MAPK signaling pathway. However, the position and relative abundance of oncogenic histone Kla remains unknown. Therefore, we sought to quantitatively map the Kla sites on histone proteins that are regulated by oncogenic signaling. To this end, we used stable isotopic labeling of amino acids in cell culture (SILAC) to quantitatively characterize the impact that different inhibitors of MAPK pathway members exert on histone Kla[221]. Histone extracts from SILAC-cultured A431 that were exposed to erlotinib or DMSO were subjected to proteolytic digestion followed by peptide immunoprecipitation using the pan-Kla antibody. The peptides were analyzed by HPLC-coupled mass spectrometric analysis to identify Kla peptides and to quantify their associated changes. Consistent with western blot analysis, exposing A431 cells to erlotinib resulted in the downregulation of a number of histone Kla sites (**Figure 2.4a**). Relative to the other sites identified in the EGFR inhibited group, Positions H2AK9 and K13 experienced the most dramatic reduction in Kla for the EGFR, losing >2.5-fold modification relative to the DMSO treated control. On the histone tails of histone H3, positions K9 and K18 both experienced a >2-fold reduction. For histone H4, K5, K8, and K12 all experienced > 2-fold reduction.

We applied the exact experimental work to quantitatively characterize the KRAS-mediated histone lysine lactylome. To this end, we exposed MiaPaca-2 cells to either DMSO or sotorasib and analyzed Kla enriched peptides by mass spectrometry. Consistent with EGFR inhibition, KRAS inhibition resulted in a dramatic decrease in H2AK9 and K13 (**Figure 2.4b**). In addition, H4K8 experienced roughly a >2.5 fold decrease in histone Kla relative to DMSO treated group. The relative loss in histone Kla was greater in KRAS inhibited cells than in EGFR

inhibited cells. However, sites on histone H3 experienced a greater loss in the EGFR inhibited group relative to the KRAS inhibited group. Curiously, position H3K56 did not experience robust changes as a result of either EGFR or KRAS inhibition.

We next evaluated BRAF using the aforementioned strategy. To characterize BRAF induced histone Kla, A375 cells were exposed to either DMSO or vemurafenib and Kla enriched peptides were analyzed by mass spectrometry. Remarkably, Histone H3 K9 experienced a greater than 4 -fold reduction as a result of BRAF inhibition (**Figure 2.4c**). This reduction was the largest loss of Kla observed on a single site with respect to EGFRi, KRASi, BRAFi, and MEKi. Unlike EGFR and KRAS inhibition, H3K14la levels were not altered by BRAF inhibition. However, H3K18, K23, and K27 were all reduced more than 2- fold, with H3K27la experiencing roughly a 3-fold loss in Kla as a result of BRAF inhibition.

Lastly, we sought to quantitatively characterize the histone lysine lactylome as a function of MEK regulation. Using the aforementioned strategy, we exposed A431 cells to either DMSO or trametinib. The resultant Kla enriched peptides were analyzed by mass spectrometry. Consistent with the inhibition of EGFR, KRAS, and BRAF, MEK inhibition resulted in dramatic (>3 fold) reduction of H4K5la (**Figure 2.4d**). By contrast, the most distinct observation that we deduced from the MEK inhibited cells was the little to no change that occurred on H4K8la. H2BK5la also experienced a greater than 3-fold reduction. We summarized the regulation of the histone lysine lactylome as it relates to each of the core members of the MAPK using a heatmap (**Figure 2.5**).

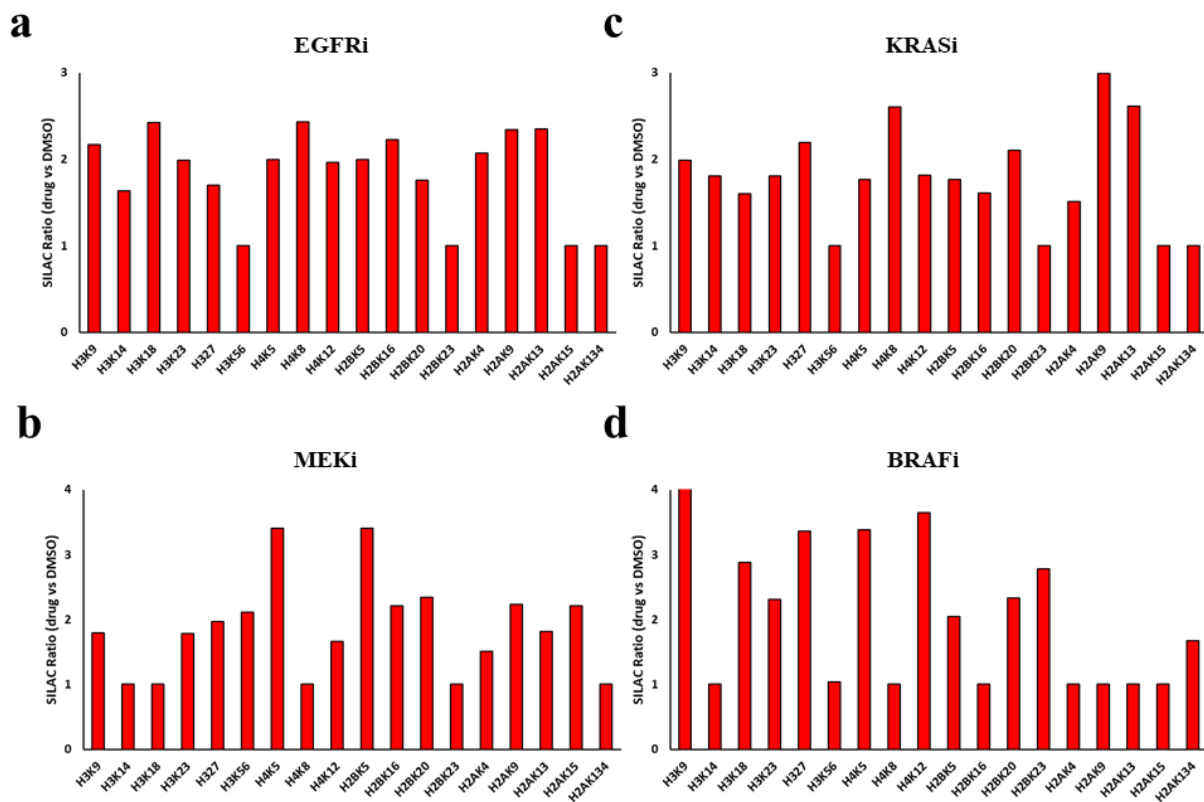


Figure 2.4 Proteomic characterization MAPK-regulated histone lysine lactylation.
Proteomic characterization of oncogenic lysine lactylation reveals differential contributions from MAPK members. (a) A431 cells were treated with 10 µM of erlotinib for 24 hours. (b) A431 cells were treated with 1 µM of trametinib for 24 hours. (c) MiaPaca-2 cells were treated with 10 µM of sotorasib for 24 hours. (d) A375 cells were treated with 10 µM of vemurafenib for 24 hours. (a-d) SILAC ratios were calculated from experimentally corresponding SILAC pairs that were exposed to either a drug or dimethylsulfoxide (DMSO).

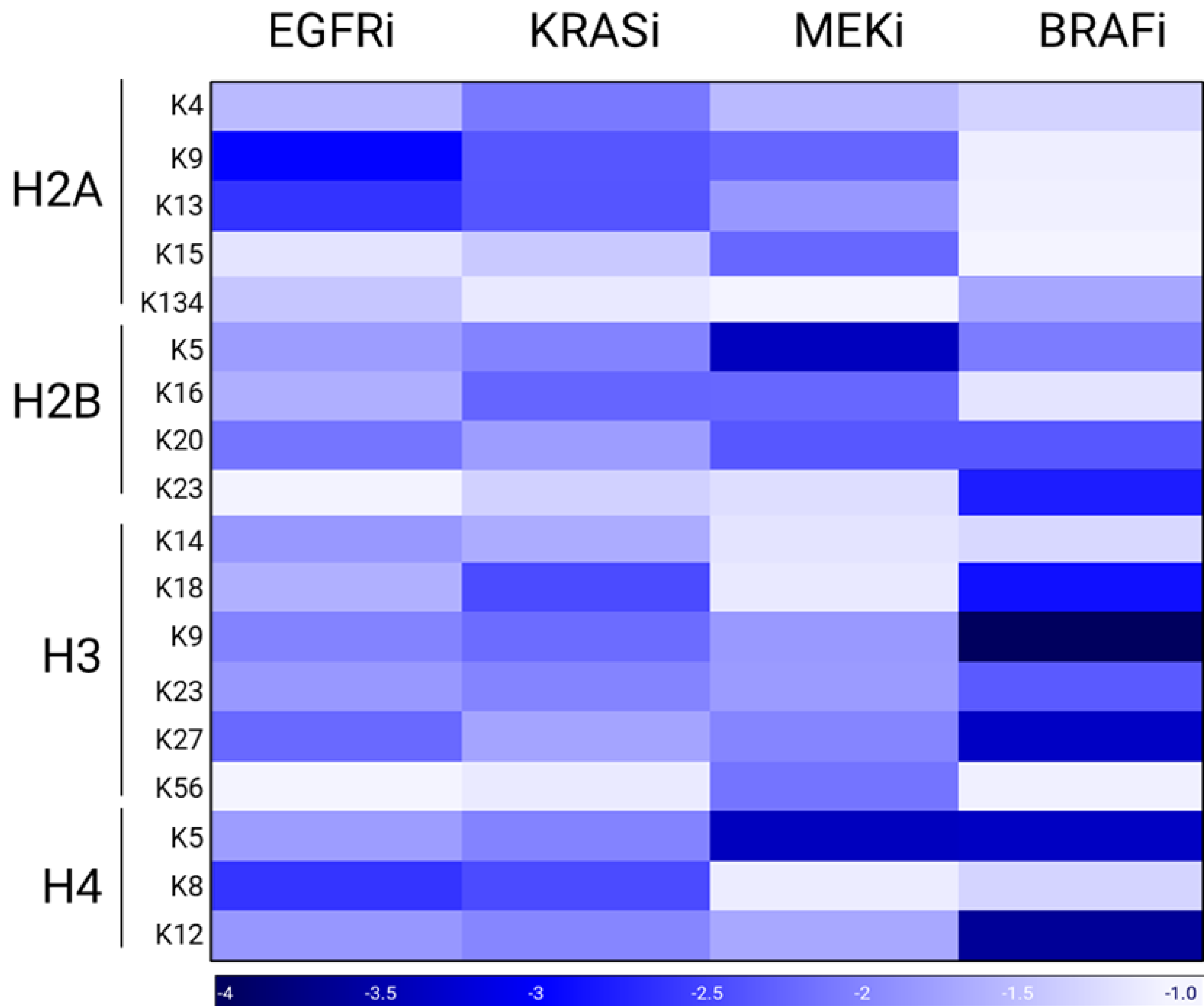


Figure 2.5 Heatmap comparison of the differential contributions observed in MAPK-regulated histone lysine lactylation.
Heatmap overview of the MAPK-regulated histone lysine lactylome. (a) Heatmap of fold change reduction of histone Kla between DMSO exposed or drug exposed cells. Scale bar represents color to fold change value. Heatmap generated in Perseus.

2.3 Discussion

In summary, we demonstrate that attenuating RAS-RAS-MEK-ERK/MAPK (MAPK) signaling at any step of the MAPK pathway resulted in decreased histone Kla levels. These data collectively demonstrate that the MAPK pathway is a novel and critical biological regulator of histone Kla. Lastly, we show that oncogenic mutations in key MAPK members can positively regulate histone Kla levels, suggesting that Kla is an oncogenic histone mark. Furthermore, we quantitatively map the oncogenic histone Kla landscape. Intriguingly, the histone site bearing the greatest downregulation of Kla was distinct for each member of the MAPK that was inhibited. For example, H2AK9, H3K18, H2BK5, and H3K9 were the most downregulated sites for EGFRi, KRASi, MEKi, and BRAFi, respectively (FIG. XX). This observation suggests that certain members of the MAPK signaling pathway may distinctly promote Kla on specific histone residues[152]. Nevertheless, several similarities between the treatments were observed. Notably, all treatments resulted in a dramatic reduction in Kla levels on H3K9, K23, and K27. Intriguingly, EGFRi and KRASi resulted in reduced Kla levels on histone H4 K5, K8 and K12, while MEKi and BRAFi only saw reduced Kla on H4 K5 and 12. These results collectively demonstrate that oncogenic histone Kla can be driven by the MAPK pathway. Given that H4 K5, K8, and K12 are known HAT1 substrates while H4 K5 and K12 are known MOF substrates, these data suggest that oncogenic histone Kla may be driven in part by a novel acyltransferase.

How oncogenic histone Kla may functionally contribute to oncogenesis remains unknown. Nevertheless, mounting evidence suggests that excessive L-lactate that is exported from cancer cells into the tumor microenvironment may have deleterious impacts on regulatory function of various non-tumor cells, such as enhancing tumor immune-suppression from CD4 t regulatory cells (Tregs) and dampening the anti-tumor responsiveness of CD8 tumor infiltrating

leukocytes[189,214,222]. However, our work details a potential intracellular function for cancer associated lactate generation. While it is clear that histone Kla can be promoted by oncogenic mechanisms, the physiological consequences of histone Kla merits further investigation. Importantly, the compounds used to inhibit the MAPK in this study are currently used on patients in the clinic. Therefore, our data reveals a potentially unforeseen molecular consequence that may stem from the use of various clinically relevant targeted therapies.

2.4 Methods

Reagents

The pan-Kla antibody (PTM-1401) was developed by PTM Biolabs (Chicago, IL). pan Kac antibody (PTM-101) was from PTM Biolabs (Chicago, IL). The anti-H3 (#4499), anti-ERK1/2 (#4695), anti-phospho-ERK1/2 (#9101) antibodies were obtained from Cell Signaling Technology. All drugs used in this study were acquired from SelleckChem. Cell lines used were purchased from ATCC and used without further authentication.

Cell culture

Cells were obtained from ATCC and used without further authentication. Cells were grown in high glucose DMEM (Thermo Fisher Scientific) supplemented with 10% FBS at 37°C in 5% CO_2. Drug

Drug treatments

All drugs used in this study were resuspended in dimethylsulfoxide (DMSO, Sigma Aldrich) and stored -20 degrees Celsius. Cells were drugged from approximately 24 hours before harvesting for western blot analysis.

Western blot analysis

Cells were washed 3 times in 1x phosphate buffered saline (PBS). Cells were harvested in 2% SDS lysis buffer (2% SDS, 60 mM Tris, 10% glycerol, 5% beta-mercaptoethanol) and incubated for 5 minutes at 100 *C. Protein lysate concentration was determined by Bradford assay. Protein lysates were resolved on 15% SDS polyacrylamide gels were us and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 3% bovine serum albumin (BSA) re-suspended in 1x tris buffered saline+tween (TBS-T: 20 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 1 hour at room temperature, with the exception for H3 antibody, which was blocked in 3% non-fat milk buffer (3% dry non-fat milk in 1x TBS-T). Membranes were incubated in primary antibody overnight at 4*C. Membranes were washed 5 times in TBS-T for 5 minutes, followed by 1 hour incubation in secondary antibody at room temperature. Membranes were washed as preciously described and probed using Pierce ECL Chemiluminescent Western Blot Detection kit (ThermoFisher, 32209).

Stable isotope labeling of cells

Cells were grown in lysine-free DMEM (ThermoFisher, #88364) supplemented with 10% dialyzed FBS, and either light (12C614N2-L-Lysine, Cambridge Isotope Laboratories, #ULM-8766PK) or heavy (13C614N2-L-Lysine, Cambridge Isotope Laboratories, #ULM-291-H-PK) lysine (100 mg/L). Cells were grown for more than seven generations to achieve more than 98% labeling efficiency. All cell lines were cultured using ATCC recommended media in a 5% CO2 atmosphere at 37 °C. Labeling efficiency was tested before experimental use of cell lines.

Peptide immunoprecipitation

Histones proteins were extracted from human cells using a standard acid-extraction protocol39, and subjected to trypsin digestion as per the manufacturer’s instructions. Pan anti-K(L-la) (PTM-

1401, PTM Bio Inc.) or pan anti-Kac antibodies (PTM-101, PTM Bio Inc.) were first conjugated to Protein A Sepharose beads (GE Healthcare BioSciences) and then incubated with tryptically digested histone peptides with gentle agitation overnight at 4 °C. The beads were then washed three times with NETN buffer (50 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40), twice with ETN buffer (50 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA) and once with water. Peptides were eluted from the beads with 0.1% TFA and dried in a SpeedVac system (ThermoFisher Scientific).

HPLC–MS/MS analysis & SILAC quantification

Tryptic peptides were loaded onto a homemade capillary column (10 cm length × 75 mm ID, 3 μm particle size) connected to an EASY-nLC 1000 system (Thermo Fisher Scientific). Peptides were separated and eluted with a gradient of 2% to 90% HPLC buffer B (0.1% formic acid in acetonitrile, v/v) in buffer A (0.1% formic acid in water, v/v) at a flow rate of 200 nl min−1 over 60 min. The eluted peptides were then ionized and analyzed by a Exploris mass spectrometer (ThermoFisher Scientific). Full mass spectrometry was acquired in the orbitrap mass analyzer over the range m/z 300 to 1,400 with a resolution of 70,000 at m/z 200. The 12 most intense ions with charge ≥2 were fragmented with normalized collision energy of 27 and tandem mass spectra were acquired with a mass resolution of 17,500 at m/z 200. Qunatification of histone K(L-la) and Kac sites was performed using Maxquant40. Heavy/light PTM peptide ratios that were calculated by Maxquant were normalized to heavy/light ratios of their unmodified peptide counter parts.

CHAPTER 3

WARBURG-DERIVED LACTATE IS A KEY SOURCE FOR ONCOGENIC HISTONE LACTYLATION

3.1 Introduction

The excessive generation of lactate by cells that exist in an oxygen replete microenvironment is a defining feature of many tumors known as the Warburg effect[181]. Initially, the Warburg effect was thought to be a condition that can only be achieved under pathophysiological conditions, such as cancer[184]. However, several lines of evidence now show that the Warburg effect can occur under various physiological conditions and is essential to these processes, including brain development or immune cell function[199,223]. Nevertheless, while not exclusive to cancer, the Warburg effect can most certainly be appreciated for its prevalence in diverse forms of cancer.

Mounting evidence suggests that the Warburg effect in cancer can be achieved by oncogenic signaling that emanates from mutant members of the RAS-RAF-MEK-ERK/MAPK (MAPK) signaling pathway[215]. In Chapter 2, we demonstrated that oncogenic signaling emanating from the MAPK pathway can sustain elevated histone Kla levels in diverse cancer cell lines. Given these two observations, we hypothesized that Warburg derived lactate is a key source for oncogenic histone Kla.

In the following chapter, we investigate the relationship between the activity of the MAPK signaling pathway and the concentration of intracellular lactate. We will correlate MAPK signaling with the activation of known Warburg associated factors that can promote glycolysis. In addition to this, we will determine whether functional glycolysis is a principal determinant of

histone Kla in cancer cells. Lastly, we will attempt to stunt downstream and upstream glycolytic flux to assess whether lactate is sufficient for inducing histone lysine lactylation.

3.2 Results

3.2.1 Oncogenic signaling induces the Warburg effect and generates lactate

We demonstrated in Chapter 2 that oncogenic activating mutations in members of the MAPK pathway can positively regulate histone lysine lactylation levels in cancer cells. Previous reports suggest that intracellular lactate levels can be elevated via the action of activating mutations found in the MAPK proteins in cancer cells[215]. The Warburg effect can be induced via signaling that originates from the MAPK pathway, which results in elevated intracellular lactate[196,224]. To this point, we observed a marked reduction in Warburg associated genes in A375 (BRAF V600E) melanoma cells that were exposed to vemurafenib (**Figure 3.1a**). Among these genes, we observed a reduction in c-Myc protein expression by western blot analysis. c-Myc is known to both upregulate hexokinase 2 (HK2) and downregulate thioredoxin-interacting protein (TXNIP)[225,226]. Hexokinase 2 is a well characterized upstream glycolytic enzyme that participates in the first step of glycolysis by phosphorylating glucose to generate glucose-6-phosphate, thereby enhancing glycolytic flux. TXNIP is a metabolically regulated protein that can function as a tumor suppressor and a redox regulatory element[227,228]. Consistent with the downregulated of c-Myc in vemurafenib treated A375 cells, we observed a marked reduction in HK2 and in significant upregulation of TXNIP by western blot analysis (**Figure 3.1a**). TXNIP has been shown to bind p53 and function as a co-activator of genes associated with cellular senescence, such as p21. Accordingly, we observed an increase in p21 protein levels in A375 cells exposed to vemurafenib **(Figure 3.1a**). In summary, BRAF inhibition results in a reduction in c-MYC, a known transcriptional activator of HK2 and a known transcriptional repressor of

TXNIP and p21. Given the widely established role that c-MYC is known to play in rewriting cancer metabolism toward the Warburg effect, we next sought to determine whether the MAPK pathway regulates intracellular lactate levels.

The MAPK pathway has been shown to activate the Warburg effect in cancer cells via an ERK-mediated negative feedback loop that suppresses oxidative phosphorylation[197]. Excessive lactate that is generated by cancer cells has long been considered a waste product of glycolysis that gets exported from cells. However, in Chapter 2 we demonstrate that oncogenic signaling from the MAPK pathway can induce histone lysine lactylation (Kla). Given that histone Kla is known to sensitive to the levels of intracellular lactate, we repurposed the same strategy used in Chapter 2 to explore the impact that the MAPK signaling pathway has on establishing intracellular lactate levels in diverse cancer cells. To measure lactate, we used a commercially available tool that couples the enzymatic oxidation of lactate, yielding NADH as a by-product. The NADH that is generated in this reaction then reacts with a bioluminescent NADH probe to produce color in the 570-nanometer range (nm) and fluorescence (excitation/emission = 537/587).

We began our exploration by examining the contributions of EGFR signaling on intracellular lactate levels. A431 cells that were exposed to increasing concentrations of FBS saw a dose dependent increase in intracellular lactate, suggesting that EGFR stimulation in cancer cells can promote lactate synthesis (**Figure 3.2a**). Accordingly, exposing A431 cells to EGFR inhibitor, erlotinib, resulted in a dose dependent decrease in intracellular lactate (**Figure 3.2b**). Taken together, these data show that EGFR activity can positively modulate the levels of intracellular lactate in cancer cells.

We evaluated whether RAS, which sits directly downstream of EGFR, could modulate intracellular lactate levels. We exposed pancreatic cancer cells lines harboring either mutant or wildtype KRAS to the KRAS G12C specific inhibitor, sotorasib. Exposing the PANC-1 cell line (wild type KRAS) to increasing sotorasib resulted in no change in intracellular lactate levels (**Figure 3.2c**). However, exposing MiaPaca-2 or H385 cells (harbor KRAS G12C) to increasing doses of sotorasib resulted in a dose dependent reduction of intracellular lactate (**Figure 3.2c**). Therefore, these data indicate that KRAS can enhance lactate generation in cancer cells. Moreover, these data demonstrate that oncogenic activating mutations in KRAS can promote lactate generation.

We next evaluated whether RAF, which is downstream of RAS, could modulate intracellular lactate levels. We exposed melanoma cell lines containing either wildtype or mutant BRAF to escalating doses of BRAF V600E specific inhibitor, vemurafinib. Exposing A431 cells to increasing concentrations of vemurafinib did not result in any observable changes in intracellular lactate levels (**Figure 3.2d**). However, exposing A375 or SKMEL-28 cells to increasing concentrations of vemurafenib resulted in a dose dependent decrease in intracellular lactate levels (**Figure 3.2d**). These results demonstrate that vemurafenib can reduce intracellular lactate levels in BRAF V600E mutated cell lines, but not in BRAF wildtype cells. Therefore, BRAF can positively drive lactate production in cancer. Similarly, oncogenic activating mutations in BRAF are sufficient to enhance lactate production in cancer cells.

These data collectively demonstrate that lactate production is coupled to oncogenic signaling that emanates from the MAPK pathway. Therefore, histone Kla, the Warburg effect, and lactate production, are intimately to the MAPK signaling pathway. In our report that describes the discovery of lactyllysine (Kla), we demonstrate that lactate can directly modulate

the abundance of Kla in cells[30]. If lactate is the primary source for histone Kla in cancer cells, we should expect that perturbing glycolysis would modulate histone Kla levels. We next sought to determine whether perturbing glycolysis could impact histone Kla levels in cancer cells.

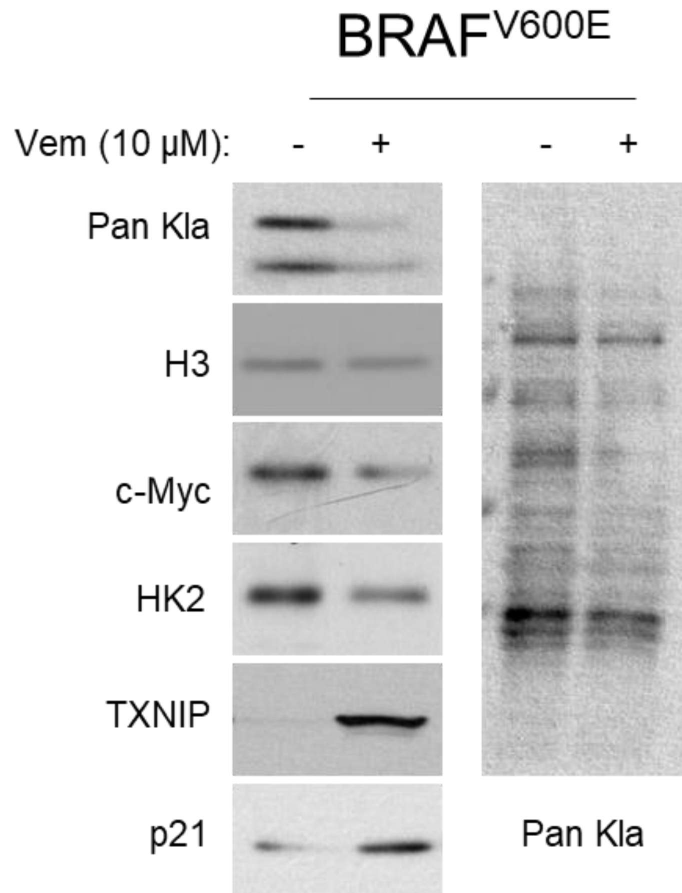


Figure 3.1 Warburg effect regulatory proteins are modulated by BRAF.
Several Warburg effect associated proteins are modulated by BRAF activity. (a) A375 cells were exposed to 10 µM of vemurafenib for 24 hours. Western blot results generated from the use of the indicated antibody (left of gel panel). Primary antibody incubated overnight at a working concentration of 1 µg/ µL.

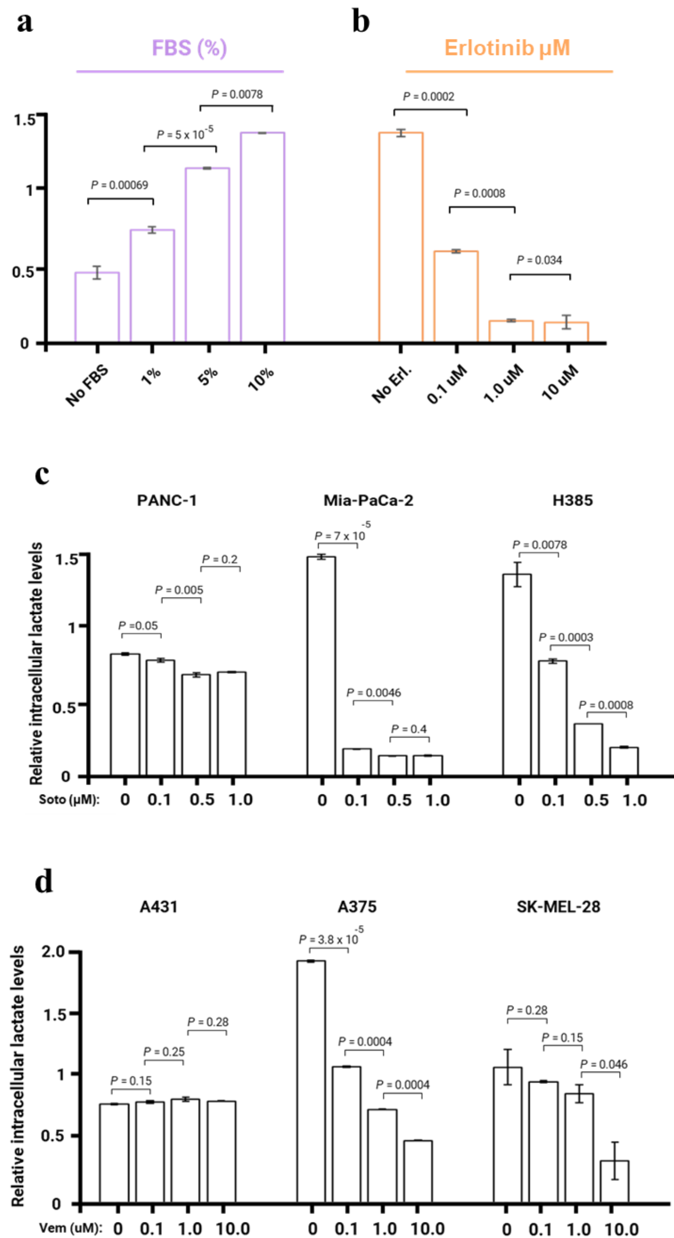


Figure 3.2 The level of intracellular lactate is controlled by the MAPK pathway.
Intercellular lactate generation is regulated by the activity of the MAPK in cancer cells. (a) A431 cells were serum starved for 24 hours before the indicated amount of serum containing media (as a percent) was introduced and the cells were cultured for an additional 24 hours. (b) A431 cells were exposed to the indicated concentration of erlotinib for 24 hours. (c) PANC-1, Miapaca-2 and H385 cells were exposed to the indicated concentration of sotorasib for 24 hours. (d) A431, A375, and SKMEL-28 cells were exposed to the indicated concentration of vemurafenib for 24 hours. (a-d) Cells were harvested and subjected to lactate assay as described in the Methods section.

3.2.2 Histone lysine lactylation is coupled to glycolysis

Given that inhibition of oncogenic BRAF or KRAS resulted in the concomitant reduction of lactate and histone Kla, we hypothesized that glycolysis, and the Warburg effect, is essential for establishing histone Kla in cancer cells. To test this hypothesis, we used small molecule inhibitors of different glycolytic proteins to evaluate the impact of their inhibition on histone Kla levels. We first assessed whether upstream glycolytic activity is necessary for establishing oncogenic histone Kla levels in cancer cells. Accordingly, exposing cells to high glucose concentrations resulted in a dose-dependent increase in histone Kla levels (**Figure 3.3a**). This result suggests that the key glycolytic substrate, glucose, is required for maintaining of histone Kla in cancer cells. Consistent with this observation, exposing cells to 2-deoxyglucose (2-DG), a non-metabolizable glucose analog that is a known to inhibit HK2, resulted in a dose dependent reduction in histone Kla levels (**Figure 3.3b**)[229]. Given that HK2 is the first enzyme in glycolysis that modifies glucose, this result suggests that glucose contributes to histone Kla through via its catabolism in glycolysis. Collectively these data demonstrate that glucose and upstream stream glycolytic activity are necessary for maintaining histone Kla levels in cancer cells.

Given that upstream glycolytic activity is required to establish oncogenic histone Kla levels in cancer cells, we next sought to evaluate whether the activity of downstream glycolytic enzymes is also required to establish oncogenic histone Kla. Exposing A375 cells to increasing concentrations of PDK1 inhibitor, dichloroacetate (DCA), resulted in a dose dependent reduction in histone Kla levels (**Figure 3.4a)**[230]. PDK is known to inactivate PDH, which converts pyruvate to acetyl-CoA for the TCA cycle[231]. Inhibiting PDK1 with DCA causes a greater pool of acetyl-CoA being generated from pyruvate, resulting in less lactate generation[230]. Therefore, these results suggest that the catabolism of final metabolic substrate of glycolysis (pyruvate) has

dire implications on the levels of histone Kla. Consistent with this observation, exposing cells to UK5099, a known inhibitor of mitochondrial pyruvate carrier (MPC), resulted in a modest increase in Kla levels (**Figure 3.4b**)[232,233]. This likely stems from increasing the pool of pyruvate that is available for reduction into lactate.

Given that histone Kla is perturbed by DCA exposure, we reasoned that perturbations that affect lactate generation would result in a reduction in histone Kla. Therefore, we evaluated whether histone Kla levels could be perturbed by inhibiting lactate dehydrogenase (LDH). Functional LDH exists predominately as a tetramer that is composed of different combinations of for LDH subunits: A, B, C, and D. The LDH complex is currently the only known regulatory source for lactate generation in human cells[234]. Consistent with our hypothesis, exposing A375 cells to increasing concentrations of oxamate, a pan-LDH inhibitor, resulted in a dose-dependent reduction in histone Kla levels (**Figure 3.5a**)[235]. Accumulating evidence suggests that LDH-A is upregulated in highly glycolytic tumors via oncogenic programs that support the Warburg effect. We next exposed A375 cells to increasing concentrations GNE-140, an LDH-A specific inhibitor[236]. GNE-140 exposure resulted in a dose dependent decrease in histone Kla. Of note, the decrease in histone Kla levels that we observed in GNE-140 exposed cells was less dramatic than the reduction in histone Kla observed in oxamate exposed cells. These results demonstrate that downstream glycolytic activity is required to establish oncogenic histone Kla. Moreover, our data suggests that Warburg-derived lactate is the key determinant of oncogenic histone Kla.

Taken together, we have shown now that lactate generation is principally driven by the activity of the MAPK pathway in cells. In addition to lactate generation, the levels of histone Kla are also tied to the activity of the MAPK pathway. Now, we have shown that unperturbed glycolysis is critical to establish histone Kla levels in cells. Therefore, we propose a model where

the MAPK maintains histone Kla levels in cancer cells via lactate generation from the Warburg effect.

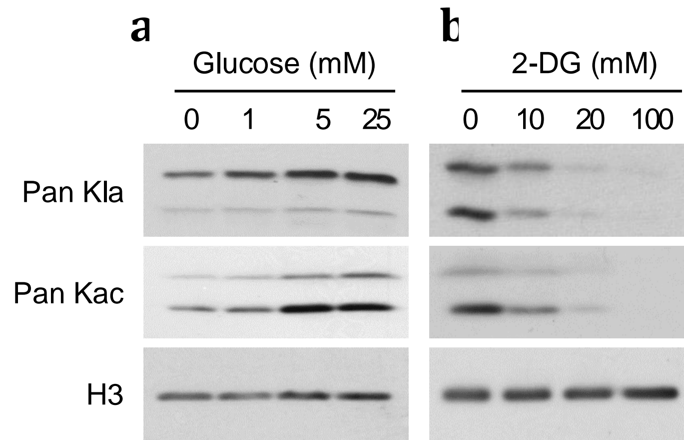


Figure 3.3 Upstream glycolysis is required for oncogenic histone lysine lactylation. Upstream glycolysis and glycolytic substrates are required for cancer cells to sustain histone lysine lactylation levels. (a) A375 cells were glucose starved for 24 hours before the indicated amount of glucose was introduced, and the cells were cultured for an additional 24 hours. (b) A375 cells were exposed to the indicated concentration of hexokinase-2 inhibitor, 2-deoxyglucose (2-DG), for 24 hours. (a, b) Western blot results generated from the use of the indicated antibody (left of gel panel). Primary antibody incubated overnight at a working concentration of 1 μg/ μL.

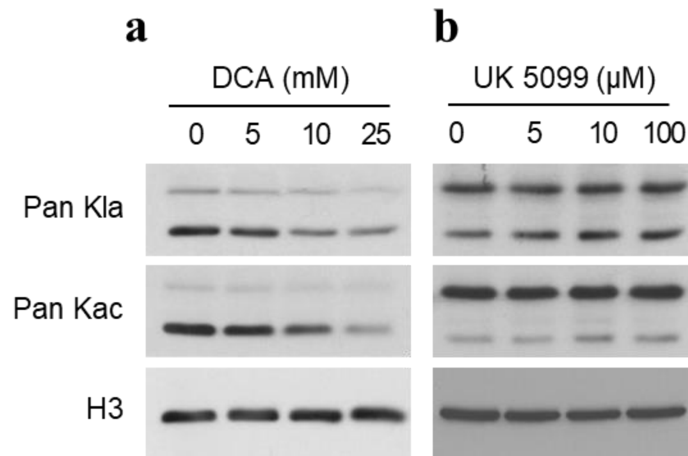


Figure 3.4 Downstream glycolysis is required for oncogenic histone lysine lactylation. Downstream glycolysis is required for cancer cells to sustain histone lysine lactylation levels. (a) A375 cells were exposed to the indicated concentration of PDK1 inhibitor, dichloroacetate (DCA), for 24 hours. (b) A375 cells were exposed to the indicated concentration of MPC inhibitor, UK 5009, for 24 hours. (a, b) Western blot results generated from the use of the indicated antibody (left of gel panel). Primary antibody incubated overnight at a working concentration of 1 μg/ μL.

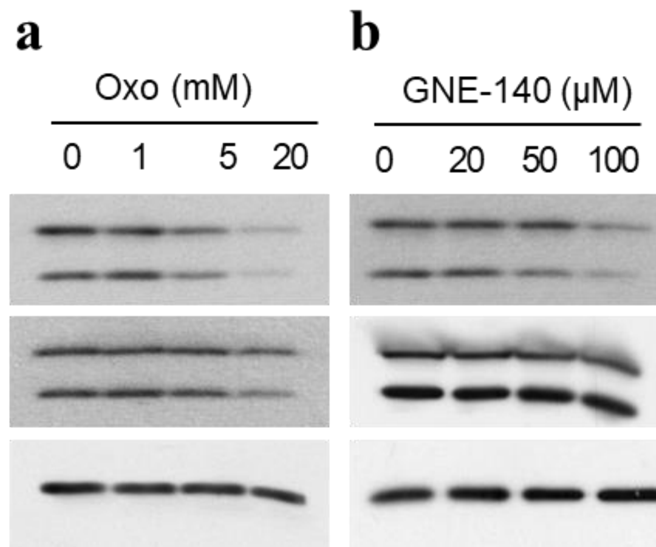


Figure 3.5 Lactate-generating machinery is required for oncogenic histone lysine lactylation.
Functional lactate dehydrogenase is required to sustain oncogenic histone lysine lactylation levels in cancer cells. (a) A375 cells were exposed to the indicated concentration of pan-LDH inhibitor, Oxamate (Oxo), for 24 hours. (b) A375 cells were exposed to the indicated concentration of LDH-A inhibitor, GNE-140, for 24 hours. (a, b) Western blot results generated from the use of the indicated antibody (left of gel panel). Primary antibody incubated overnight at a working concentration of 1 µg/ µL.

3.2.3 Lactate is sufficient for oncogenic histone Kla

We have demonstrated that the MAPK signaling pathway can control the levels of intracellular lactate and the levels of histone lysine lactylation. We further demonstrated that functional glycolysis is necessary to maintain histone Kla levels in cancer cells. However, we have not directly shown whether lactate is the key precursor molecule to generate histone Kla in cancer cells. Since it was shown that the levels of histone Kla can be modulated by the concentration of intracellular lactate, we first hypothesized that ectopic lactate could sufficiently drive histone Kla levels, regardless of functionally active MAPK signaling[30].

To test whether ectopic lactate could drive histone Kla in cancer cells independently of active MAPK signaling, we attempted to restore histone Kla levels in A375 cells that were exposed to vemurafenib (BRAFi). Consistent with our findings in Chapter 2, we observed a reduction in histone Kla levels in cells exposed to vemurafenib (**Figure 3.6a**). In addition, and consistent with previously published work, we observed an increase in histone Kla levels in A375 cells that were exposed to 20 mM of lactate (**Figure 3.6a**). Alas, we could restore histone Kla levels in A375 cells that were exposed to vemurafenib (**Figure 3.6a**). This result supports our hypothesis that lactate is sufficient to drive histone Kla in cancer cells. Furthermore, these results suggest that lactate generation may be down stream of the MAPK pathway. Therefore, the MAPK pathway is sufficient, but not necessary for histone Kla.

We previously demonstrated that glycolysis is necessary for histone Kla in cancer cells, which is independent of active MAPK signaling. To test whether ectopic lactate could drive histone Kla in cancer cells in a manner that occurs independently of active glycolytic activity, we attempted another restoration experiment. The two primary cellular fates for lactate in cells are considered to be1) its export from cell 2) its conversion into pyruvate by LDH. We previously

demonstrated that glucose is necessary for maintaining histone Kla in cancer cells. Therefore, we hypothesized that lactate could directly contribute to histone Kla levels in a manner that occurs independently of glycolysis. To explore this possibility, we used combinations of glycolytic perturbations to functionally limit glycolytic and gluconeogenic flux, and then determined whether ectopic lactate could directly induce histone Kla levels. Note, MAPK signaling was not perturbed as a result of any of the tested glycolytic perturbations (**Figure 3.7a**). Consistent with previous results, glucose starving A375 cells resulted in a decrease in histone Kla levels (**Figure 3.7a**). Likewise, exposing A375 cells to pan-LDH inhibitor, oxamate, resulted in decreased histone Kla levels. Strikingly, cells that were cultured in glucose deprived media containing exogenous lactate and oxamate had fully restored histone Kla levels (**Figure 3.7a**). These results indicate that lactate can drive histone Kla in a manner that is independent of active glycolysis. Therefore, this finding highly suggests that lactate is a direct and primary precursor metabolite that is responsible for histone Kla.

Collectively, these data show that the MAPK signaling pathway regulates histone Kla and lactate levels in cancer cells. We found that the MAPK regulates the level of lactate in cells through activating the Warburg effect. We determined that lactate that is generated by the Warburg effect is then used as a precursor metabolite for histone Kla in cancer cells. We have elucidated a novel cellular regulatory axis between the MAPK signaling pathway, glycolysis, and histone Kla. This axis is particularly interesting because it describes the paradigm where the flow of information moves from a central cell signaling pathway into a metabolic pathway that is transduced into an epigenetic pathway.

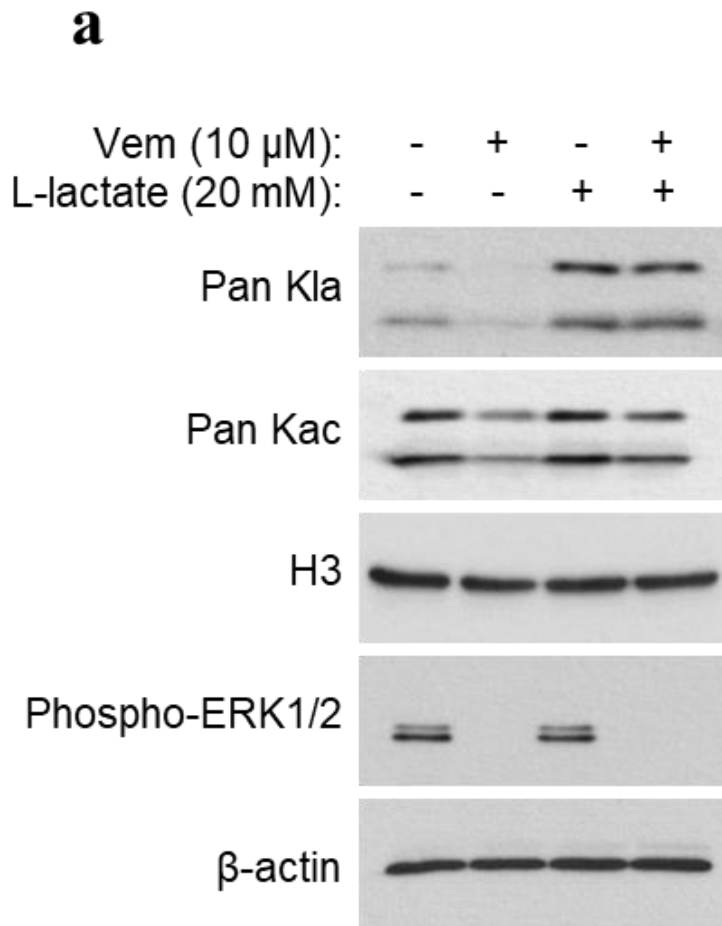


Figure 3.6 Lactate drives histone lysine lactylation independently of active MAPK signaling.
Ectopic lactate can restore the diminished histone Kla levels caused by MAPK perturbation. (a) A375 cells were exposed to the following conditions: DMSO, 10 µM of vemurafenib, 20 mM of ectopic lactate, or 10 µM of vemurafenib and 20 mM of lactate. All tests were terminated 24 hours post-exposure and prepared for western blot analysis. Western blot results generated from the use of the indicated antibody (left of gel panel). Primary antibody incubated overnight at a working concentration of 1 µg/ µL.

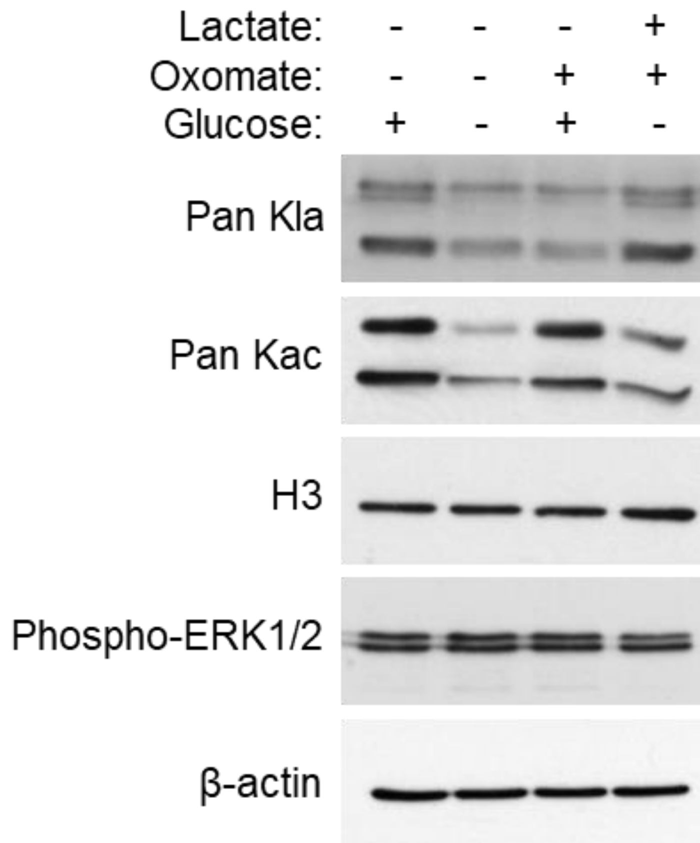


Figure 3.7 Lactate drives histone lysine lactylation independently of active glycolysis. Ectopic lactate can restore the diminished histone Kla levels caused perturbed glycolysis. (a) A375 cells were exposed to the following conditions: No treatment, 25 mM glucose, 20 mM Oxo and 25 mM glucose, and 20 mM Oxo and 20 mM lactate. All tests were terminated 24 hours post-exposure and prepared for western blot analysis. Western blot results generated from the use of the indicated antibody (left of gel panel). Primary antibody incubated overnight at a working concentration of 1 μg/ μL.

3.3 Discussion

In Chapter 3, we mechanistically dissect the principal components that regulate histone Kla levels in cancer cells. We determine that in addition to histone Kla levels, the MAPK signaling pathway regulates intracellular lactate levels. Furthermore, using drugs to inhibit oncogenic BRAF or KRAS results in decreased intracellular lactate, highlighting that this mechanism is likely to be involved in both homeostatic and disease associated processes. Having determined that the MAPK could promote lactate generation in cells, we next determined that glycolysis is essential for sustaining oncogenic histone Kla levels. Using a variety of methods to perturb upstream and downstream glycolysis, we determined that any perturbation to glycolysis can disrupt oncogenic histone Kla levels. Inhibiting PDK1 with DCA yielded particularly intriguing results that allowed us to conclude that the final step of glycolysis may be essential for oncogenic histone Kla levels, e.g. the production of lactate. Alas, we determined that exposing cancer cells to exogenous lactate circumvented the loss of histone Kla that was associated with either perturbed MAPK signaling or perturbed glycolysis. Therefore, lactate was sufficient to rescue the loss of histone Kla in the conditions we tested. This observation suggests strongly that lactate is the key precursor metabolite that drives oncogenic histone levels. These collective observations indicate that oncogenic MAPK signaling can drive histone lysine lactylation via Warburg-derived lactate. This signaling axis is particularly novel because it describes a mechanism where the flow of information moves through a well characterized signaling pathway into a metabolic pathway that eventually moves into an epigenetic pathway.

3.4 Methods

Reagents

The pan-Kla antibody (PTM-1401) was developed by PTM Biolabs (Chicago, IL). pan Kac antibody (PTM-101) was from PTM Biolabs (Chicago, IL). The anti-H3 (#4499), anti-ERK1/2 (#4695), anti-phospho-ERK1/2 (#9101) antibodies were obtained from Cell Signaling Technology. The anti-p21 (#2974), anti-c-Myc, anti-TXNIP (#18583), and anti-HK2 (#2867) were all obtained from Cell Signaling Technology. All drugs used in this study were acquired from SelleckChem. Cell lines used were purchased from ATCC and used without further authentication.

Cell culture

Cells were obtained from ATCC and used without further authentication. Cells were grown in high glucose DMEM (Thermo Fisher Scientific) supplemented with 10% FBS at 37°C in 5% CO_2. High glucose DMEM was used in FBS dosing experiments. 10% FBS was used in low glucose experiments.

Drug treatments

All drugs used in this study were resuspended according to the suppliers' instructions (SelleckChem) -20 degrees Celsius until use. Cells were drugged from approximately 24 hours before harvesting for western blot or lactate analysis.

Western blot analysis

Cells were washed 3 times in 1x phosphate buffered saline (PBS). Cells were harvested in 2% SDS lysis buffer (2% SDS, 60 mM Tris, 10% glycerol, 5% beta-mercaptoethanol) and incubated for 5 minutes at 100 *C. Protein lysate concentration was determined by Bradford assay. Protein lysates were resolved on 15% SDS polyacrylamide gels were us and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 3% bovine serum albumin (BSA) re-suspended in 1x tris buffered saline+tween (TBS-T: 20 mM Tris, 150 mM

NaCl, 0.05% Tween 20) for 1 hour at room temperature, with the exception for H3 antibody, which was blocked in 3% non-fat milk buffer (3% dry non-fat milk in 1x TBS-T). Membranes were incubated in primary antibody overnight at 4*C. Membranes were washed 5 times in TBS-T for 5 minutes, followed by 1 hour incubation in secondary antibody at room temperature. Membranes were washed as preciously described and probed using Pierce ECL Chemiluminescent Western Blot Detection kit (ThermoFisher, 32209).

Lactate assay measurement

To measure intracellular lactate, we used the Biovision Lactate Colorimetric Assay Kit II (Catalog #K627-100). All experiments were performed in accordance to the manufacturers' protocol. Biological triplicates were measured using three technical replicates. Cells were washed three times in cold PBS and harvested in Lactate Assay Buffer. For n number of samples a master mix was prepared for n*1 by combining 46 uL of Lactate Assay Buffer with 2 uL of Lactate Substrate Mix and 2 uL of Lactate Enzyme Mix. 2 uL of test lysate was added to to 48 uL of Lactate Assay Buffer and added to the well of 96 well plate. 50 uL of master mix was added to the lysate in the 96 well, bring the final reaction volume to 100 uL. The plate was protected from light and incubated at room temperature for 30 minutes before measuring.

CHAPTER 4

MOF IS A NOVEL LACTYLTRANSFERASE

4.1 Introduction

The reversible nature of protein phosphorylation, acetylation, and many other types of protein PTMs is essential for diverse forms of cellular signaling. In many cases, the reversibility of protein PTMs is orchestrated by enzymes that can either install or remove them[98]. In the case of protein phosphorylation, kinases are known to install the phosphoryl group to select residues on proteins, while phosphatases are enzymes that are known to remove these phosphoryl groups[107,108,237,238]. Similar, KATs are enzymes that are known to install acetyl groups to lysine side chains, while KDACs are enzymes known to remove these acetyl groups[98,239]. In Chapter 1, we reviewed the ever-expanding universe of acyltransferases: KATs that can install more than just acetyl groups to lysine side chains[240]. In the case of lysine lactylation (Kla), it was reported that Kla can be added to histone proteins by the well-known acyltransferase, p300[30,241]. In this reaction, p300 was shown to lactylate a variety of lysine residues on histone proteins by using the co-factor, lactyl-CoA[30,242]. Lysine lactylation of histone proteins by p300 was shown to directly activate gene transcription from an in vitro chromatin template[30]. This observation was consistent with observations made in vivo where lactylated genes that are identified by ChIP-Seq correlate with elevated mRNA levels identified in paired RNA-Seq data[32]. These two pieces of evidence suggest that Kla can directly activate gene transcription, and that this process is likely to occur in vivo. However, the finding that p300 can possess lactyltransferase ability is important to claim that Kla is enzyme catalyzed, this enzyme was likely chosen to prove this concept due to the fact that p300 is known to exhibit a significant degree of ‘promiscuity’[243]. Promiscuity refers to an enzyme’s ability to non-selectively catalyze the addition or removal of a given chemical

modification. Indeed, p300 has been observed to catalyze a number of chemically distinct modifications across a variety of different substrates. To date, p300 has been reported to install Kac, Kpr, Kbu, Kcr, Kbhb, Khib, and Kla marks on histone proteins and non-histone proteins[30,244–248]. While some degree of promiscuity can be observed in other KATs, none have been identified that rival the robust acyltransferase ability of p300. To this end, no other enzyme has been reported to possess lactyltransferase activity at this time.

In an in vitro screen to identify novel lactyltransferases, we observed that MOF1 could exhibit lactyltransferase activity. Like other members of the MYST family, MOF exists exclusively within a protein complex in cells[73,79]. Beyond histone proteins, acetylation has been shown to regulate the activity of a number of nuclear regulatory proteins, including the activity of several KATs[249,250]. MOF is one such enzyme that can itself be acetylated, and MOF acetylation has been demonstrated to significantly impact its function[72]. Liang and colleagues were the first demonstrate that MOF could acetylate itself (autoacetylation) on K274[251]. In this report, they also determine that MOF K274ac could be removed by SIRT1[251]. In this same report, Liang and colleagues claim that K274ac induces a loss in MOF chromatin occupancy and a reduction in its main substrate, H4K16ac[251]. A few months after Liang and colleagues initial report, Marmorstien, Côté, and colleagues published a similar report that confirmed MOF K274ac autoacetylation[252]. However, they find that MOF K274ac increases H4K16ac in cells. They determine the structure of MOF and find that in the unacetylated state, K274 engages with the catalytic glutamine residue, thereby causing partial occupancy of the catalytic binding pocket[252]. This partial occupancy observed in MOFs unacetylated state is thought to dramatically reduce the enzyme’s ability to engage with its cognate substrate[252]. After these two-initial

reports, several reports from independent groups confirmed that MOF 274Kac autoacetylation can activate MOF enzymatic activity[253].

We will demonstrate that MOF1 can possess lactyltransferase activity in vitro and in vivo. We quantitatively characterize the MOF1 regulated histone lactylome by using SILAC and proteomics. Finally, we use molecular simulations to generate models that can provide biochemical clues regarding how the CoA binding pocket can accommodate lactyl-CoA and acetyl-CoA. To our knowledge, this is the first report of a lactyltransferase that is not p300.

4.2 Results

4.2.1 MOF is a lactyltransferase in vitro and in vivo

KATs require the use of a high energy co-factor (CoA) to facilitate the enzymatic installation of an acyl species to the side chain of lysine. However, as we reviewed in Chapter 4.1, the presence of a co-factor is not always sufficient to facilitate the enzymatic activity of KATs. As is the case for the majority of MYST family KATs, MOF exists exclusively in a protein complex. In addition to this requisite condition, MOF requires the autoacetylation of K274 to enable cognate substrate recognition[253].

With these considerations in mind, we developed two plasmids to evaluate whether MOF could function as a lactyltransferase in vitro: Flag-tagged MOF plasmid (Flag-MOF) and a Flag-tagged MOF K274R plasmid (Flag-MOF K274R). Flag was selected due to its small nature and so that MOF could be purified from human cellular extract, thereby preserving its native complexes. The K274R mutation was utilized to mimic and preserve an unmodified lysine state, thereby resulting in an inactivated MOF. Using purified native MOF and MOF K274R, we performed a series of biochemical reactions to determine whether MOF could possess

lactyltransferase activity. The reactions we tested consisted of differing combinations of Flag-MOF, Flag-MOF K274R, lactyl-CoA, and purified core histone proteins.

As expected, incubating lactyl-CoA and Flag-MOF without histone proteins results in zero detection of lactyltaion on histone proteins (**Figure 4.1a**). The same is true for this reaction when Flag-MOF K274R is used. These controls demonstrate the purity of the biochemical reactions that were conducted. In addition, purified core histone proteins that were incubated with Flag-MOF where lactyl-CoA was absent saw little to no change in histone Kla signal (**Figure 4.1a**). Many acyl-CoA species have been shown to non-enzymatically react with lysine residues that are present on proteins[254,255]. To control for these phenomena, we incubated lactyl-CoA with purified core histone proteins. We observed that lactyl-CoA could non-enzymatically react with histones (**Figure 4.1a**). However, when we incubated lactyl-CoA and purified core histone proteins with purified Flag-MOF, we observed significantly greater Kla signal compared to the non-enzymatic lactylation that we observed between lactyl-CoA and purified core histone proteins (**Figure 4.1a**). To further confirm that the elevated histone Kla signal that we observed by adding Flag-MOF to lactyl-CoA and purified core histone proteins, we performed the same reaction using Flag-MOF K274R. MOF K274R cannot be autoacetylated and is therefore trapped in a perpetual state of catalytic inactivity, e.g. catalytically dead[253]. Adding purified Flag-MOF K274R to lactyl-CoA and purified core histones resulted in roughly the same amount of Kla observed in the reaction between only lactyl-CoA and purified histones (**Figure 4.1a**). This observation strongly suggests that the increased histone Kla observed in the reaction between, Flag-MOF, lactyl-CoA, and purified core histones, is enzyme catalyzed. Therefore, these data strongly support our hypothesis that MOF can possess novel lactyltransferase activity and uses lactyl-CoA as a co-factor to generate Kla on histone proteins.

Should MOF be a lactyltrasnferase in cells, we hypothesized that its loss in cells would result in a reduction in histone Kla abundance. To test this hypothesis, we performed shRNA knockdown experiments followed by assessing the levels of Kla by western blot analysis. Using HEK 293 cells, we generated a stable MOF knockdown cell line using lentivirus that expresses shRNA against MOF. shMOF expressing HEK 293 cells experienced a dramatic loss of endogenous MOF, as assessed by western blot analysis (**Figure 4.2a**). Interestingly, we observed little to no change in histone Kac levels in the shMOF cells. However, the shMOF cells had an observable reduction in histone Kla when compared to the wildtype MOF cells (shNT) **Figure 4.2a**. This observation suggests that MOF can contribute to maintaining histone Kla levels in cells. Furthermore, this observation supports our hypothesis that MOF is lactyltransferase in cells.

Applying the same logic as before, should MOF be a lactyltranserase in cells, we hypothesized that greater MOF protein abundance in cells would result in an increase in histone Kla abundance. To test this hypothesis, we overexpressed Flag-MOF in human cells to examine its impact on the levels of histone Kla. Flag-MOF overexpression in cells was validated by western blot analysis using an anti-Flag antibody (**Figure 4.2b**). We observed an increase in histone Kla levels in Flag-MOF overexpressing cells relative to the empty vector control group. Consistent with our previous results and with previous reports, exposing these cells to exogenous lactate resulted in greater histone Kla levels relative to control (**Figure 4.2b**). We next exposed Flag-MOF overexpressing cells to exogenous lactate and observed an even greater increase in histone Kla levels relative to Flag-MOF overexpression alone or lactate exposure alone (**Figure 4.2b**). In conclusion, these results support our hypothesis that overexpressing MOF will result in greater histone Kla levels. Furthermore, adding exogenous lactate to MOF overexpressing cells further boosts histone Kla levels, which has implications that we will discuss in discuss in Chapter 4.3.

Taken together, these data strongly support our hypothesis that MOF is a novel lysine lactyltransferase both *in vitro* and *in vivo*. Given that the overexpression of MOF resulted in a significant increase in histone Kla levels in cells, we next sought to quantitatively characterize MOF lactyltransferase activity in cells using proteomics to identify and quantify MOF regulated lactyllysine substrates on histone proteins.

4.2.2 Comprehensive characterization of MOF regulated histone Kla

We have demonstrated that MOF can possess lactyltransferase both *in vitro*. MOF can lactylate lysine residues on histone proteins using lactyl-CoA as a co-factor. We further demonstrated that loss of MOF in cells results in lower histone Kla levels relative to wildtype. Alas, we show that overexpression of MOF in cells can substantially increase histone Kla levels relative to wildtype. Taken together, these pieces of evidence support the claim that MOF is a novel lactyltransferase.

We therefore sought to expand upon the novel lactyltransferase activity of MOF by comprehensively characterizing its lactyltransferase activity in cells. We chose to only focus on the lactylation of histone proteins by MOF. Our comprehensive characterization of MOF regulated histone Kla was accomplished using proteomic strategy that leverages high performance liquid chromatography coupled mass spectrometry. This technique allowed us to both identify and quantify MOF regulated Kla sites on histone proteins.

We initiated this experiment by culturing HEK 293T cells in media that contained either 'heavy' lysine ($^{13}C_6{}^{14}N_2$-L-Lysine) or 'light' lysine ($^{12}C_6{}^{14}N_2$-L-Lysine). These cells were cultured in their respective heavy or light medias for 8 generations[221,256]. We overexpressed Flag-MOF in the light conditioned cells and empty vector in the heavy cells, and the cells were allowed to culture for an additional 48 hours. Histone extracts from MOF overexpressing cells

and from empty-vector control cells were harvested, measured, and pooled together at equal concentrations. The combined peptide pool was trypsinized into proteolytic peptides and resultant peptides were subjected to peptide immunoprecipitation using the pan-Kla antibody. The enriched peptides were analyzed by HPLC-MS/MS.

We identified a total of 16 Kla sites on core histone proteins (**Figure 4.3a**). Of the core histone subunits, we identified the most sites on histone H3. On histone H3, H3K27la was the most upregulated residue that was regulated by MOF, experiencing roughly a 4 -fold change relative to empty vector control. Histone H3K23la was the next most upregulated Kla site that was enhanced by MOF, observing a slightly greater than 2 -fold more Kla abundance relative to empty (**Figure 4.3a**).

To our surprise, many of the canonical MOF regulated Kac sites were not recapitulated in terms of Kla. We detected 4 Kla sites on histone H4: K5, K8, K12, K16. MOF is best known for its propensity to acetylate H4K16 during sex linked dosage compensation[74]. Curiously, H4K16la experienced the least dramatic change relative to the other histone H4 sites, exhibiting roughly no change relative to empty vector control (**Figure 4.3a**). H4K8 and H4K12, which have also been reported to be MOF Kac sites, were the most dynamically changed histone H4 sites, with both just shy of increasing by 2 -fold relative to empty vector control.

Very little change was observed in H2AKla sites between MOF overexpressing and empty vector control (**Figure 4.3a**). However, H2B experienced some dramatic changes in Kla abundance due to MOF. In particular, H2BK5la and K16la experienced the most dynamic change, with K5 increasing by 2 -fold and K16 increasing by roughly 2.5 fold, respectively. H2B K20la also increased by approximately 2 -fold in response to MOF overexpression.

These data provide a quantitative overview of the MOF regulated histone lysine lactylome. We discover that the histone lysine lactylome is quantitatively distinct from the MOF regulated histone lysine acetylome. Curiously, we find that MOF regulated histone lysine lactylation sites share considerable overlap with MOF regulated histone lysine crotonylation sites[96]. This overlap could suggest that MOF acyltransferase substrate preference is distinct from its acetyltransferase substrates.

4.2.3 Biochemical modeling of MOF acyl-CoA interaction

After initially determining that MOF is a lactyltransferase both *in vitro* and *in vivo*, we characterized the MOF regulated histone lysine lactylome using quantitative proteomics to identify and quantify putative MOF histone Kla substrates. Given the distinct structural differences that exist between acetyl-CoA, crotonyl-CoA, and lactyl-CoA, we used modeling to investigate the potential biochemical binding mechanism present in MOF that enables its lactyltransferase ability[242].

We find that the MOF binding pocket could accommodate the extended chain length of lactyl-CoA (**Figure 4.4a**). Beyond merely steric hinderance resulting from size, a unique feature present in the CoA binding region of MOF was the hydrophobic pocket that can be formed. Curiously, this hydrophobic pocket is made available to certain CoAs based on their size. In this case, the lactyl moiety of lactyl-CoA could be stably held in a hydrophobic pocket formed by Val141, Ala142, Pro176, Pro179, and Leu180 (**Figure 4.4a**). One feature that makes lactyl distinct from acetyl and likely crotonyl is the hydroxy group present in lactyl. The hydroxy group of the lactyl moiety can form a hydrogen bonding interaction with the backbone nitrogen atom of Ile144, which we suspect is a critical chemical position that coordinates the molecular recognition of lactyl-CoA by MOF (**Figure 4.4a**).

The discovery of MOF as a lactyltransferase adds to the growing body of evidence that suggests various KATs can bind non-acetyl acyl-CoA species, which subsequently imbues them with acyltransferase activity. Several reports now exist that detail biochemical mechanisms that enable the selective utilization of distinct acyl-CoA by specific KATs.

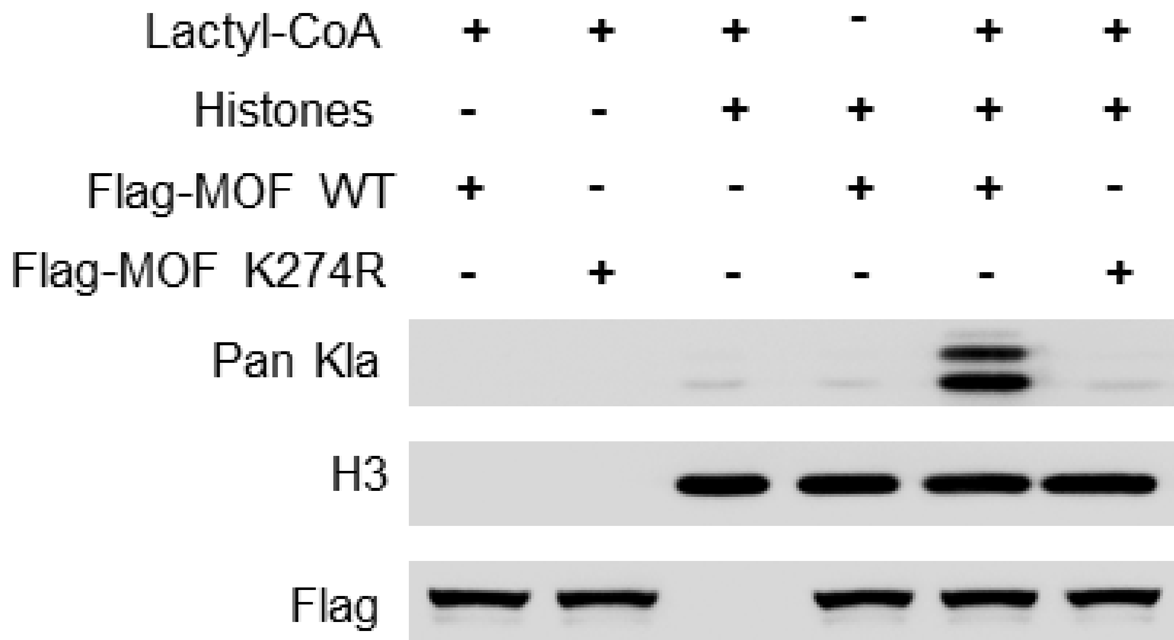


Figure 4.1 MOF can enzymatically lactylate histone proteins in vitro.
MOF can enzymatically lactylate histone proteins in vitro using lactyl-CoA as a co-factor. (a) Flag-MOF or Flag-MOF K274R were immuno-precipitated and purified from HEK 293 cells. Reaction components for each reaction are indicated by a plus symbol (+). For any given component that was used in a reaction, the concentration of said component is as follows: 10 μM lactyl-CoA, 1 μg Flag-MOF, 1 μg Flag MOF K274R, and 2 μg of purified histone. Reactions were terminated after 30 minutes post assembly by the addition of protein loading buffer. Western blot results generated from the use of the indicated antibody (left of gel panel). Primary antibody incubated overnight at a working concentration of 1 μg/ μL.

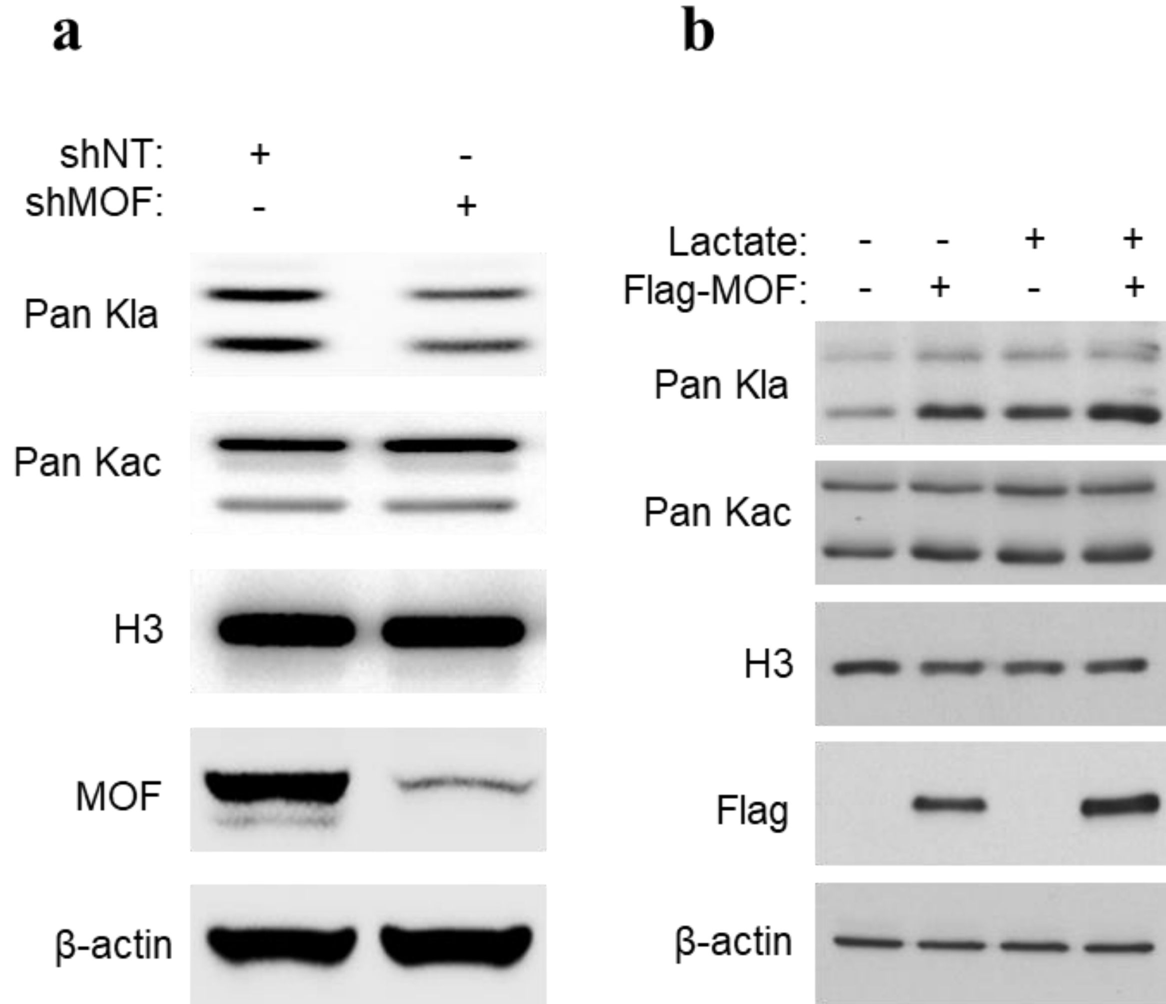


Figure 4.2 MOF regulates histone lysine lactylation levels in cells.
Loss of MOF in cells results in reduced histone Kla levels. (a) Parental HEK 293 cells and shMOF HEK 293 cells were cultured to 80% confluency and harvested for western blot analysis. (b) HEK 293 cells were transfected with plasmid containing containing no gene in expression cassette or with plasmid containing Flag MOF in the expression cassette. Cells were harvested 48 hours after plasmid transfection for western blot analysis. (a, b) Western blot results generated from the use of the indicated antibody (left of gel panel). Primary antibody incubated overnight at a working concentration of 1 μg/ μL.

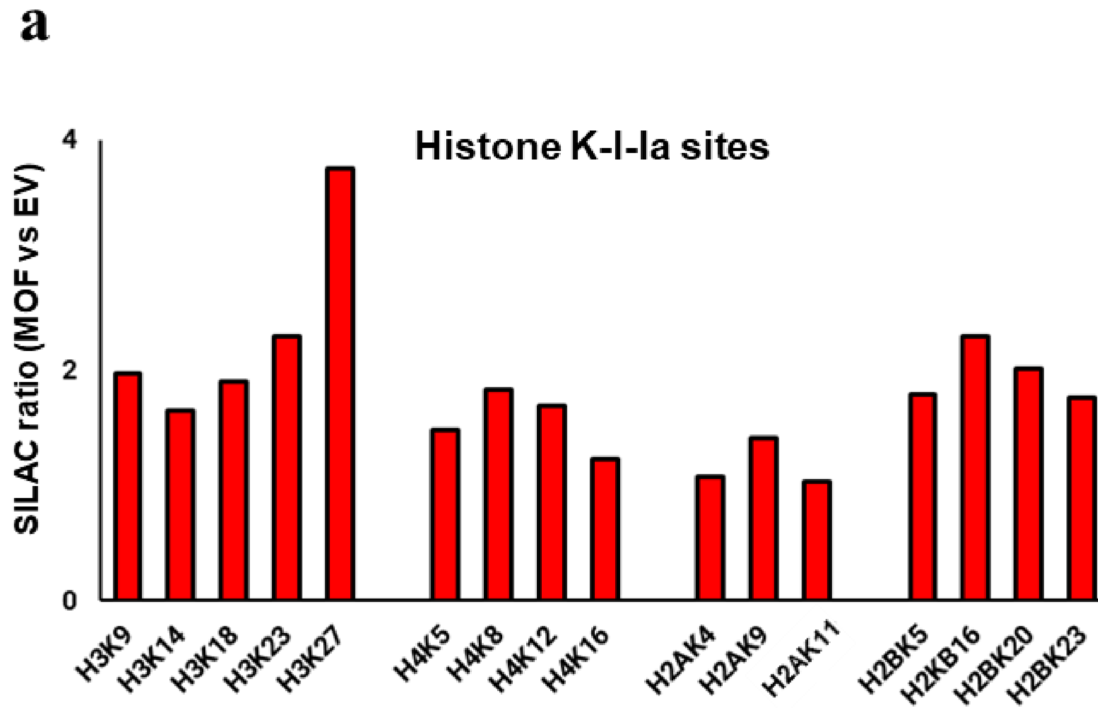


Figure 4.3 MOF Proteomic characterization of MOF-regulated histone lysine lactylation. Proteomic characterization of MOF regulated lysine lactylation. (a) HEK 293 cells were transfected with plasmid containing no gene in expression cassette or with plasmid containing Flag MOF in the expression cassette. SILAC ratio for identified histone Kla sites were calculated using Perseus.

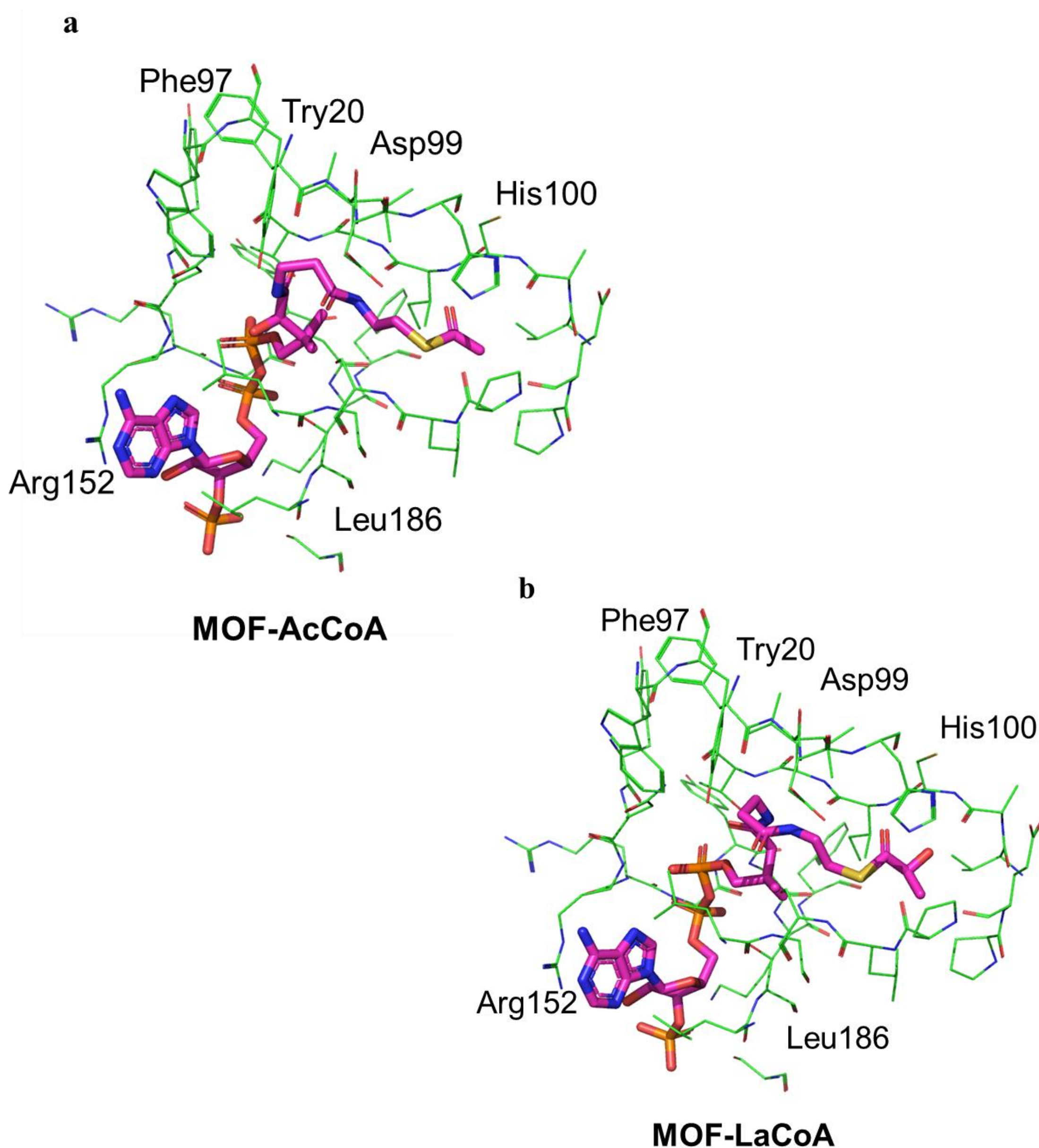


Figure 4.4 Molecular modeling of MOF in complex with acetyl-CoA and lactyl-CoA. Modeling of MOF mechanism of molecular recognition of acetyl-CoA and lactyl-CoA. (a) Ball and stick model of MYST CoA binding domain from MOF in complex with acetyl-CoA. (b) Ball and stick model of MYST CoA binding domain from MOF in complex with lactyl-CoA. (c) Model of MOF in complex with acetyl-CoA using electrostatic surface potential landscape. (d) Model of MOF in complex with lactyl-CoA using electrostatic surface potential landscape. (e) Molecular docking simulations were performed using AutoDock 4.2.6 software equipped with AutoDock Tools. The following parameters were used: 100 docking trials, population size of 150, mutation rate of 0.02, crossover rate of 0.8, local search rate of 0.06, and 25 million energy evaluations

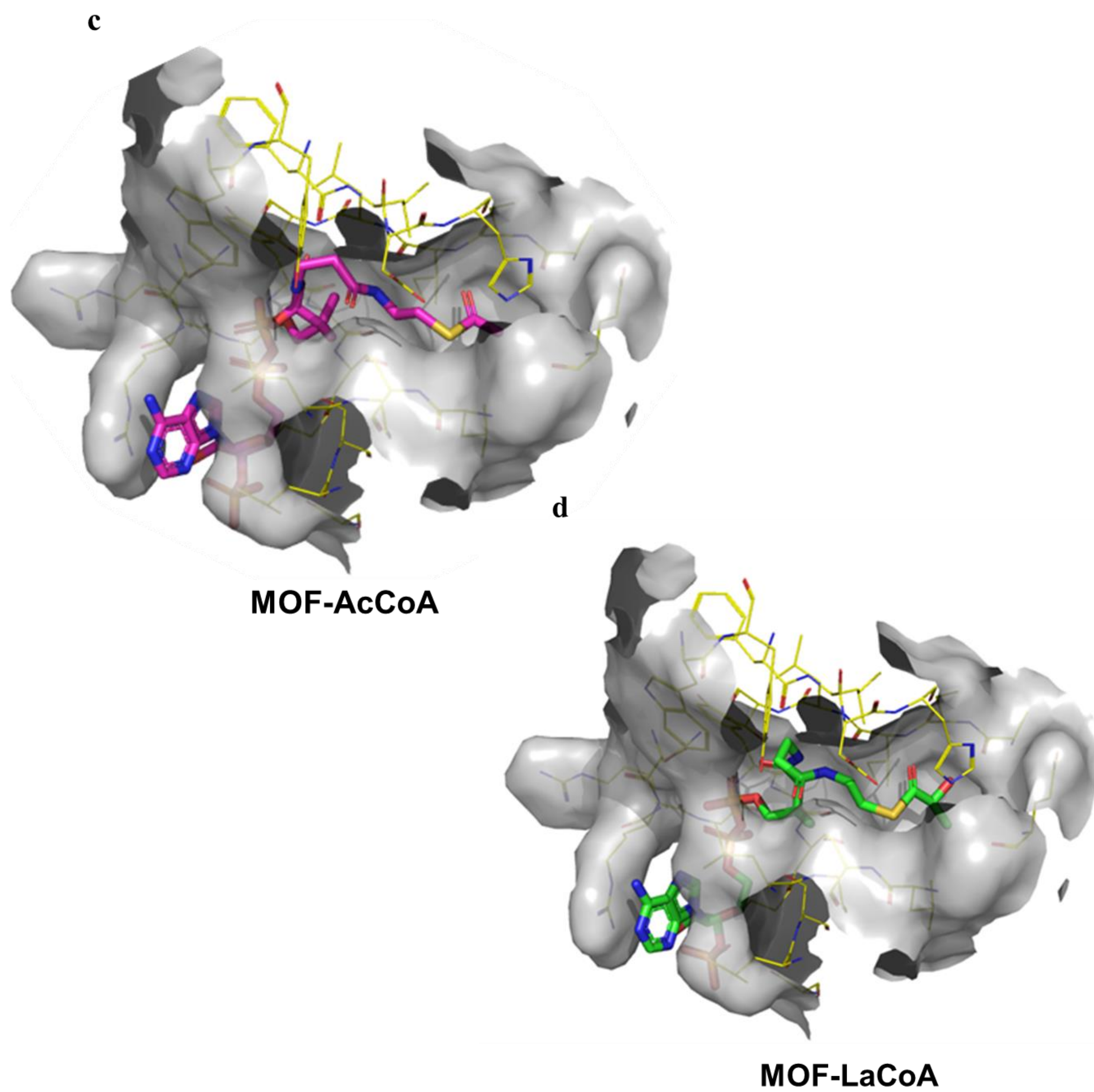


Figure 4.4, continued.

4.3 Discussion

In Chapter 4, we reported the novel discovery that MOF can possess significant lactyltransferase activity. We demonstrated that MOF possesses lactyltransfersae activity in vitro and by using catalytically dead MOF, we further demonstrated that the catalytic activity of MOF is necessary for its lactyltransferase activity. We show that reducing MOF in cells results in a concomitant reduction in histone Kla levels, suggesting that MOF establishes basal histone Kla level in cells. Conversely, we show that increasing MOF levels in cells results in a concomitant increase in histone Kla levels. We therefore characterized putative MOF regulated lysine lactylation sites on histone proteins. We determined that MOF histone Kla substrates differ from MOF Kac substrates. Lastly, we modeled the molecular binding of lactyl-CoA and MOF to determine the mechanism of molecular recognition that is afforded to MOF via its protein structure.

The discovery that MOF is a lactyltransferase adds both to MOFs repertoire to acylate lysines and adds to the number of previously annotate KATs that are capable of acylating proteins[41,96,97]. Several reports now exist that provide evidence for novel biochemical mechanisms that exist to enable the selective utilization of distinct acyl-CoA by specific HATs. One such example stems from the work of Lu and colleagues, KAT2A was discovered in a unique complex that contains the metabolic enzyme, α-KGDH[257]. In this complex, α-KGDH is thought to generate succinyl-CoA at local concentrations that push the reaction kinetics of KAT2A to a state where, instead of using acetyl-CoA, succinyl-CoA is utilized as a co-factor to succinylate lysine on histone H3[257,258]. These data were some of the first evidence of a model that had been used to rationalize a scenario in which a KAT would selectively utilize one acyl-CoA species over acetyl-CoA[43,51]. Whether lactate can be generated within a supramolecular

protein complex that also contains a KAT is not known. However, LDH is known to exist in the nucleus at levels that exceed those of α-KGDH. Nevertheless, lactate exists at concentrations in cells that are comparable to those of acetate and pyruvate.

4.4 Methods

Reagents

Antibodies and cell lines

The antibodies used here were anti-Pan Kac (PTM Biolabs, PTM-101), anti-Pan K(L-la) (PTM Biolabs, PTM-1401), anti-H3 (Cell Signaling Technology, #4499) and anti-Flag (Cell Signaling Technology, #2368), . Cell lines were purchased from ATCC (www.atcc.org) and used without further authentication. Mycoplasma contamination was routinely tested using a MycoAlert Mycoplasma Detection Kit (Lonza, LT07-118).

Cell culture & stable isotope labeling of cells

Cells were grown in lysine-free DMEM (ThermoFisher, #88364) supplemented with 10% dialyzed FBS, and either light (12C614N2-L-Lysine, Cambridge Isotope Laboratories, #ULM-8766PK) or heavy (13C614N2-L-Lysine, Cambridge Isotope Laboratories, #ULM-291-H-PK) lysine (100 mg/L). Cells were grown for more than seven generations to achieve more than 98% labeling efficiency. All cell lines were cultured using ATCC recommended media in a 5% CO2 atmosphere at 37 °C. Labeling efficiency was tested before experimental use of cell lines.

Preparation of cell lysate

Cells were sonicated for 3 min on ice using a high-intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea, 2 mM EDTA, 3 μM TSA, 50 mM NAM, 5 mM DTT and 1% Protease Inhibitor Cocktail III). The remaining debris was removed by centrifugation at 18 000× g at 4 oC

for 3 min. The protein concentration was determined using Bradford assay according to the manufacturers protocol (Biorad #5000205).

Peptide immunoprecipitation

Histone proteins were extracted from human cells using a standard acid-extraction protocol, and subjected to trypsin digestion as per the manufacturer's instructions. Pan anti-K(L-la) (PTM-1401, PTM Bio Inc.) or pan anti-Kac antibodies (PTM-101, PTM Bio Inc.) were first conjugated to Protein A Sepharose beads (GE Healthcare BioSciences) and then incubated with tryptically digested histone peptides with gentle agitation overnight at 4 °C. The beads were then washed three times with NETN buffer (50 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40), twice with ETN buffer (50 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA) and once with water. Peptides were eluted from the beads with 0.1% TFA and dried in a SpeedVac system (ThermoFisher Scientific).

HPLC–MS/MS analysis & SILAC quantification

Tryptic peptides were loaded onto a homemade capillary column (10 cm length × 75 mm ID, 3 μm particle size) connected to an EASY-nLC 1000 system (Thermo Fisher Scientific). Peptides were separated and eluted with a gradient of 2% to 90% HPLC buffer B (0.1% formic acid in acetonitrile, v/v) in buffer A (0.1% formic acid in water, v/v) at a flow rate of 200 nl min−1 over 60 min. The eluted peptides were then ionized and analyzed by a Exploris mass spectrometer (ThermoFisher Scientific). Full mass spectrometry was acquired in the orbitrap mass analyzer over the range m/z 300 to 1,400 with a resolution of 70,000 at m/z 200. The 12 most intense ions with charge ≥2 were fragmented with normalized collision energy of 27 and tandem mass spectra were acquired with a mass resolution of 17,500 at m/z 200. Qunatification of histone K(L-la) and Kac sites was performed using Maxquant40. Heavy/light PTM peptide ratios that were

calculated by Maxquant were normalized to heavy/light ratios of their unmodified peptide counter parts.

Western Blot analysis

Cells were washed 3 times in 1x phosphate buffered saline (PBS). Cells were harvested in 2% SDS lysis buffer (2% SDS, 60 mM Tris, 10% glycerol, 5% beta-mercaptoethanol) and incubated for 5 minutes at 100 *C. Protein lysate concentration was determined by Bradford assay. Protein lysates were resolved on 15% SDS polyacrylamide gels were us and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 3% bovine serum albumin (BSA) re-suspended in 1x tris buffered saline+tween (TBS-T: 20 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 1 hour at room temperature, with the exception for H3 antibody, which was blocked in 3% non-fat milk buffer (3% dry non-fat milk in 1x TBS-T). Membranes were incubated in primary antibody overnight at 4*C. Membranes were washed 5 times in TBS-T for 5 minutes, followed by 1 hour incubation in secondary antibody at room temperature. Membranes were washed as preciously described and probed using Pierce ECL Chemiluminescent Western Blot Detection kit (ThermoFisher, 32209). For whole l-lactylome western blot analysis, a 10% SDS polyacrylamide gel was used.

In vitro lactylation assay

Flag-MOF and histones were purified from HEK 293T cells. For each reaction, 2 μg of purified core histone proteins histones, 10 μM of lactyl-CoA, with or without purified Flag-MOF were added in the reaction buffer (50 mM Tris-Cl, pH = 8.0, 10% glycerol, 10 mM butyric acid, 0.1 mM EDTA, 1 mM DTT and 1 mM PMSF). The reaction mixture was incubated for 30 min at room temperature, then protein loading buffer was added to stop the reaction. Samples were

heated at 95 DEGREE C for 10 min. Immunoblotting was carried out to measure the histone lactylation signals.

Molecular Docking

The crystal structure of MOF (aa 174-449) complexed with acetyl-CoA (PDB ID: 2GIV) was used to construct a molecular docking model. Lactyl-CoA was built basing on the structure of acetyl-CoA. Molecular docking simulations were performed using AutoDock 4.2.6 software equipped with AutoDock Tools (Scripps Research Institute). Lamarckian genetic algorithm (LGA) was selected for ligand conformational searching. For each ligand, the docking parameters were set as follows: trials of 100 dockings, population size of 150, mutation rate of 0.02, crossover rate of 0.8, local search rate of 0.06, and 25 million energy evaluations.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

In summary, we report the discovery of a novel physiological and pathological regulatory mechanism that can drive histone Kla in cells via growth factor signaling. We connect the MAPK signaling pathway to histone lysine lactylation, and demonstrate that the MAPK signaling pathway regulates histone Kla via promoting the Warburg effect. We demonstrate that lactate that is generated from the Warburg effect is necessary for promoting oncogenic histone Kla and that exogenous lactate is sufficient to restore histone Kla levels in cells that have perturbed MAPK signaling and in cells that have perturbed glycolytic activity. We use quantitative proteomics techniques to characterize the histone lysine lactylome as it is regulated by each member of the MAPK signaling pathway. Finally, we discovery the novel lactyltransferase activity of the MYST family member, MOF. We show that MOF requires K274 functionality to retain its lactyltransferase activity *in vitro*. One again using quantitative proteomics, we characterize the MOF regulated histone lysine lactylome. Finally, we use molecular modeling to predict the possible mechanism of molecular recognition between lactyl-CoA and MOF.

While our work answers several questions, it raises more questions that it seems to have answered. This admission has more to do with the incredibly comprehensive and deep body of knowledge that has been brought into existence before and while we carried out this work. For example, we find it particularly intriguing that histone Kla dynamics were shown to be differentially governed by distinct members of the MAPK pathway. One possibility that could explain this phenomenon is the inherent complexity that is known to be involved in MAPK signaling networks[117]. Many signaling networks are known to be activated by distinct members

of the MAPK pathway itself, such as the PI3K signaling pathway or PIN1/PDK1 pathway[259]. Whether the PI3K pathway can promote histone Kla has not been shown. However, given the mechanism that we use to describe how the MAPK regulates histone Kla, it is fair to suggest that any pathway that is capable of modulating the levels of intracellular lactate is then capable of modulating the levels of histone Kla[30]. Therefore, it is likely that other oncogenic pathways, and by contrast, other types of anti-cancer therapies, can directly impact the levels of histone Kla. Looking beyond disease for a moment, a number of cellular processes require coordinated adjustments to cellular metabolism. Coordinated changes in cellular metabolism can allow cells to generate process-specific biomaterials and pay for the energy costs associated with their generation. For example, the cell cycle has been shown to coordinate short term changes in cell metabolism that involves lactate generation[260]. This particular example is tantalizing, as it suggests that histone Kla dynamics may be cell cycle regulated. We have already observed that other PTMs can be cell cycle regulated[109].

While lactate is central to the modulation of histone Kla in cells, the unique quantitative differences that we observed across histone Kla sites after inhibition of specific MAPK members suggest that histone Kla may be regulated in a non-stochastic manner. The discovery of MOF provided some evidence that Kla may be regulated in a non-stochastic manner.

Appreciating the abstract significance of our work for moment, the differential regulation of histone lysine lactylation by unique MAPK members offers a novel conceptual perspective wherein the flow of information is occurring across diverse and seemingly distant signaling paradigms. We see that the flow of information moves through a growth factor signaling cascade into cellular metabolism, and into epigenetics. While most experts would agree that information can traverse diverse signaling paradigms to achieve a specified outcome, the theoretical depth of

each of the defined biological processes that we examined can often mirage them to appear disparate and remote from the true interconnectedness that defines biology. In this way, our work and our findings highlight a pragmatic conceptual strategy that emphasizes the reactivity of a certain process in response to a perturbation or alternation in another distally associated and discrete process. Put differently, we measured the interconnected of several discrete and distally associated biological processes by perturbing one process and examining the other processes sensitivity to said perturbation.

Another unique observation that can be appreciate from our work is the intracellular role that lactate may play on tumor cell function, thus representing a novel cell autonomous epigenetic program driven by lactate. Mounting evidence suggests that excessive lactate that is generated by cancer cells is exported into the tumor microenvironment. Exported lactate has been shown to support pro-tumor functions of diverse cell types in the tumor[261,262]. For example, tumor associated lactate was shown to enhance tumor immune suppression from CD4 t-regulatory cells (Tregs) and dampening the anti-tumor responsiveness of CD8 tumor infiltrating leukocytes[214]. However, our work details a potential intracellular function for cancer associated lactate generation. While it is clear that histone Kla can be promoted by oncogenic mechanisms, the physiological consequences of histone Kla merits further investigation. Importantly, the compounds used to inhibit the MAPK in this study are actively used on patients in the clinic. Therefore, our data reveals a potentially unforeseen molecular consequence that various clinically relevant targeted therapies may have.

The discovery of MOF as a lactyltransferases raises more questions than it answers. The MOF K274R mutant is particularly intriguing. The K274R mutation essentially functions as an enzyme-dead form of MOF by disabling the MOF K274 autoacetylation which is required for its

acetyltransferase activity. In the initial report that described the autoacetylation of MOF at K274, Liang and colleagues also report that SIRT1 deacetylates this residue. A report published by Zhao and colleagues that focused on quantitatively mapping the SIRT1-regulated acetylome also confirmed that SIRT1 has significant deacetylation activity toward MOF K274ac[263]. We demonstrate that K274 is essential for MOFs lactyltransferasive activity. Whether MOF K274 was acetylated or acylated was not determined in this study. However, a recent report from Galligan and colleagues found that SIRT1 had little activity toward Kla[264]. Therefore, in a situation where K274 of MOF were lactylated, SIRT1 would not be able to restore MOF to an unmodified state[264]. It's intriguing to speculate the functional impacts that acylated K274 may have on MOF function. Furthermore, it is not known whether acylation of MOF on K274 can impact its acyl-CoA preference and utilization. Nevertheless, our discovery herein provides the rationale to explore the underlying mechanisms that govern MOF lactyltransferase activity.

REFERENCES:

1. Alberts, B. *et al. Molecular Biology of the Cell*. (Garland Science, 2002).
2. Smith, L. M. & Kelleher, N. L. Proteoform: a single term describing protein complexity. *Nat Methods* **10**, 186–187 (2013).
3. Piovesan, A. *et al.* Human protein-coding genes and gene feature statistics in 2019. *BMC Research Notes* **12**, 315 (2019).
4. Aebersold, R. *et al.* How many human proteoforms are there? *Nat Chem Biol* **14**, 206–214 (2018).
5. Walsh, C. T., Garneau-Tsodikova, S. & Gatto, G. J. Protein posttranslational modifications: the chemistry of proteome diversifications. *Angew Chem Int Ed Engl* **44**, 7342–7372 (2005).
6. Socolich, M. *et al.* Evolutionary information for specifying a protein fold. *Nature* **437**, 512–518 (2005).
7. Wilk-Blaszczak, M. Posttranslational modifications of proteins.
8. Barber, K. W. & Rinehart, J. The ABCs of PTMs. *Nat Chem Biol* **14**, 188–192 (2018).
9. Prabakaran, S., Lippens, G., Steen, H. & Gunawardena, J. Post-translational modification: nature's escape from genetic imprisonment and the basis for dynamic information encoding: Information encoding by post-translational modification. *WIREs Syst Biol Med* **4**, 565–583 (2012).
10. Xu, H. *et al.* PTMD: A Database of Human Disease-associated Post-translational Modifications. *Genomics Proteomics Bioinformatics* **16**, 244–251 (2018).
11. Datta, S. R. *et al.* Akt Phosphorylation of BAD Couples Survival Signals to the Cell-Intrinsic Death Machinery. *Cell* **91**, 231–241 (1997).

12. Cheung, P., Allis, C. D. & Sassone-Corsi, P. Signaling to Chromatin through Histone Modifications. *Cell* **103**, 263–271 (2000).

13. Sandman, K. & Reeve, J. N. Archaeal histones and the origin of the histone fold. *Current Opinion in Microbiology* **9**, 520–525 (2006).

14. Luger, K., Rechsteiner, T. J., Flaus, A. J., Waye, M. M. Y. & Richmond, T. J. Characterization of nucleosome core particles containing histone proteins made in bacteria11Edited by A. Klug. *Journal of Molecular Biology* **272**, 301–311 (1997).

15. Zhou, K., Gaullier, G. & Luger, K. Nucleosome structure and dynamics are coming of age. *Nat Struct Mol Biol* **26**, 3–13 (2019).

16. Mattiroli, F. *et al.* Structure of histone-based chromatin in Archaea. *Science* **357**, 609–612 (2017).

17. Liu, Y. *et al.* Virus-encoded histone doublets are essential and form nucleosome-like structures. *Cell* **184**, 4237-4250.e19 (2021).

18. Goldberg, A. D., Allis, C. D. & Bernstein, E. Epigenetics: A Landscape Takes Shape. *Cell* **128**, 635–638 (2007).

19. Nakamura, K. *et al.* H4K20me0 recognition by BRCA1–BARD1 directs homologous recombination to sister chromatids. *Nat Cell Biol* **21**, 311–318 (2019).

20. Paquin, K. L. & Howlett, N. G. The histone DNA repair code: H4K20me2 makes its mark. *Mol Cancer Res* **16**, 1335–1345 (2018).

21. Rea, S. *et al.* Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406**, 593–599 (2000).

22. Jenuwein, T. & Allis, C. D. Translating the Histone Code. *Science* **293**, 1074–1080 (2001).

23. Bloch, K. & Borek, E. Biological acetylation of natural amino acids. *J Biol Chem* **164**, 483 (1946).

24. Lysine Propionylation and Butyrylation Are Novel Post-translational Modifications in Histones* - Molecular & Cellular Proteomics. https://www.mcponline.org/article/S1535-9476(20)31724-2/fulltext.

25. Huang, H. *et al.* Lysine benzoylation is a histone mark regulated by SIRT2. *Nat Commun* **9**, 3374 (2018).

26. Dai, L. *et al.* Lysine 2-hydroxyisobutyrylation is a widely distributed active histone mark. *Nature Chemical Biology* **10**, 365–370 (2014).

27. Tan, M. *et al.* Lysine Glutarylation Is a Protein Posttranslational Modification Regulated by SIRT5. *Cell Metabolism* **19**, 605–617 (2014).

28. Tan, M. *et al.* Identification of 67 Histone Marks and Histone Lysine Crotonylation as a New Type of Histone Modification. *Cell* **146**, 1016–1028 (2011).

29. Delaney, K. A. Discovery and Validation of Methacryllysine Modification on Histone Proteins. 160 doi:10.6082/uchicago.2232.

30. Zhang, D. *et al.* Metabolic regulation of gene expression by histone lactylation. *Nature* **574**, 575–580 (2019).

31. Xie, Z. *et al.* Metabolic Regulation of Gene Expression by Histone Lysine β-hydroxybutyrylation. *Mol Cell* **62**, 194–206 (2016).

32. Zhang, D. *et al.* Metabolic regulation of gene expression by histone lactylation. *Nature* **574**, 575–580 (2019).

33. Luger, K. & Richmond, T. J. The histone tails of the nucleosome. *Current Opinion in Genetics & Development* **8**, 140–146 (1998).

34. Shechter, D., Dormann, H. L., Allis, C. D. & Hake, S. B. Extraction, purification and analysis of histones. *Nat Protoc* **2**, 1445–1457 (2007).

35. Gruppuso, P. A. *et al.* Stability of histone post-translational modifications in samples derived from liver tissue and primary hepatic cells. *PLoS One* **13**, e0203351 (2018).

36. Zhang, Z. *et al.* Identification of lysine succinylation as a new post-translational modification. *Nature Chemical Biology* **7**, 58–63 (2011).

37. Peng, C. *et al.* The First Identification of Lysine Malonylation Substrates and Its Regulatory Enzyme *. *Molecular & Cellular Proteomics* **10**, (2011).

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

39. Chen, Y., Chen, W., Cobb, M. H. & Zhao, Y. PTMap—A sequence alignment software for unrestricted, accurate, and full-spectrum identification of post-translational modification sites. *PNAS* **106**, 761–766 (2009).

40. Zhang, K., Chen, Y., Zhang, Z. & Zhao, Y. Identification and Verification of Lysine Propionylation and Butyrylation in Yeast Core Histones Using PTMap Software. *J. Proteome Res.* **8**, 900–906 (2009).

41. Zhu, Z. *et al.* Identification of lysine isobutyrylation as a new histone modification mark. *Nucleic Acids Res* **49**, 177–189 (2021).

42. Sabari, B. R., Zhang, D., Allis, C. D. & Zhao, Y. Metabolic regulation of gene expression through histone acylations. *Nature Reviews Molecular Cell Biology* **18**, 90–101 (2017).

43. Trefely, S., Lovell, C. D., Snyder, N. W. & Wellen, K. E. Compartmentalised acyl-CoA metabolism and roles in chromatin regulation. *Molecular Metabolism* **38**, 100941 (2020).

44. Lipmann, F. & Kaplan, N. O. Coenzyme for acetylation, a pantothenic acid derivative. *J Biol Chem* **167**, 869 (1947).

45. Lipmann, F. Development of the acetylation problem, a personal account. *Science* **120**, 855–865 (1954).

46. Shi, L. & Tu, B. P. Acetyl-CoA and the Regulation of Metabolism: Mechanisms and Consequences. *Curr Opin Cell Biol* **33**, 125–131 (2015).

47. Kori, Y. *et al.* Proteome-wide acetylation dynamics in human cells. *Sci Rep* **7**, 10296 (2017).

48. Gao, X. *et al.* Acetate functions as an epigenetic metabolite to promote lipid synthesis under hypoxia. *Nature Communications* **7**, 11960 (2016).

49. Evertts, A. G. *et al.* Quantitative Dynamics of the Link between Cellular Metabolism and Histone Acetylation. *Journal of Biological Chemistry* **288**, 12142–12151 (2013).

50. Yucel, N. *et al.* Glucose Metabolism Drives Histone Acetylation Landscape Transitions that Dictate Muscle Stem Cell Function. *Cell Reports* **27**, 3939-3955.e6 (2019).

51. Campbell, S. L. & Wellen, K. E. Metabolic Signaling to the Nucleus in Cancer. *Molecular Cell* **71**, 398–408 (2018).

52. Dai, X., Lv, X., Thompson, E. W. & Ostrikov, K. (Ken). Histone lactylation: epigenetic mark of glycolytic switch. *Trends in Genetics* (2021) doi:10.1016/j.tig.2021.09.009.

53. Varner, E. L. *et al. Quantification of lactoyl-CoA by liquid chromatography mass spectrometry in mammalian cells and tissues*. 2020.03.15.992859 https://www.biorxiv.org/content/10.1101/2020.03.15.992859v2 (2020) doi:10.1101/2020.03.15.992859.

54. Irizarry-Caro, R. A. *et al.* TLR signaling adapter BCAP regulates inflammatory to reparatory macrophage transition by promoting histone lactylation. *PNAS* **117**, 30628–30638 (2020).

55. Pougovkina, O., Brinke, H. te, Wanders, R. J. A., Houten, S. M. & Boer, V. C. J. de. Aberrant protein acylation is a common observation in inborn errors of acyl-CoA metabolism. *Journal of Inherited Metabolic Disease* **37**, 709–714 (2014).

56. Smestad, J., Erber, L., Chen, Y. & Maher, L. J. Chromatin Succinylation Correlates with Active Gene Expression and Is Perturbed by Defective TCA Cycle Metabolism. *iScience* **2**, 63–75 (2018).

57. SUCLA2 mutations cause global protein succinylation contributing to the pathomechanism of a hereditary mitochondrial disease | Nature Communications. https://www.nature.com/articles/s41467-020-19743-4.

58. Gaffney, D. O. *et al.* Non-enzymatic Lysine Lactoylation of Glycolytic Enzymes. *Cell Chemical Biology* **27**, 206-213.e6 (2020).

59. Maksimovic, I., Zheng, Q., Trujillo, M. N., Galligan, J. J. & David, Y. An Azidoribose Probe to Track Ketoamine Adducts in Histone Ribose Glycation. *J Am Chem Soc* **142**, 9999–10007 (2020).

60. Galligan, J. J. *et al.* Methylglyoxal-derived posttranslational arginine modifications are abundant histone marks. *PNAS* **115**, 9228–9233 (2018).

61. Zheng, Q., Maksimovic, I., Upad, A. & David, Y. Non-enzymatic covalent modifications: a new link between metabolism and epigenetics. *Protein Cell* **11**, 401–416 (2020).

62. O’Neill, L. A. J. & Artyomov, M. N. Itaconate: the poster child of metabolic reprogramming in macrophage function. *Nature Reviews Immunology* **19**, 273–281 (2019).

63. Marmorstein, R. & Zhou, M.-M. Writers and Readers of Histone Acetylation: Structure, Mechanism, and Inhibition. *Cold Spring Harb Perspect Biol* **6**, a018762 (2014).

64. Musselman, C. A., Lalonde, M.-E., Côté, J. & Kutateladze, T. G. Perceiving the epigenetic landscape through histone readers. *Nat Struct Mol Biol* **19**, 1218–1227 (2012).

65. Zhao, S., Yue, Y., Li, Y. & Li, H. Identification and characterization of 'readers' for novel histone modifications. *Current Opinion in Chemical Biology* **51**, 57–65 (2019).

66. The interplay of histone modifications – writers that read. *EMBO reports* **16**, 1467–1481 (2015).

67. Colino-Sanguino, Y. *et al.* A Read/Write Mechanism Connects p300 Bromodomain Function to H2A.Z Acetylation. *iScience* **21**, 773–788 (2019).

68. Ragvin, A. *et al.* Nucleosome Binding by the Bromodomain and PHD Finger of the Transcriptional Cofactor p300. *Journal of Molecular Biology* **337**, 773–788 (2004).

69. Vidali, G., Boffa, L. C., Bradbury, E. M. & Allfrey, V. G. Butyrate suppression of histone deacetylation leads to accumulation of multiacetylated forms of histones H3 and H4 and increased DNase I sensitivity of the associated DNA sequences. *Proc Natl Acad Sci U S A* **75**, 2239–2243 (1978).

70. Brownell, J. E. *et al.* Tetrahymena Histone Acetyltransferase A: A Homolog to Yeast Gcn5p Linking Histone Acetylation to Gene Activation. *Cell* **84**, 843–851 (1996).

71. Avvakumov, N. & Côté, J. The MYST family of histone acetyltransferases and their intimate links to cancer. *Oncogene* **26**, 5395–5407 (2007).

72. Voss, A. K. & Thomas, T. MYST family histone acetyltransferases take center stage in stem cells and development. *BioEssays* **31**, 1050–1061 (2009).

73. Thomas, T. & Voss, A. K. The diverse biological roles of MYST histone acetyltransferase family proteins. *Cell Cycle* **6**, 696–704 (2007).

74. Kadlec, J. *et al.* Structural basis for MOF and MSL3 recruitment into the dosage compensation complex by MSL1. *Nature Structural & Molecular Biology* **18**, 142–149 (2011).

75. Kamine, J., Elangovan, B., Subramanian, T., Coleman, D. & Chinnadurai, G. Identification of a Cellular Protein That Specifically Interacts with the Essential Cysteine Region of the HIV-1 Tat Transactivator. *Virology* **216**, 357–366 (1996).

76. Brady, M. E. *et al.* Tip60 Is a Nuclear Hormone Receptor Coactivator *. *Journal of Biological Chemistry* **274**, 17599–17604 (1999).

77. Tang, Y., Luo, J., Zhang, W. & Gu, W. Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. *Mol Cell* **24**, 827–839 (2006).

78. Legube, G. *et al.* Role of the Histone Acetyl Transferase Tip60 in the p53 Pathway *. *Journal of Biological Chemistry* **279**, 44825–44833 (2004).

79. Liu, N. *et al.* ING5 Is a Tip60 Cofactor That Acetylates p53 in Response to DNA Damage. *Cancer Res* **73**, 3749–3760 (2013).

80. Huang, H. *et al.* Landscape of the regulatory elements for lysine 2-hydroxyisobutyrylation pathway. *Cell Res* **28**, 111–125 (2018).

81. Borrow, J. *et al.* The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. *Nat Genet* **14**, 33–41 (1996).

82. Champagne, N. *et al.* Identification of a human histone acetyltransferase related to monocytic leukemia zinc finger protein. *J Biol Chem* **274**, 28528–28536 (1999).

83. Champagne, N., Pelletier, N. & Yang, X. J. The monocytic leukemia zinc finger protein MOZ is a histone acetyltransferase. *Oncogene* **20**, 404–409 (2001).

84. Thomas, T., Voss, A. K., Chowdhury, K. & Gruss, P. Querkopf, a MYST family histone acetyltransferase, is required for normal cerebral cortex development. *Development* **127**, 2537–2548 (2000).

85. Klein, B. J. *et al.* Recognition of Histone H3K14 Acylation by MORF. *Structure* **25**, 650-654.e2 (2017).

86. Klein, B. J. *et al.* Histone H3K23-specific acetylation by MORF is coupled to H3K14 acylation. *Nat Commun* **10**, 4724 (2019).

87. Xiong, X. *et al.* Selective recognition of histone crotonylation by double PHD fingers of MOZ and DPF2. *Nat Chem Biol* **12**, 1111–1118 (2016).

88. Iizuka, M. & Stillman, B. Histone Acetyltransferase HBO1 Interacts with the ORC1 Subunit of the Human Initiator Protein *. *Journal of Biological Chemistry* **274**, 23027–23034 (1999).

89. Burke, T. W., Cook, J. G., Asano, M. & Nevins, J. R. Replication Factors MCM2 and ORC1 Interact with the Histone Acetyltransferase HBO1*. *Journal of Biological Chemistry* **276**, 15397–15408 (2001).

90. Miotto, B. & Struhl, K. HBO1 histone acetylase is a coactivator of the replication licensing factor Cdt1. *Genes Dev* **22**, 2633–2638 (2008).

91. Xiao, Y. *et al.* HBO1 is a versatile histone acyltransferase critical for promoter histone acylations. *Nucleic Acids Research* **49**, 8037–8059 (2021).

92. mof, a putative acetyl transferase gene related to the Tip60 and MOZ human genes and to the SAS genes of yeast, is required for dosage compensation in Drosophila. *The EMBO Journal* **16**, 2054–2060 (1997).
93. Dosage compensation in Drosophila melanogaster: epigenetic fine-tuning of chromosome-wide transcription | Nature Reviews Genetics. https://www.nature.com/articles/nrg3124.
94. Chen, Q. Y. *et al.* Structure and function of histone acetyltransferase MOF. *AIMS Biophysics* **2**, 555–569 (2015).
95. Shogren-Knaak, M. *et al.* Histone H4-K16 Acetylation Controls Chromatin Structure and Protein Interactions. *Science* **311**, 844–847 (2006).
96. Liu, X. *et al.* MOF as an evolutionarily conserved histone crotonyltransferase and transcriptional activation by histone acetyltransferase-deficient and crotonyltransferase-competent CBP/p300. *Cell Discovery* **3**, 1–17 (2017).
97. Han, Z. *et al.* Revealing the protein propionylation activity of the histone acetyltransferase MOF (males absent on the first). *J Biol Chem* **293**, 3410–3420 (2018).
98. Verdin, E. & Ott, M. 50 years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. *Nat Rev Mol Cell Biol* **16**, 258–264 (2015).
99. Cori, C. F. & Cori, G. T. Polysaccharide phosphorylase. 21 (1947).
100. Swanson, M. A. & Cori, C. F. Studies on the structure of polysaccharides; relation of structure to activation of phosphorylases. *J Biol Chem* **172**, 815–824 (1948).
101. Krebs, E. G. & Fischer, E. H. The phosphorylase b to a converting enzyme of rabbit skeletal muscle. *Biochimica et Biophysica Acta* **20**, 150–157 (1956).

102. Fischer, E. H. & Krebs, E. G. THE ISOLATION AND CRYSTALLIZATION OF RABBIT SKELETAL MUSCLE PHOSPHORYLASE b. *Journal of Biological Chemistry* **231**, 65–71 (1958).

103. Krebs, E. G., Kent, A. B. & Fischer, E. H. THE MUSCLE PHOSPHORYLASE b KINASE REACTION. *Journal of Biological Chemistry* **231**, 73–83 (1958).

104. Hunter, T. Discovering the first tyrosine kinase. *PNAS* **112**, 7877–7882 (2015).

105. Hunter, T. Tyrosine phosphorylation: thirty years and counting. *Curr Opin Cell Biol* **21**, 140–146 (2009).

106. Lemmon, M. A. & Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117–1134 (2010).

107. Pawson, T. & Scott, J. D. Protein phosphorylation in signaling – 50 years and counting. *Trends in Biochemical Sciences* **30**, 286–290 (2005).

108. Hunter, T. Signaling—2000 and Beyond. *Cell* **100**, 113–127 (2000).

109. Eckerdt, F., Perez-Neut, M. & Colamonici, O. R. LIN-9 Phosphorylation on Threonine-96 Is Required for Transcriptional Activation of LIN-9 Target Genes and Promotes Cell Cycle Progression. *PLOS ONE* **9**, e87620 (2014).

110. Cell Signaling | Learn Science at Scitable. https://www.nature.com/scitable/topicpage/cell-signaling-14047077/.

111. De Meyts, P. The Insulin Receptor and Its Signal Transduction Network. in *Endotext* (eds. Feingold, K. R. et al.) (MDText.com, Inc., 2000).

112. Quandt, J. *et al.* Wnt-β-catenin activation epigenetically reprograms Treg cells in inflammatory bowel disease and dysplastic progression. *Nat Immunol* **22**, 471–484 (2021).

113. Wnt/beta-catenin pathway: modulating anticancer immune response | Journal of Hematology & Oncology | Full Text. https://jhoonline.biomedcentral.com/articles/10.1186/s13045-017-0471-6.

114. Harvey, K. F., Zhang, X. & Thomas, D. M. The Hippo pathway and human cancer. *Nat Rev Cancer* **13**, 246–257 (2013).

115. Plotnikov, A., Zehorai, E., Procaccia, S. & Seger, R. The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochim Biophys Acta* **1813**, 1619–1633 (2011).

116. Cargnello, M. & Roux, P. P. Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. *Microbiol Mol Biol Rev* **75**, 50–83 (2011).

117. Kholodenko, B. N. & Birtwistle, M. R. Four-dimensional dynamics of MAPK information processing systems. *Wiley Interdiscip Rev Syst Biol Med* **1**, 28–44 (2009).

118. Yang, W. *et al.* ERK1/2-dependent phosphorylation and nuclear translocation of PKM2 promotes the Warburg effect. *Nature Cell Biology* **14**, 1295–1304 (2012).

119. Plotnikov, A. *et al.* The nuclear translocation of ERK1/2 as an anticancer target. *Nat Commun* **6**, 6685 (2015).

120. Chesterman, F. C., Harvey, J. J., Dourmashkin, R. R. & Salaman, M. H. The pathology of tumors and other lesions induced in rodents by virus derived from a rat with Moloney leukemia. *Cancer Res* **26**, 1759–1768 (1966).

121. Harvey, J. J. An Unidentified Virus which causes the Rapid Production of Tumours in Mice. *Nature* **204**, 1104–1105 (1964).

122. Scolnick, E. M. & Parks, W. P. Harvey sarcoma virus: a second murine type C sarcoma virus with rat genetic information. *J Virol* **13**, 1211–1219 (1974).

123. Avruch, J. MAP kinase pathways: The first twenty years. *Biochim Biophys Acta* **1773**, 1150–1160 (2007).

124. Ahn, N. G., Weiel, J. E., Chan, C. P. & Krebs, E. G. Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. *J Biol Chem* **265**, 11487–11494 (1990).

125. Ahn, N. G. *et al.* Multiple components in an epidermal growth factor-stimulated protein kinase cascade. In vitro activation of a myelin basic protein/microtubule-associated protein 2 kinase. *J Biol Chem* **266**, 4220–4227 (1991).

126. Cox, A. D. & Der, C. J. Ras history. *Small GTPases* **1**, 2–27 (2010).

127. Wennerberg, K., Rossman, K. L. & Der, C. J. The Ras superfamily at a glance. *J Cell Sci* **118**, 843–846 (2005).

128. Rapp, U. R. *et al.* Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. *Proc Natl Acad Sci U S A* **80**, 4218–4222 (1983).

129. Huebner, K. *et al.* Actively transcribed genes in the raf oncogene group, located on the X chromosome in mouse and human. *Proc Natl Acad Sci U S A* **83**, 3934–3938 (1986).

130. Ikawa, S. *et al.* B-raf, a new member of the raf family, is activated by DNA rearrangement. *Mol Cell Biol* **8**, 2651–2654 (1988).

131. Yoon, S. & Seger, R. The extracellular signal-regulated kinase: Multiple substrates regulate diverse cellular functions. *Growth Factors* **24**, 21–44 (2006).

132. Colicelli, J. Human RAS superfamily proteins and related GTPases. *Sci STKE* **2004**, RE13 (2004).

133. Wittinghofer, A. & Nassar, N. How Ras-related proteins talk to their effectors. *Trends Biochem Sci* **21**, 488–491 (1996).

134. Stewart, S. *et al.* Kinase suppressor of Ras forms a multiprotein signaling complex and modulates MEK localization. *Mol Cell Biol* **19**, 5523–5534 (1999).

135. Nguyen, A. *et al.* Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. *Mol Cell Biol* **22**, 3035–3045 (2002).

136. Lavoie, H. & Therrien, M. Regulation of RAF protein kinases in ERK signalling. *Nat Rev Mol Cell Biol* **16**, 281–298 (2015).

137. Mason, C. S. *et al.* Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. *EMBO J* **18**, 2137–2148 (1999).

138. Zhang, B. H. & Guan, K. L. Activation of B-Raf kinase requires phosphorylation of the conserved residues Thr598 and Ser601. *EMBO J* **19**, 5429–5439 (2000).

139. Marais, R., Light, Y., Paterson, H. F., Mason, C. S. & Marshall, C. J. Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. *J Biol Chem* **272**, 4378–4383 (1997).

140. Li, S. & Sedivy, J. M. Raf-1 protein kinase activates the NF-kappa B transcription factor by dissociating the cytoplasmic NF-kappa B-I kappa B complex. *Proc Natl Acad Sci U S A* **90**, 9247–9251 (1993).

141. Baumann, B. *et al.* Raf induces NF-kappaB by membrane shuttle kinase MEKK1, a signaling pathway critical for transformation. *Proc Natl Acad Sci U S A* **97**, 4615–4620 (2000).

142. Galaktionov, K., Jessus, C. & Beach, D. Raf1 interaction with Cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation. *Genes Dev* **9**, 1046–1058 (1995).

143. Wang, S., Ghosh, R. N. & Chellappan, S. P. Raf-1 physically interacts with Rb and regulates its function: a link between mitogenic signaling and cell cycle regulation. *Mol Cell Biol* **18**, 7487–7498 (1998).

144. Bcl-2 Targets the Protein Kinase Raf-1 to Mitochondria - ScienceDirect. https://www-sciencedirect-com.proxy.uchicago.edu/science/article/pii/S0092867400813835.

145. Papin, C., Denouel, A., Calothy, G. & Eychène, A. Identification of signalling proteins interacting with B-Raf in the yeast two-hybrid system. *Oncogene* **12**, 2213–2221 (1996).

146. Moodie, S. A., Paris, M. J., Kolch, W. & Wolfman, A. Association of MEK1 with p21ras.GMPPNP is dependent on B-Raf. *Mol Cell Biol* **14**, 7153–7162 (1994).

147. Legault-Poisson, S., Jolivet, J., Poisson, R., Beretta-Piccoli, M. & Band, P. R. Tamoxifen-induced tumor stimulation and withdrawal response. *Cancer Treat Rep* **63**, 1839–1841 (1979).

148. Caunt, C. J., Sale, M. J., Smith, P. D. & Cook, S. J. MEK1 and MEK2 inhibitors and cancer therapy: the long and winding road. *Nature Reviews Cancer* **15**, 577–592 (2015).

149. von Kriegsheim, A. *et al.* Cell fate decisions are specified by the dynamic ERK interactome. *Nat Cell Biol* **11**, 1458–1464 (2009).

150. Liu, F., Yang, X., Geng, M. & Huang, M. Targeting ERK, an Achilles' Heel of the MAPK pathway, in cancer therapy. *Acta Pharm Sin B* **8**, 552–562 (2018).

151. Hanahan, D. & Weinberg, R. A. Hallmarks of Cancer: The Next Generation. *Cell* **144**, 646–674 (2011).

152. Braicu, C. *et al.* A Comprehensive Review on MAPK: A Promising Therapeutic Target in Cancer. *Cancers (Basel)* **11**, 1618 (2019).

153. Davies, H. *et al.* Mutations of the BRAF gene in human cancer. *Nature* **417**, 949–954 (2002).

154. Cancer Statistics - National Cancer Institute. https://www.cancer.gov/about-cancer/understanding/statistics (2015).

155. Kobayashi, S. *et al.* EGFR Mutation and Resistance of Non–Small-Cell Lung Cancer to Gefitinib. *New England Journal of Medicine* **352**, 786–792 (2005).

156. Pao, W. *et al.* Acquired Resistance of Lung Adenocarcinomas to Gefitinib or Erlotinib Is Associated with a Second Mutation in the EGFR Kinase Domain. *PLOS Medicine* **2**, e73 (2005).

157. Frampton, J. E. Lapatinib: a review of its use in the treatment of HER2-overexpressing, trastuzumab-refractory, advanced or metastatic breast cancer. *Drugs* **69**, 2125–2148 (2009).

158. Dhillon, S. Gefitinib: a review of its use in adults with advanced non-small cell lung cancer. *Target Oncol* **10**, 153–170 (2015).

159. McKeage, K. & Perry, C. M. Trastuzumab: a review of its use in the treatment of metastatic breast cancer overexpressing HER2. *Drugs* **62**, 209–243 (2002).

160. Prior, I. A., Lewis, P. D. & Mattos, C. A comprehensive survey of Ras mutations in cancer. *Cancer Res* **72**, 2457–2467 (2012).

161. Almoguera, C. *et al.* Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell* **53**, 549–554 (1988).

162. Cox, A. D., Fesik, S. W., Kimmelman, A. C., Luo, J. & Der, C. J. Drugging the undruggable RAS: Mission possible? *Nat Rev Drug Discov* **13**, 828–851 (2014).

163. Canon, J. *et al.* The clinical KRAS(G12C) inhibitor AMG 510 drives anti-tumour immunity. *Nature* **575**, 217–223 (2019).

164. Sidaway, P. Sotorasib effective in KRAS-mutant NSCLC. *Nat Rev Clin Oncol* **18**, 470–470 (2021).

165. Weerapana, E. *et al.* Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature* **468**, 790–795 (2010).

166. Zambaldo, C. *et al.* 2-Sulfonylpyridines as Tunable, Cysteine-Reactive Electrophiles. *J. Am. Chem. Soc.* **142**, 8972–8979 (2020).

167. Abbasov, M. E. *et al.* A proteome-wide atlas of lysine-reactive chemistry. *Nat. Chem.* **13**, 1081–1092 (2021).

168. KRASG12C Inhibition with Sotorasib in Advanced Solid Tumors | NEJM. https://www-nejm-org.proxy.uchicago.edu/doi/full/10.1056/NEJMoa1917239.

169. Research, C. for D. E. and. FDA grants accelerated approval to sotorasib for KRAS G12C mutated NSCLC. *FDA* (2021).

170. Parmenter, T. J. *et al.* Response of BRAF-Mutant Melanoma to BRAF Inhibition Is Mediated by a Network of Transcriptional Regulators of Glycolysis. *Cancer Discov* **4**, 423–433 (2014).

171. Bollag, G. *et al.* Vemurafenib: the first drug approved for BRAF -mutant cancer. *Nature Reviews Drug Discovery* **11**, 873–886 (2012).

172. Chapman, P. B. *et al.* Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* **364**, 2507–2516 (2011).

173. Bansal, A., Ramirez, R. D. & Minna, J. D. Mutation analysis of the coding sequences of MEK-1 and MEK-2 genes in human lung cancer cell lines. *Oncogene* **14**, 1231–1234 (1997).

174. Marks, J. L. *et al.* Novel MEK1 mutation identified by mutational analysis of epidermal growth factor receptor signaling pathway genes in lung adenocarcinoma. *Cancer Res* **68**, 5524–5528 (2008).

175. Murugan, A. K., Dong, J., Xie, J. & Xing, M. MEK1 mutations, but not ERK2 mutations, occur in melanomas and colon carcinomas, but none in thyroid carcinomas. *Cell Cycle* **8**, 2122–2124 (2009).

176. Nikolaev, S. I. *et al.* Exome sequencing identifies recurrent somatic MAP2K1 and MAP2K2 mutations in melanoma. *Nat Genet* **44**, 133–139 (2011).

177. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. & Saltiel, A. R. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci U S A* **92**, 7686–7689 (1995).

178. Gilmartin, A. G. *et al.* GSK1120212 (JTP-74057) is an inhibitor of MEK activity and activation with favorable pharmacokinetic properties for sustained in vivo pathway inhibition. *Clin Cancer Res* **17**, 989–1000 (2011).

179. Hatzivassiliou, G. *et al.* Mechanism of MEK inhibition determines efficacy in mutant KRAS- versus BRAF-driven cancers. *Nature* **501**, 232–236 (2013).

180. Kroemer, G. & Pouyssegur, J. Tumor Cell Metabolism: Cancer's Achilles' Heel. *Cancer Cell* **13**, 472–482 (2008).

181. Warburg, O. H. & 1883-. Über den Stoffwechsel der Tumoren. (1926).

182. What Is Cancer? - National Cancer Institute. https://www.cancer.gov/about-cancer/understanding/what-is-cancer (2007).

183. Pavlova, N. N. & Thompson, C. B. The Emerging Hallmarks of Cancer Metabolism. *Cell Metabolism* **23**, 27–47 (2016).

184. Vander Heiden, M. G., Cantley, L. C. & Thompson, C. B. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science* **324**, 1029–1033 (2009).

185. Berg, J. M., Tymoczko, J. L. & Stryer, L. Glycolysis and Gluconeogenesis. *Biochemistry. 5th edition* (2002).

186. Krebs, H. A. Rate control of the tricarboxylic acid cycle. *Adv. Enzyme Regul.* **8**, 335–353 (1970).

187. Bubici, C. & Papa, S. Editorial: The Warburg Effect Regulation Under Siege: the Intertwined Pathways in Health and Disease. *Frontiers in Cell and Developmental Biology* **7**, 80 (2019).

188. DeBerardinis, R. J. & Chandel, N. S. We need to talk about the Warburg effect. *Nature Metabolism* **2**, 127–129 (2020).

189. Vardhana, S. A. *et al.* Impaired mitochondrial oxidative phosphorylation limits the self-renewal of T cells exposed to persistent antigen. *Nat Immunol* **21**, 1022–1033 (2020).

190. Buck, M. D., Sowell, R. T., Kaech, S. M. & Pearce, E. L. Metabolic Instruction of Immunity. *Cell* **169**, 570–586 (2017).

191. Pearce, E. L. & Pearce, E. J. Metabolic Pathways in Immune Cell Activation and Quiescence. *Immunity* **38**, 633–643 (2013).

192. Dang, C. V., Kim, J., Gao, P. & Yustein, J. The interplay between MYC and HIF in cancer. *Nature Reviews Cancer* **8**, 51–56 (2008).

193. Lum, J. J. *et al.* The transcription factor HIF-1α plays a critical role in the growth factor-dependent regulation of both aerobic and anaerobic glycolysis. *Genes Dev.* **21**, 1037–1049 (2007).

194. Fukuda, R. *et al.* HIF-1 Regulates Cytochrome Oxidase Subunits to Optimize Efficiency of Respiration in Hypoxic Cells. *Cell* **129**, 111–122 (2007).

195. Dang, C. V. c-Myc Target Genes Involved in Cell Growth, Apoptosis, and Metabolism. *Molecular and Cellular Biology* **19**, 1–11 (1999).

196. Papa, S., Choy, P. M. & Bubici, C. The ERK and JNK pathways in the regulation of metabolic reprogramming. *Oncogene* **38**, 2223–2240 (2019).

197. Li, X. *et al.* Mitochondria-Translocated PGK1 Functions as a Protein Kinase to Coordinate Glycolysis and the TCA Cycle in Tumorigenesis. *Molecular Cell* **61**, 705–719 (2016).

198. San-Millán, I. & Brooks, G. A. Reexamining cancer metabolism: lactate production for carcinogenesis could be the purpose and explanation of the Warburg Effect. *Carcinogenesis* **38**, 119–133 (2017).

199. Brooks, G. A. Lactate as a fulcrum of metabolism. *Redox Biology* **35**, 101454 (2020).

200. Sousa Silva, M., Gomes, R. A., Ferreira, A. E. N., Ponces Freire, A. & Cordeiro, C. The glyoxalase pathway: the first hundred years… and beyond. *Biochemical Journal* **453**, 1–15 (2013).

201. Walenta, S. *et al.* High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. *Cancer Res* **60**, 916–921 (2000).

202. de Vrese, M. & Barth, C. A. Postprandial plasma D-lactate concentrations after yogurt ingestion. *Z Ernährungswiss* **30**, 131–137 (1991).

203. Ohmori, S. & Iwamoto, T. Sensitive determination of d-lactic acid in biological samples by high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications* **431**, 239–247 (1988).

204. D-Lactate in Human and Ruminant Metabolism | The Journal of Nutrition | Oxford Academic. https://academic.oup.com/jn/article/135/7/1619/4663874.

205. Waterhouse, C. & Keilson, J. Cori cycle activity in man. *J Clin Invest* **48**, 2359–2366 (1969).

206. Katz, J. & Tayek, J. A. Gluconeogenesis and the Cori cycle in 12-, 20-, and 40-h-fasted humans. *American Journal of Physiology-Endocrinology and Metabolism* **275**, E537–E542 (1998).

207. CORI, C. F. GLYCOGEN BREAKDOWN AND SYNTHESIS IN ANIMAL TISSUES. *Endocrinology* **26**, 285–296 (1940).

208. Hui, S. *et al.* Glucose feeds the TCA cycle via circulating lactate. *Nature* **551**, 115–118 (2017).

209. Faubert, B. *et al.* Lactate Metabolism in Human Lung Tumors. *Cell* **171**, 358-371.e9 (2017).

210. Baltazar, F., Afonso, J., Costa, M. & Granja, S. Lactate Beyond a Waste Metabolite: Metabolic Affairs and Signaling in Malignancy. *Frontiers in Oncology* **10**, 231 (2020).

211. Brooks, G. A. *et al.* Lactate in contemporary biology: a phoenix risen. *The Journal of Physiology* **n/a**,.

212. Colegio, O. R. *et al.* Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* **513**, 559–563 (2014).

213. Zappasodi, R. *et al.* CTLA-4 blockade drives loss of T reg stability in glycolysis-low tumours. *Nature* **591**, 652–658 (2021).

214. Watson, M. J. *et al.* Metabolic support of tumour-infiltrating regulatory T cells by lactic acid. *Nature* **591**, 645–651 (2021).

215. Mukhopadhyay, S., Vander Heiden, M. G. & McCormick, F. The metabolic landscape of RAS-driven cancers from biology to therapy. *Nature Cancer* **2**, 271–283 (2021).

216. Nagarajan, A., Malvi, P. & Wajapeyee, N. Oncogene-directed alterations in cancer cell metabolism. *Trends Cancer* **2**, 365–377 (2016).

217. Levine, A. J. & Puzio-Kuter, A. M. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. *Science* **330**, 1340–1344 (2010).

218. Hulea, L. *et al.* Translational and HIF-1α-Dependent Metabolic Reprogramming Underpin Metabolic Plasticity and Responses to Kinase Inhibitors and Biguanides. *Cell Metabolism* **28**, 817-832.e8 (2018).

219. Cunningham, T. W., Kuppuswamy, D. & Pike, L. J. Treatment of A431 cells with epidermal growth factor (EGF) induces desensitization of EGF-stimulated phosphatidylinositol turnover. *J Biol Chem* **264**, 15351–15356 (1989).

220. Grünwald, V. & Hidalgo, M. Development of the epidermal growth factor receptor inhibitor Tarceva (OSI-774). *Adv Exp Med Biol* **532**, 235–246 (2003).

221. Ong, S.-E. *et al.* 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 (2002).

222. Quinn, W. J. *et al.* Lactate Limits T Cell Proliferation via the NAD(H) Redox State. *Cell Reports* **33**, 108500 (2020).

223. O'Neill, L. A. J., Kishton, R. J. & Rathmell, J. A guide to immunometabolism for immunologists. *Nat Rev Immunol* **16**, 553–565 (2016).

224. Gehart, H., Kumpf, S., Ittner, A. & Ricci, R. MAPK signalling in cellular metabolism: stress or wellness? *EMBO Rep* **11**, 834–840 (2010).

225. Kim, J., Gao, P., Liu, Y.-C., Semenza, G. L. & Dang, C. V. Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. *Mol Cell Biol* **27**, 7381–7393 (2007).

226. Qu, X. *et al.* c-Myc-driven glycolysis via TXNIP suppression is dependent on glutaminase-MondoA axis in prostate cancer. *Biochem Biophys Res Commun* **504**, 415–421 (2018).

227. Ji, S. *et al.* FBW7 (F-box and WD Repeat Domain-Containing 7) Negatively Regulates Glucose Metabolism by Targeting the c-Myc/TXNIP (Thioredoxin-Binding Protein) Axis in Pancreatic Cancer. *Clin Cancer Res* **22**, 3950–3960 (2016).

228. Kaadige, M. R., Looper, R. E., Kamalanaadhan, S. & Ayer, D. E. Glutamine-dependent anapleurosis dictates glucose uptake and cell growth by regulating MondoA transcriptional activity. *PNAS* **106**, 14878–14883 (2009).

229. Pajak, B. *et al.* 2-Deoxy-d-Glucose and Its Analogs: From Diagnostic to Therapeutic Agents. *Int J Mol Sci* **21**, 234 (2019).

230. Stacpoole, P. W. & Greene, Y. J. Dichloroacetate. *Diabetes Care* **15**, 785–791 (1992).

231. Gagliardi, P. A., Puliafito, A. & Primo, L. PDK1: At the crossroad of cancer signaling pathways. *Semin Cancer Biol* **48**, 27–35 (2018).

232. Hildyard, J. C. W., Ammälä, C., Dukes, I. D., Thomson, S. A. & Halestrap, A. P. Identification and characterisation of a new class of highly specific and potent inhibitors of the mitochondrial pyruvate carrier. *Biochim Biophys Acta* **1707**, 221–230 (2005).

233. Vacanti, N. M. *et al.* Regulation of substrate utilization by the mitochondrial pyruvate carrier. *Mol Cell* **56**, 425–435 (2014).

234. Farhana, A. & Lappin, S. L. Biochemistry, Lactate Dehydrogenase. in *StatPearls* (StatPearls Publishing, 2021).

235. Zhai, X., Yang, Y., Wan, J., Zhu, R. & Wu, Y. Inhibition of LDH-A by oxamate induces G2/M arrest, apoptosis and increases radiosensitivity in nasopharyngeal carcinoma cells. *Oncol Rep* **30**, 2983–2991 (2013).

236. Boudreau, A. *et al.* Metabolic plasticity underpins innate and acquired resistance to LDHA inhibition. *Nat Chem Biol* **12**, 779–786 (2016).

237. Lim, W. A. & Pawson, T. Phosphotyrosine Signaling: Evolving a New Cellular Communication System. *Cell* **142**, 661–667 (2010).

238. Graves, L. M., Duncan, J. S., Whittle, M. C. & Johnson, G. L. The dynamic nature of the kinome. *Biochemical Journal* **450**, 1–8 (2013).

239. Gräff, J. & Tsai, L.-H. Histone acetylation: molecular mnemonics on the chromatin. *Nat Rev Neurosci* **14**, 97–111 (2013).

240. Barnes, C. E., English, D. M. & Cowley, S. M. Acetylation & Co: an expanding repertoire of histone acylations regulates chromatin and transcription. *Essays Biochem* **63**, 97–107 (2019).

241. Yang, K. *et al.* Lactate promotes macrophage HMGB1 lactylation, acetylation, and exosomal release in polymicrobial sepsis. *Cell Death Differ* 1–14 (2021) doi:10.1038/s41418-021-00841-9.

242. Patel, R. *et al.* Molecular Docking and Simulation Studies Predict Lactyl-CoA as the Substrate for P300 Directed Lactylation. (2020) doi:10.26434/chemrxiv.12770360.v1.

243. Kaczmarska, Z. *et al.* Structure of p300 in complex with acyl-CoA variants. *Nature Chemical Biology* **13**, 21–29 (2017).

244. Cheng, Z. *et al.* Molecular Characterization of Propionyllysines in Non-histone Proteins. *Molecular & Cellular Proteomics* **8**, 45–52 (2009).

245. Sabari, B. R. *et al.* Intracellular Crotonyl-CoA Stimulates Transcription through p300-Catalyzed Histone Crotonylation. *Molecular Cell* **58**, 203–215 (2015).

246. Bhattacharya, A. *et al. EP300 (p300) mediated histone butyrylation is critical for adipogenesis*. 2021.08.01.454641 https://www.biorxiv.org/content/10.1101/2021.08.01.454641v1 (2021) doi:10.1101/2021.08.01.454641.

247. Huang, H. *et al.* p300-Mediated Lysine 2-Hydroxyisobutyrylation Regulates Glycolysis. *Mol Cell* **70**, 663-678.e6 (2018).

248. Liu, K. *et al.* p53 β-hydroxybutyrylation attenuates p53 activity. *Cell Death & Disease* **10**, 1–13 (2019).

249. Black, J. C., Mosley, A., Kitada, T., Washburn, M. & Carey, M. The SIRT2 deacetylase regulates autoacetylation of p300. *Mol Cell* **32**, 449–455 (2008).

250. Wang, J. & Chen, J. SIRT1 Regulates Autoacetylation and Histone Acetyltransferase Activity of TIP60 *. *Journal of Biological Chemistry* **285**, 11458–11464 (2010).

251. Lu, L. *et al.* Modulations of hMOF autoacetylation by SIRT1 regulate hMOF recruitment and activities on the chromatin. *Cell Res* **21**, 1182–1195 (2011).

252. Yuan, H. *et al.* MYST protein acetyltransferase activity requires active site lysine autoacetylation. *The EMBO Journal* **31**, 58–70 (2012).

253. McCullough, C. E., Song, S., Shin, M. H., Johnson, F. B. & Marmorstein, R. Structural and Functional Role of Acetyltransferase hMOF K274 Autoacetylation. *J Biol Chem* **291**, 18190–18198 (2016).

254. Wagner, G. R. *et al.* A Class of Reactive Acyl-CoA Species Reveals the Non-Enzymatic Origins of Protein Acylation. *Cell Metab* **25**, 823-837.e8 (2017).

255. Simithy, J. *et al.* Characterization of histone acylations links chromatin modifications with metabolism. *Nat Commun* **8**, 1141 (2017).

256. Cox, J. *et al.* A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nature Protocols* **4**, 698–705 (2009).

257. Wang, Y. *et al.* KAT2A coupled with the α-KGDH complex acts as a histone H3 succinyltransferase. *Nature* **552**, 273–277 (2017).

258. Wang, Y., Guo, Y. R., Xing, D., Tao, Y. J. & Lu, Z. Supramolecular assembly of KAT2A with succinyl-CoA for histone succinylation. *Cell Discov* **4**, 1–4 (2018).

259. Cantley, L. C. The Phosphoinositide 3-Kinase Pathway. *Science* **296**, 1655–1657 (2002).

260. Cai, L. & Tu, B. P. Driving the Cell Cycle Through Metabolism. *Annu. Rev. Cell Dev. Biol.* **28**, 59–87 (2012).

261. de la Cruz-López, K. G., Castro-Muñoz, L. J., Reyes-Hernández, D. O., García-Carrancá, A. & Manzo-Merino, J. Lactate in the Regulation of Tumor Microenvironment and Therapeutic Approaches. *Front Oncol* **9**, 1143 (2019).

262. Li, F. & Simon, M. C. Cancer Cells Don't Live Alone: Metabolic Communication within Tumor Microenvironments. *Developmental Cell* **0**, (2020).

263. Chen, Y. *et al.* Quantitative Acetylome Analysis Reveals the Roles of SIRT1 in Regulating Diverse Substrates and Cellular Pathways. *Mol Cell Proteomics* **11**, 1048–1062 (2012).

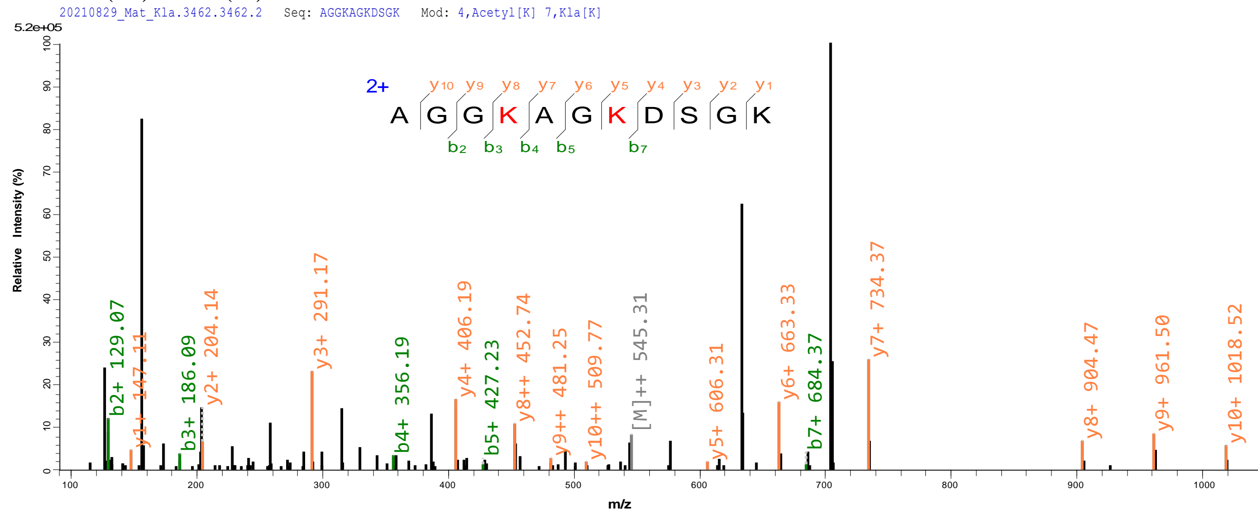
264. Jennings, E. Q. *et al.* Sirtuin 2 Regulates Protein LactoylLys Modifications. *ChemBioChem* **22**, 2102–2106 (2021).

APPENDIX

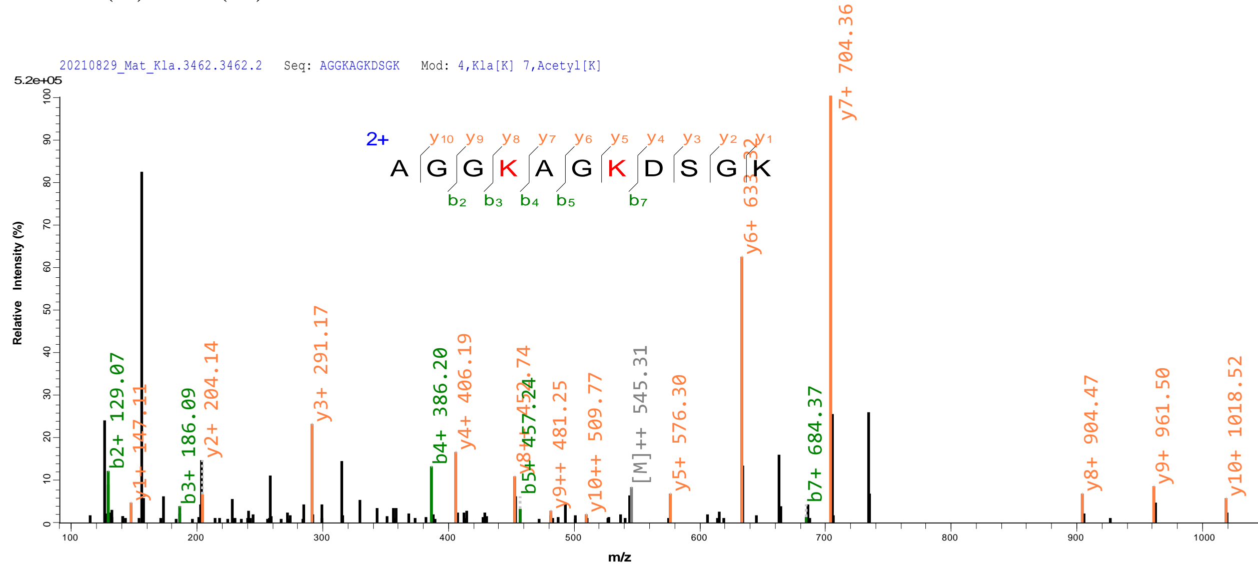
Spectra for Kla sites identified by IP-MS/MS.

Annotated MASCOT images for the 22 histone Kla sites identified by IP-MS/MS using pan Kla antibody. The parameters for the search included the following modifications: Kla (lysine +72.021 Da), Kac, N-terminal acetylation, K/Rme1, K/Rme2, and Kme3. Histone peptide matches with Kmea sites were manually verified for correctness for all spectra with MASCOT scores of at least 20. All spectra were manually verified for quality.

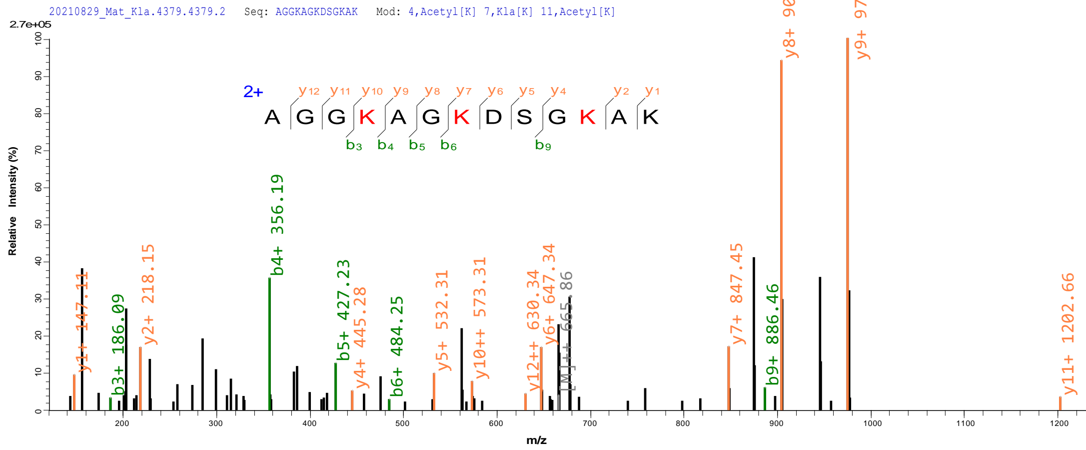
AGGK(ac)AGK(la)DSGK



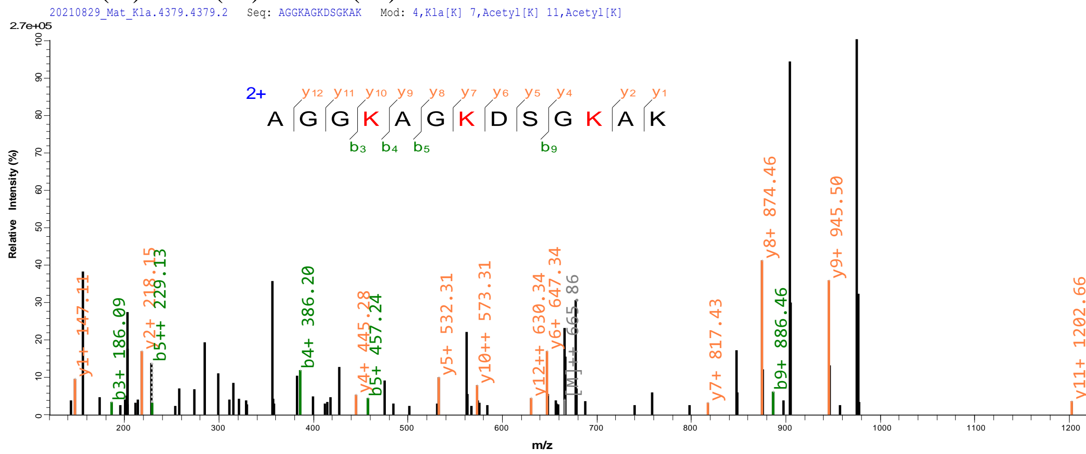
AGGK(la)AGK(ac)DSGK



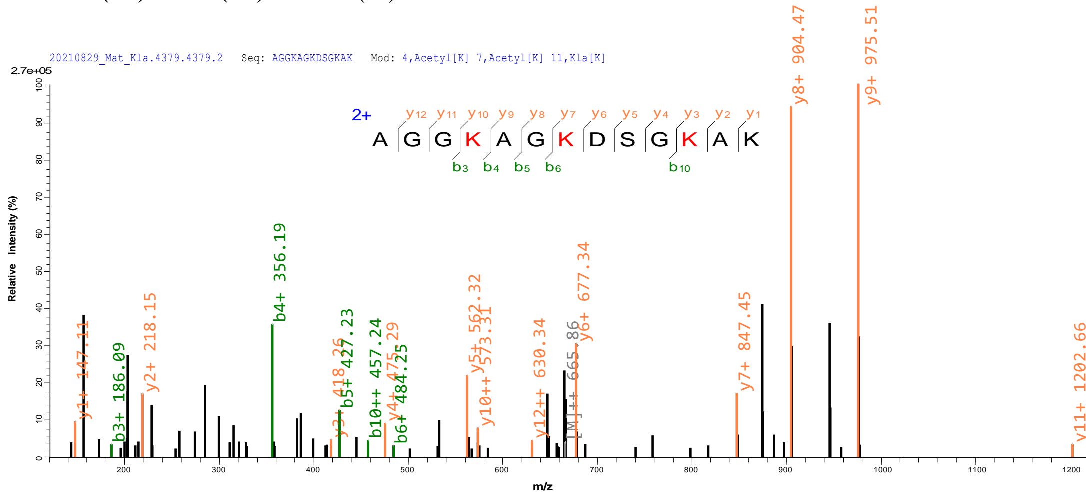
AGGK(ac)AGK(la)DSGK(ac)AK



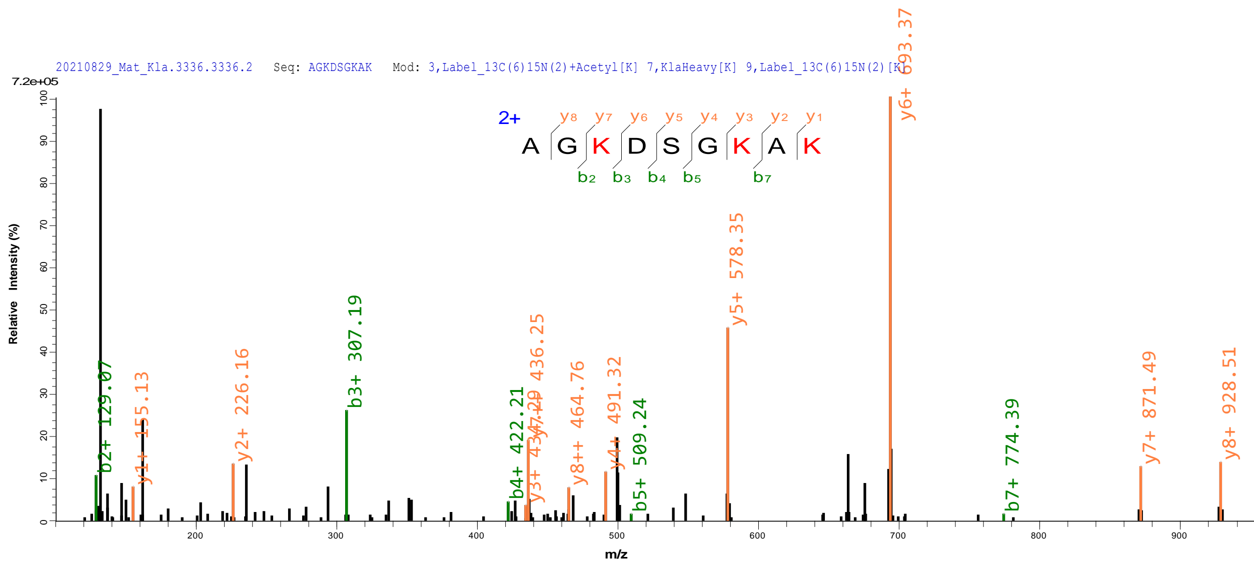
AGGK(la)AGK(ac)DSGK(ac)AK



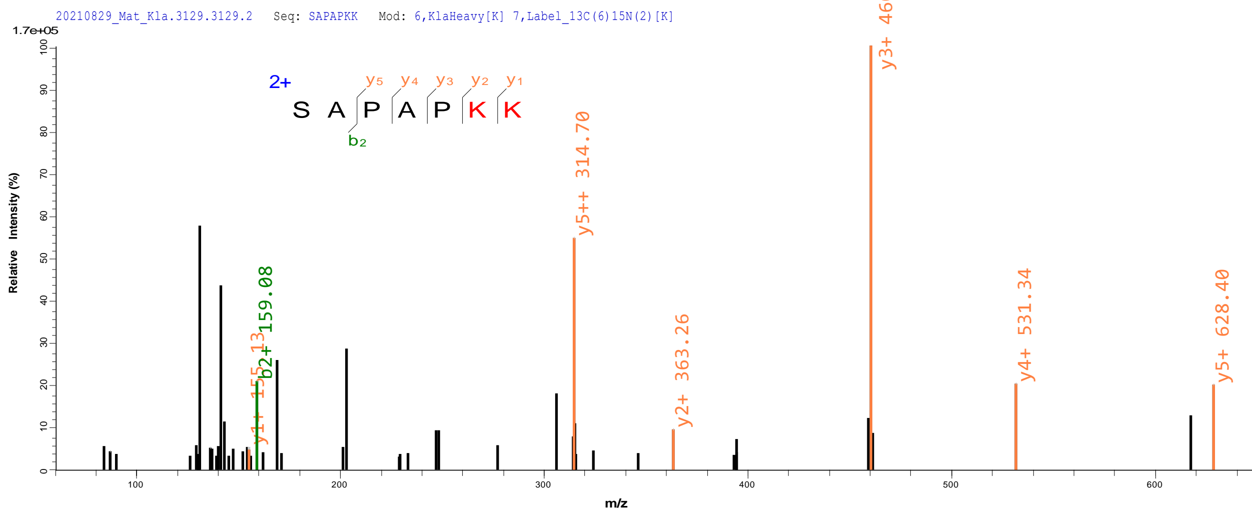
AGGK(ac)AGK(ac)DSGK(la)AK



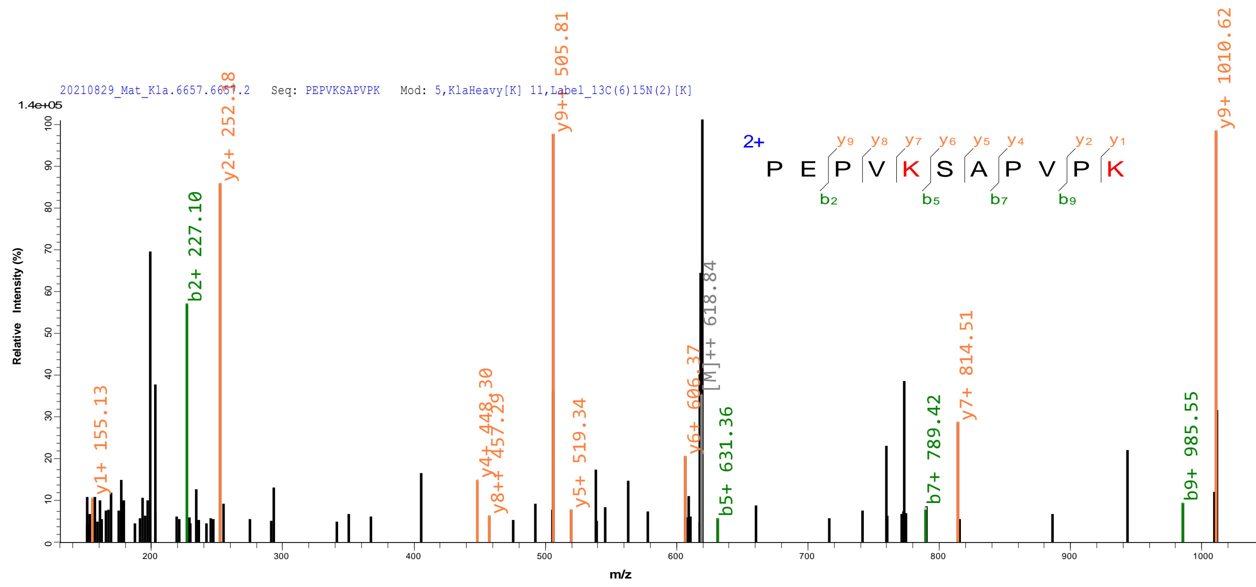
AGK(ac)DSGK(la)AK



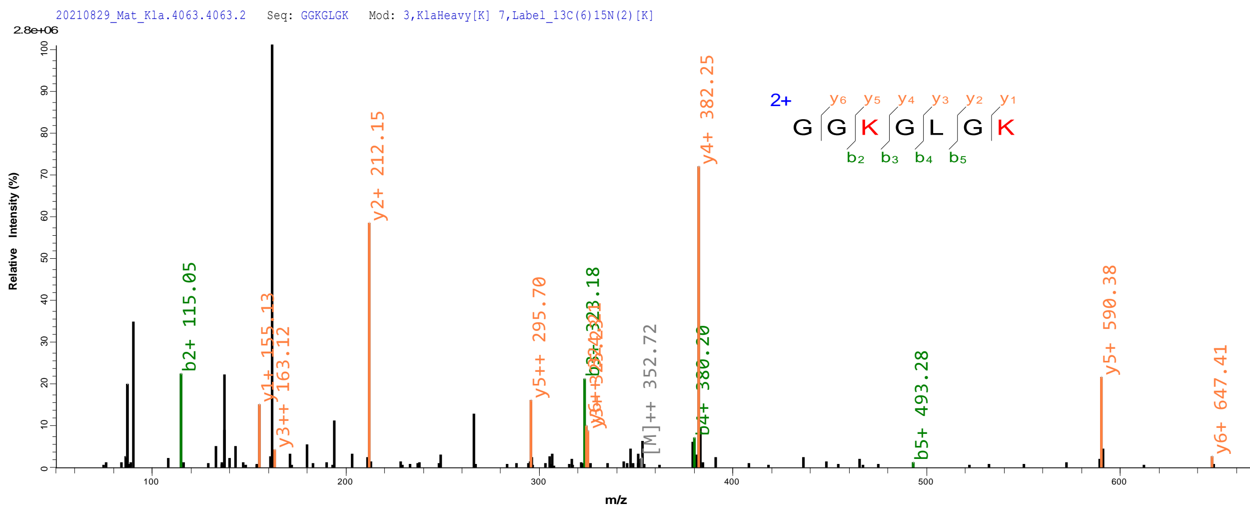
SAPAPK(la)K



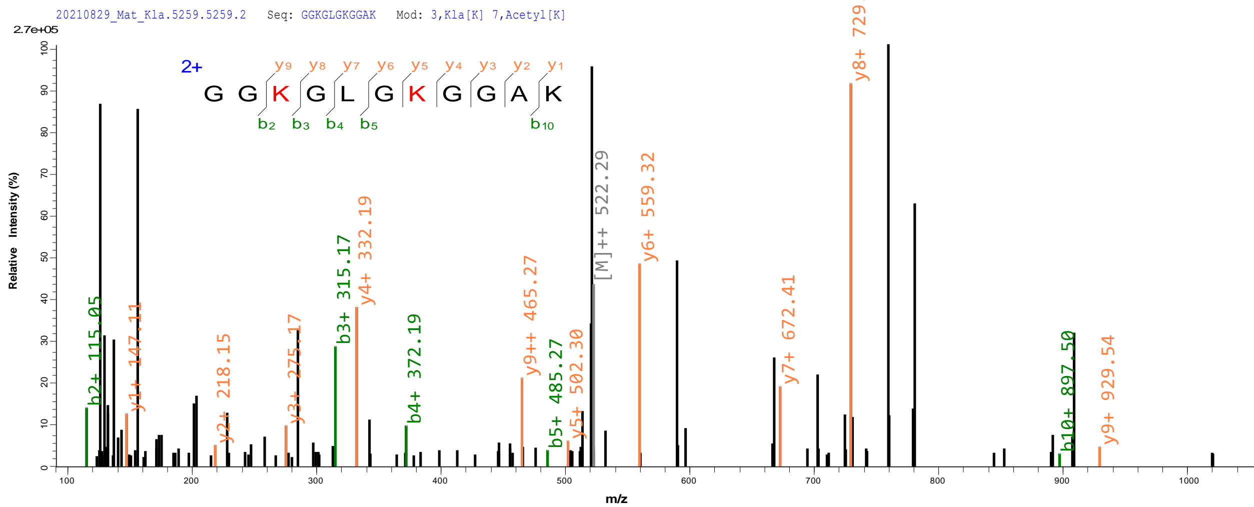
PEPVK(la)SAPVPK



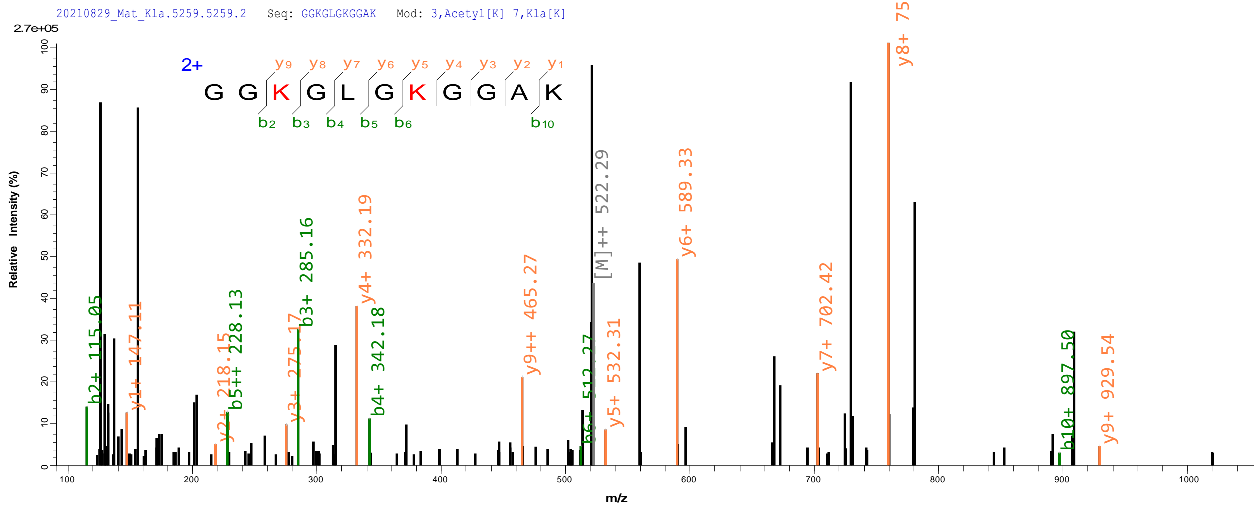
GGK(la)GLGK



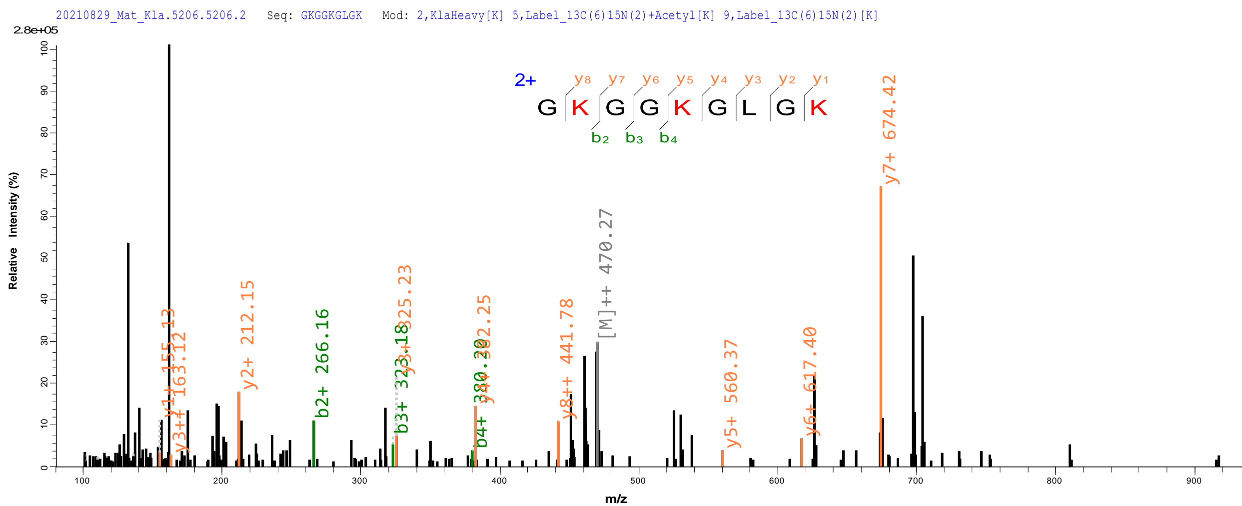
GGK(la)GLGK(ac)GGAK



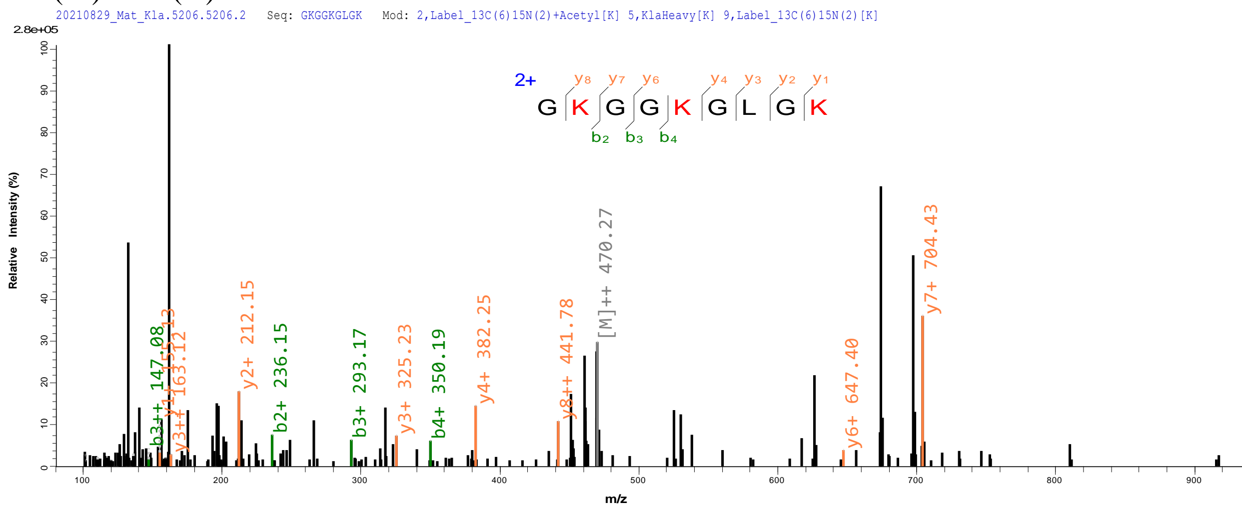
GGK(ac)GLGK(la)GGAK



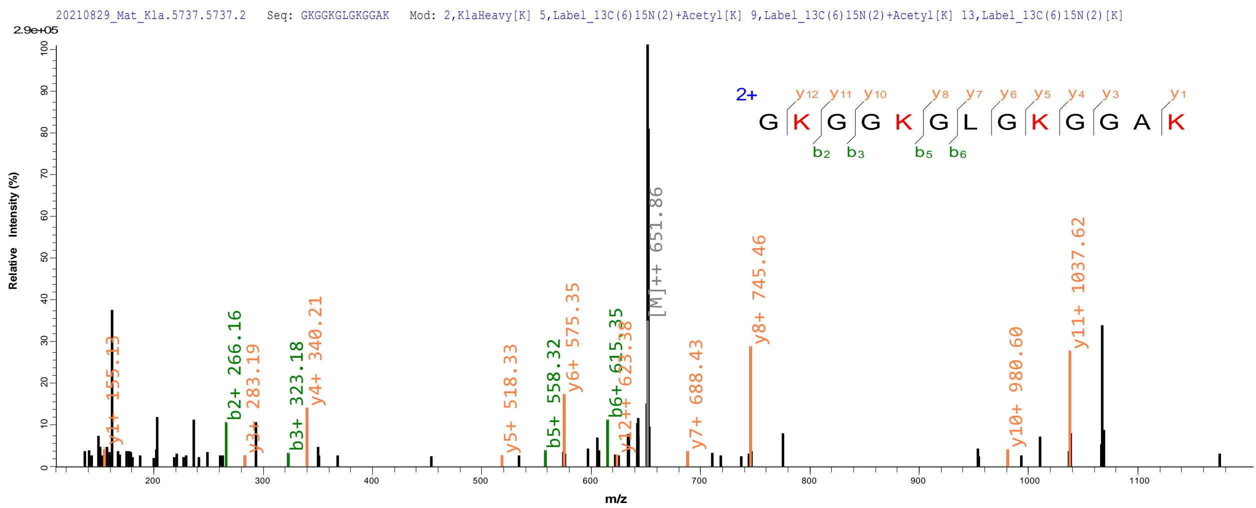
GK(la)GGK(ac)GLGK



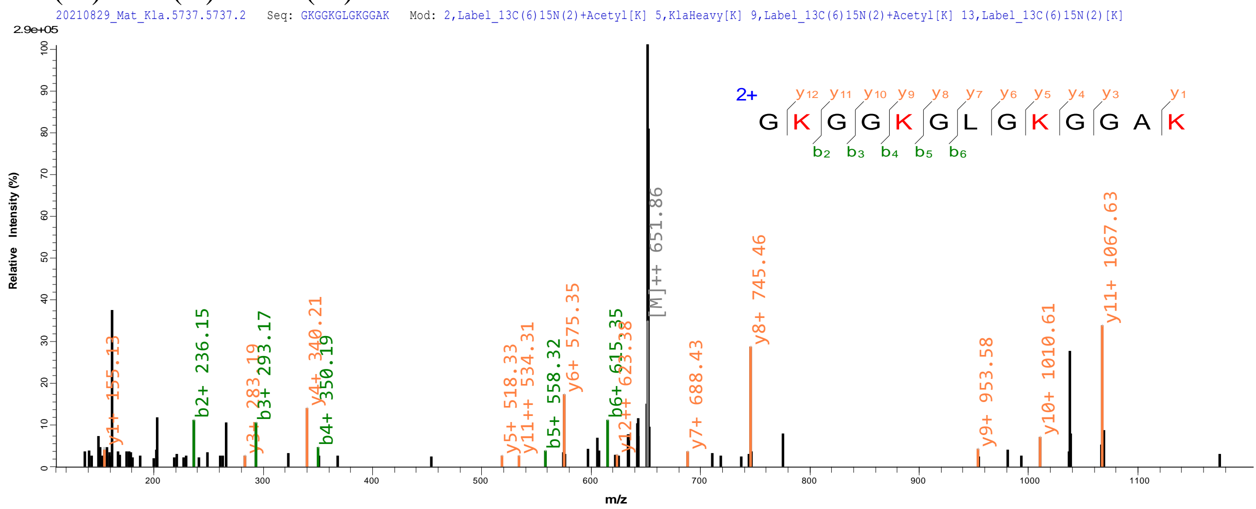
GK(ac)GGK(la)GLGK



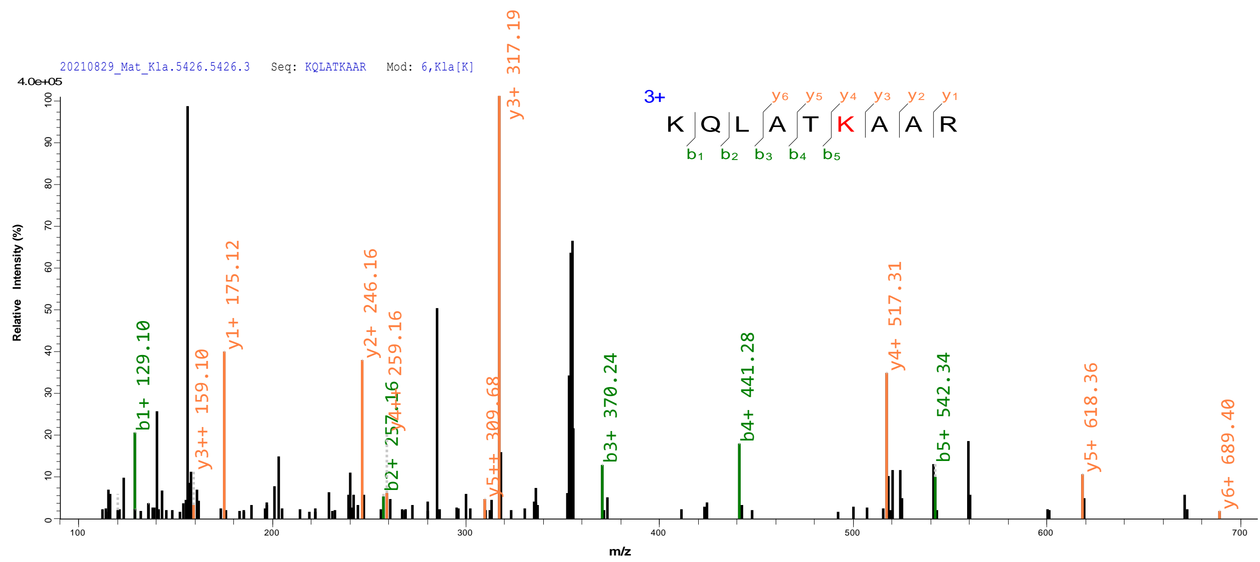
GK(la)GGK(ac)GLGK(ac)GGAK



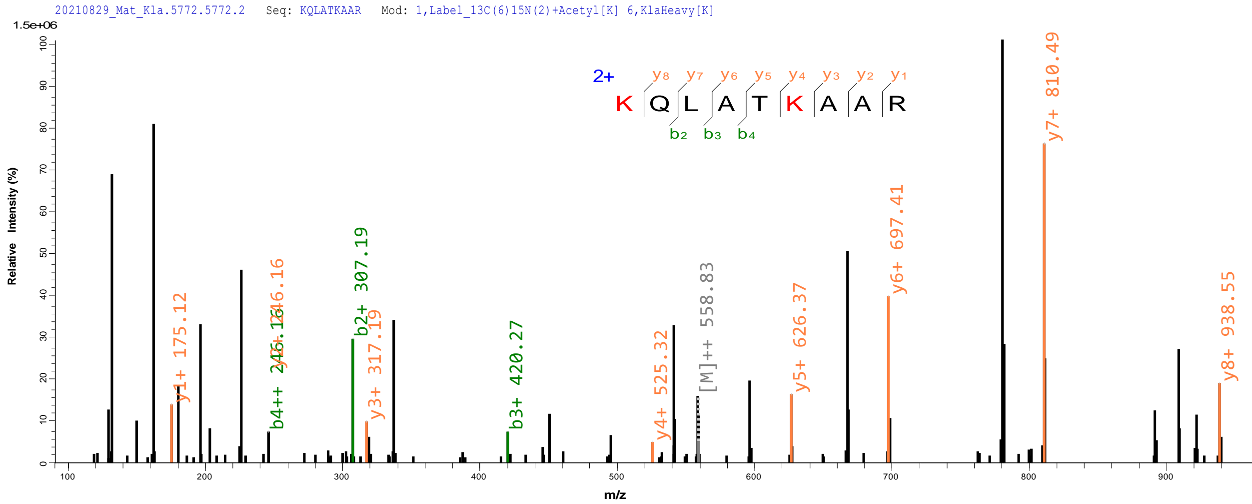
GK(ac)GGK(la)GLGK(ac)GGAK



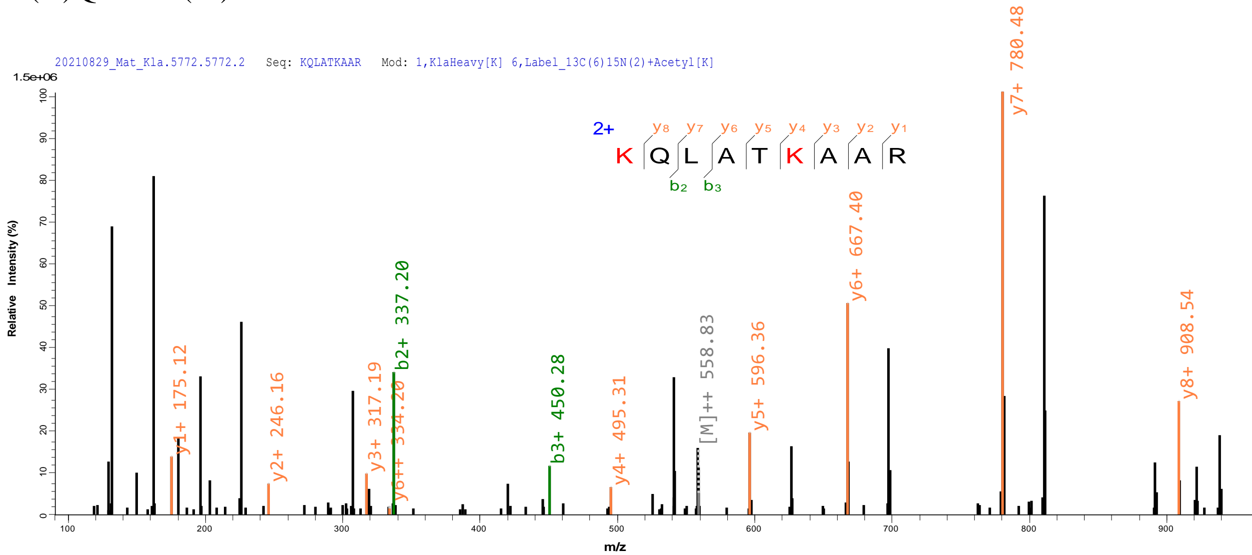
KQLATK(la)AAR



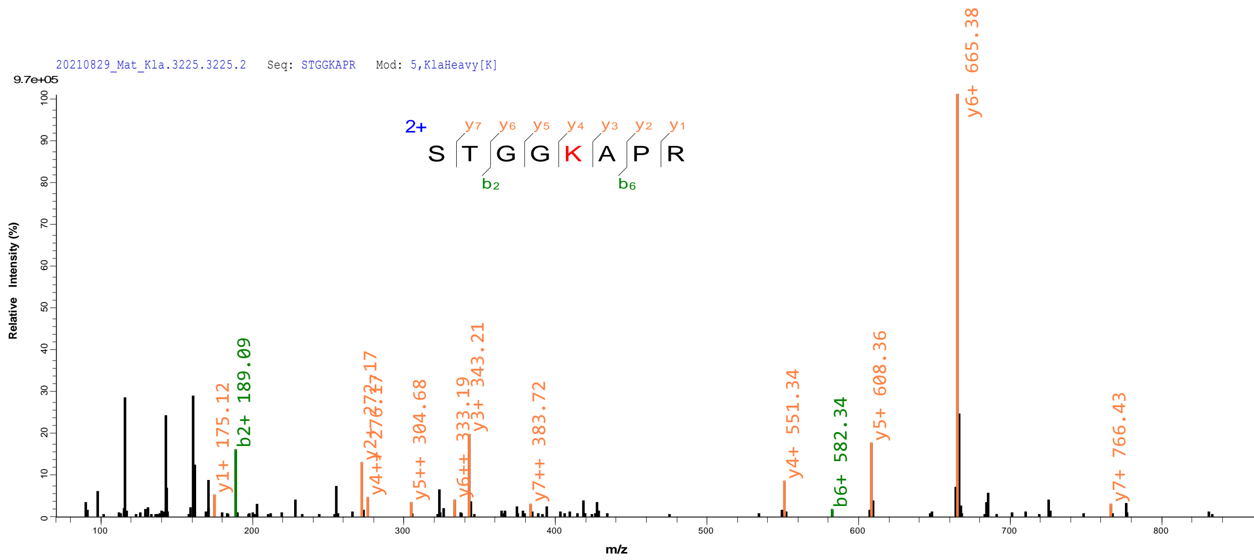
K(ac)QLATK(la)AAR



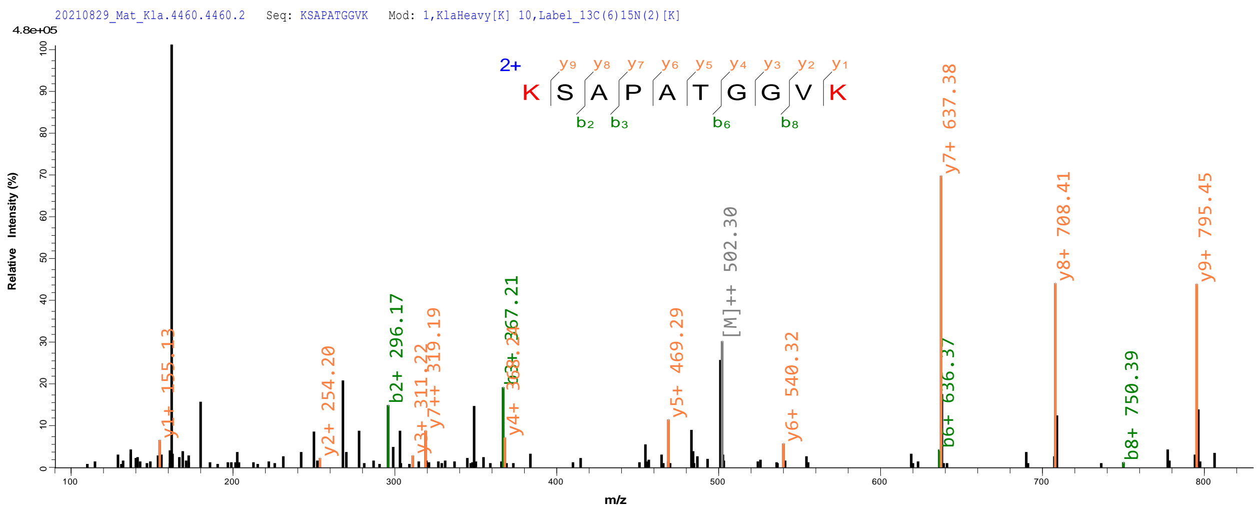
K(la)QLATK(ac)AAR



STGGK(la)APR



K(la)SAPATGGVK


20210829_Mat_Kla.4460.4460.2 Seq: KSAPATGGVK Mod: 1,KlaHeavy[K] 10,Label_13C(6)15N(2)[K]
4.8e+05
Relative Intensity (%)
m/z
2+
K S A P A T G G V K
y1+ 155.13
y2+ 254.20
b2+ 296.17
y3+ 311.22
y7++ 319.19
b3+ 367.21
y4+ 368.24
y5+ 469.29
[M]++ 502.30
y6+ 540.32
b6+ 636.37
y7+ 637.38
y8+ 708.41
b8+ 750.39
y9+ 795.45