July 2017

Regulation of Extracellular Signal-Regulated Kinase by Histone Deacetylase 6

Jheng-Yu Wu
University of South Florida, jhengyu.wu@gmail.com

Follow this and additional works at: http://scholarcommons.usf.edu/etd
Part of the Biochemistry Commons, and the Cell Biology Commons

Scholar Commons Citation
http://scholarcommons.usf.edu/etd/6985

This Dissertation is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.
Regulation of Extracellular Signal-Regulated Kinase by Histone Deacetylase 6

by

Jheng-Yu Wu

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Department of Pathology and Cell Biology
Morsani College of Medicine
University of South Florida

Major Professor: Xiaohong Zhang, Ph.D.
Santo V. Nicosia, M.D.
Patricia Kruk, Ph.D.
Jiandong Chen, Ph.D.

Date of Approval
July 3, 2017

Keywords: Acetylation, MAPK, PTM, inhibitor

Copyright © 2017, Jheng-Yu Wu
Dedication

I dedicate this work to my wife, Ning Xu, for being there with me at every step of this journey. I also dedicate this work to my parents, Jui-Nan Wu and Jui-Hsin Wu Keng, and all of my family. I would like to thank them for supporting me emotionally, and giving me the confidence to embark upon this journey.
Acknowledgements

I would like to sincerely thank my mentor Dr. Xiaohong (Mary) Zhang for her great guidance and patience in my graduate education, and for providing a nurturing research environment. I will be always grateful to her for all her encouragement and advice. I would like to sincerely acknowledge my committee members Drs. Patricia Kruk, Santo Nicosia, and Jiandong Chen for all the great suggestions and scientific discussion they cheerfully provided.

I want to express my sincere appreciation to former laboratory members Drs. Shengyan (Kevin) Xiang, Huiqin Dong, and Kendra Williams, and present member, Dr. Mu Zhang, for guiding me as a graduate student in the laboratory, as well as other members including Nicolette Moses, Joshua Haakenson, Drs. Chen Hu and Wei Liu for their support. I would like to thank all undergraduate students including Madison Schmidtmann, Xin Ke, Ianne Itchon, and Kabir Kewalramani for their help and for creating a wonderful working atmosphere.

I would like to thank the faculty and staff of Department of Pathology and Cell Biology in Morsani College of Medicine at University of South Florida for their educational and financial support. I would also like to thank the faculty and staff of the Medical Science Ph.D. program for all their support. I also express my gratitude to the North America Changhua Association for providing financial support through the Jackson Yang Scholarship. I would like to wholeheartedly thank all faculty and staff of The Cancer Biology Graduate Program at the Wayne State University School of Medicine and the Karmanos Cancer Institute, especially Drs. Larry Matherly, George Brush, Manohar Ratnam, and Stephan Patrick, and Nadia Daniel and Jean Guerin for providing the excellent research environment and all their support.

Finally, I am thankful to my family and my friends for all their unwavering backing and encouragement.
# Table of Contents

- List of Figures ........................................................................................................ iii
- List of Tables ........................................................................................................... v
- Abstract .................................................................................................................. vi

Chapter 1: Introduction .............................................................................................. 1
  - Overview of Histone Deacetylase ...................................................................... 2
  - Overview of HDAC6 ......................................................................................... 2
    - Clinical Relevance of HDAC6 ....................................................................... 4
    - Inhibitors of HDAC6 ................................................................................... 5
  - Overview of the MAPK pathway ....................................................................... 5
    - Post-Translational Modification of the MAPK Pathway .............................. 7
    - Post-Translational Modification of ERK1/2 .................................................. 7
  - Overview of MAPKs ......................................................................................... 8
    - p38 ............................................................................................................... 9
    - JNK ........................................................................................................... 10
    - ERK5 ......................................................................................................... 11
  - MAPK Pathway and Cancer ............................................................................. 12
    - RAF ........................................................................................................... 12
    - MEK ........................................................................................................... 13
    - ERK1/2 ....................................................................................................... 14
    - p38 .............................................................................................................. 15
    - JNK ........................................................................................................... 16
    - ERK5 ......................................................................................................... 17
  - Inhibitors of MAPKs ......................................................................................... 17
  - Interaction of HDACs and MAPKs ................................................................... 20
  - Combination Treatment of HDAC Inhibitors and MAPK Pathway Inhibitors .... 20

Chapter 2: Materials and Methods .......................................................................... 22
  - Antibodies ....................................................................................................... 22
  - Chemicals and Reagents ............................................................................... 22
  - Plasmids construction ................................................................................... 23
  - Cell Culture and Cell Lines ........................................................................... 25
  - Establishment of HDAC6 Stable Knock-Down A549 Cells ........................ 25
  - Establishment of ERK1 Stable Knock-Down H292 and A549 Cells ............. 26
  - Immunoprecipitation and Immunoblotting ...................................................... 26
  - Purification of GST-Tagged ERK Proteins from 293T Cells ......................... 27
  - Purification of His-ERK1/2 Proteins ............................................................... 27
  - GST Pull-Down assay ..................................................................................... 28
  - Radioactive *In Vitro* Kinase Assay ............................................................... 28
  - Non-Radioactive *In Vitro* Kinase Assay ..................................................... 29
In Vitro Acetylation Assay ................................................................. 29

Chapter 3: ERK1/2 are Acetylated Proteins ........................................... 31
  ERK1/2 Interacts with HDAC6 Directly ............................................. 31
  Inhibition or Depletion of HDAC6 Increases ERK1/2 Acetylation .......... 33

Chapter 4: ERK1/2 Acetylation is Regulated by CBP/p300 and HDAC6 .......... 38
  ERK1/2 Acetylation is Controlled by CBP/p300 In Vivo ......................... 38
  ERK1/2 Acetylation is Controlled by CBP/p300 In Vitro ......................... 38
  HDAC6 Deacetylates ERK1 ............................................................. 39

Chapter 5: Deacetylation of ERK1/2 Affects Its Kinase Activity .................. 45
  Acetylation Sites are Identified in ERK1 and ERK2 ............................ 45
  Acetylation Mimetic of Part of ERK1 Lysine Residues Decrease ERK1 Kinase Activity on ELK1 .......................................................... 46
  ERK1 Knock-Down Decreases Colony Formation and Cell Migration in NSCLC H292 ................................................................. 55

Chapter 6: Discussion and Future Directions ......................................... 58

References ..................................................................................... 63
List of Figures

FIGURE 1. ERK1/2 interacts with HDAC6 physically. ................................................................. 32
FIGURE 2. HDAC6 interacts with both N-terminus and C-terminus of ERK1/2 .......... 32
FIGURE 3. Inhibition of HDAC6 increases ERK1/2 acetylation. ............................... 34
FIGURE 4. Nicotinamide treatment does not increase ERK1/2 acetylation. .......... 35
FIGURE 5. Depletion or knockdown of HDAC6 increases ERK1/2 acetylation .... 37
FIGURE 6. ERK1 is acetylated by CBP and p300. ................................................................. 40
FIGURE 7. ERK2 is acetylated by CBP and p300 ................................................................. 42
FIGURE 8. CBP acetylates ERK1 and ERK2 in vitro. ......................................................... 43
FIGURE 9. HDAC6 deacetylates ERK1. .............................................................................. 44
FIGURE 10. Acetylation sites of ERK1 and ERK2. ......................................................... 47
FIGURE 11. The Lysine 72 site of ERK1 is acetylated. .................................................. 48
FIGURE 12. The Lysine 287 site of ERK1 is acetylated. ................................................ 48
FIGURE 13. The Lysine 294 site of ERK1 is acetylated. ................................................ 49
FIGURE 14. Acetylation-mimetic mutation change the phosphorylation level in ERK1 and ERK2. ................................................................. 50
FIGURE 15. The acetylation-mimicking mutant of ERK1(K65Q) decreases the ERK1 kinase activity ................................................................. 51
FIGURE 16. The acetylation-mimicking mutant of ERK1(K72Q) decreases the ERK1 kinase activity. ................................................................. 52
FIGURE 17. Potential structural effects of K72 mutation and acetylation. .......................53

FIGURE 18. ERK1 Lysine 72 and Lysine 65 show conservation in animals.....................55

FIGURE 19. ERK1 knockdown in H292 and A549.........................................................56

FIGURE 20. Knockdown of ERK1 in H292 weakens its ability of colony formation and migration.................................................................57
List of Tables

Table 1. The acetylation sites detected by mass-spectrometry ........................................47
Abstract

Extracellular signal-regulated kinases 1/2 (ERK1/2) are important kinases regulating cell proliferation and cell migration, and have been established as therapeutic targets for cancer treatment. Previously, we found that ERK1 phosphorylates histone deacetylase 6 (HDAC6) to regulate its enzymatic activity. However, whether HDAC6 reciprocally modulates ERK1 activity is unknown. Here, we have discovered that ERK1/2 are acetylated proteins and shown that HDAC6 manipulates ERK1’s kinase activity via deacetylation. We demonstrated that both ERK1 and ERK2 interact with HDAC6 physically. We showed that the acetylation level of GST-ERK1/2 increased in a dose- and time-dependent manner upon treatment with a pan-HDAC inhibitor, Trichostatin A. Furthermore, the treatment by HDAC6-specific inhibitor, ACY-1215, also increased the level of acetylated GST-ERK1/2. We also noted that ERK1/2 acetylation levels increased in HDAC6-knockout mouse embryonic fibroblasts and in HDAC6-knockdown A549 cell lines compared with controls. In addition, we determined that acetyltransferases CBP and p300 acetylate ERK1/2. We have identified novel acetylation sites located in ERK1 and ERK2 by mass-spectrometry analysis. Among these acetylation sites, ERK1 lysine 72 acetylation status is related to ERK1 phosphorylation. The acetylation-mimicking mutant exhibits a decreased kinase activity toward ELK1, while the deacetylation-mimicking mutant exhibits a similar level of kinase activity as the wild-type ERK1, suggesting that acetylation/deacetylation alters ERK1 enzymatic activities. Taken
together, our results suggest that HDAC6 may regulate ERK1’s kinase activity via deacetylation of its lysine 72 residue.
Chapter 1
Introduction

Overview of Histone Deacetylases

Histone deacetylases (HDACs) are a family of enzymes that remove acetyl groups from ε-lysine residues of core histones and non-histone proteins. HDACs work in conjunction with histone acetyltransferases (HATs), which add the acetyl groups to ε-lysine residues of core histones and non-histone proteins, thus governing the acetylation status of their substrates. HDACs and HATs work as erasers and writers, respectively, to deacetylate and acetylate proteins in the cell (1,2). Because more and more non-histone targets have been identified for these enzymes, HDACs and HATs are also termed as lysine deacetylases (KDACs) and lysine acetyltransferases (KATs) respectively to emphasize their functions (1).

A total of 18 histone deacetylases have been identified in humans, and are grouped into four different classes, I - IV, according to their homology to yeast (S. cerevisiae)'s prototype. Class I includes HDAC 1, 2, 3 and 8, and these HDACs are homologous to yeast Rpd3. Class II is homologous to yeast Hdal and is further divided to class IIa and class IIb. Class IIa contains HDAC 4, 5, 7 and 9, and HDAC 6 and 10 belong to class IIb. HDAC11 is the only member of class IV. The deacetylase activity of all members of classes I, II and IV is zinc-dependent. The final class of HDACs, class III, consists of
SIRT1-7. These Class III HDACs are homologous to yeast Sir2, and are alternatively termed the Sirtuin family. The deacetylase activity of the Sirtuin family is nicotinamide adenine dinucleotide (NAD$^+$)-dependent (1,2).

Some pan-HDAC inhibitors are widely used in the laboratory as a tool to study these HDACs. Trichostatin A (TSA) is a pan-HDAC inhibitor targeting class I, II and IV HDACs, while class III Sirtuins are sensitive to nicotinamide. Sodium butyrate (NaB) is an interesting HDACi considered to have pan-inhibitory activity, but excludes HDAC6, HDAC10 and all class III HDACs (1,2).

In the past, most studies of HDAC activity has focused their modulation of core histones; however recent studies have revealed non-histone and non-nuclear substrates of HDACs. Numerous of acetylated non-histone proteins have been identified in the nucleus, cytosol, and organelles (1-3). Hence, HDACs have a much wider spectrum of substrates than what we originally thought.

**Overview of HDAC6**

Unlike other HDACs, HDAC6 is unique in that it has two successive deacetylase domains, both of which are necessary for HDAC6 deacetylase function (4). This protein contains two nuclear export signals (NES), one located on its N-terminus and the other on its C-terminus. A nuclear localization signal (NLS) is present on the N-terminus, and a Ser-Glu- containing tetradecapeptide repeat region (SE14) is identified at the C-terminus. HDAC6 also has a unique ubiquitin-binding domain, zinc finger (ZnF) that is not found in
other HDACs (5,6).

Although the reports have shown that both deacetylase domains of HDAC6 are needed for its activity, the mutation of the similar residues of these two domains gives different outcomes. The substitution of histidine 216 in the first deacetylase domain to alanine (H216A) decreases catalytic activity by no more than threefold, however, the mutation of histidine 611 in the second domain (H611A) dramatically decreases activity by over 5000-fold. This data suggests that the second domain plays a critical role in HDAC6’s deacetylase action (4). Data published by our lab in 2014 has shown that the first domain of HDAC6 also has ubiquitin E3 ligase activity, whereas the second domain is purely a deacetylase (7). The unique SE14 domain serves as a cytoplasmic anchor of HDAC6. ZnF can bind to polyubiquitinated proteins when the cell is under misfolded protein stress, and HDAC6 leads the proteins to an aggresome for degradation (8-10).

HDAC6 plays a variety of roles in normal cellular functions, such as migration, gene transcription, and stress response mediation. By interacting with cytoskeleton proteins and their association proteins, α-tubulin and cortactin, HDAC6 shows the ability to control cell mobility (11,12). Runx2 is the first transcription factors found to bind HDAC6. Through interacting with p21, the function of Runx2 is manipulated by HDAC6 (13). Ligand-dependent corepressor (LCoR) is another transcription factor affected by HDAC6, and further changes the transcription of target genes (14,15). By interacting with numerous stress response proteins, such as heat shock protein 90 (HSP90), dynein, Tax, p62, Tripartite motif-containing 50 (TRIM50), and Ras-GTPase-activating protein SH3 domain-binding protein 1 (G3BP), HDAC6 participates in the cellular stress response
HDAC6 interrupts the complex of heat shock factor protein1 (HSF1) and HSP90, and further activates HSF1 for assistance in folding midfolded proteins. Through interacting with G3BP, Tax, p62, HDAC6 is involved in the formation and storage of stress granules (20). HDAC6’s activity during the stress response process is also increased after phosphorylation by casein kinase 2 (CK2) (21).

HDAC6 regulates other types of cellular functions, including autophagy and cell cycle (22,23). Furthermore, HDAC6 also regulates DNA mismatch repair protein MSH2 by deacetylation and ubiquitinating it to retain MutSα homeostasis (7). In addition, HDAC6 has been shown to be involved in signal transduction. Cell signaling regulator K-Ras and β-catenin are both deacetylated by HDAC6, further altering their oncogenic activity and nuclear localization, respectively (24-26).

**Clinical Relevance of HDAC6**

HDAC6 shows higher messenger RNA (mRNA) expression and higher protein expression in multiple cancers and cancer cell lines. **HDAC6 mRNA exhibits higher level in cancers such as colon, pancreas, breast, ovarian, brain, oral squamous cell carcinoma and acute myeloid leukemia (27,28).** In leukemia cells, HL60, K562, and KG1a, the mRNA status of **HDAC6 is unregulated** (29). However, some clinical cases have shown an intriguing phenomenon; patients with higher levels of HDAC6 display improved survival rates due to better response to HDAC inhibitors (30).
HDAC6 function has also been linked to the process of tumorigenesis. Knockdown of HDAC6 prompts the shrinkage of xenograft in mice (31). Additionally, HDAC6 is found to be essential for oncogenensis, through deacetylating survivin and further incapacitating apoptosis, as observed in cancer cell lines such as SKOV3, SKBR3, and MCF7, as well as mouse embryonic fibroblasts (MEFs) (31-33). By interacting with Ku70 and Tat, HDAC6 also serves important roles in cancer-associated signaling pathways (34). Furthermore, inhibition of HDAC6 can induce DNA damage and sensitizes transformed cell to anticancer agents (35). Our lab’s previous work shows that HDAC6 confers cisplatin resistance upon non-small cell lung cancer (NSCLC) and ovarian cancer (36).

Inhibitors of HDAC6

HDAC inhibitors have been used in the treatment of cancer. The first FDA-approved HDAC inhibitor, Vorinostat (SAHA), was used to treat the cutaneous T-cell lymphoma (CTCL) patients with progressive, persistent or recurrent disease. Several pan-HDAC inhibitors, such as Panobinostat (LBH589) and Pracinostat (SB939), have also gained FDA approval. However, the single-target compounds, which can specifically inhibit HDAC6, are necessary to avoid the off-target effects associated with pan-HDACi. Rocilinostat (ACY-1215) and Citarinostat (ACY-241) are two HDAC6-specific inhibitors currently in clinical trials. They are either used alone or in combination with other anti-cancer drug to treat multiple myeloma, malignant melanoma, leukemia, NSCLC, and breast cancer. However, the mechanisms of antitumor activity of these HDAC6 inhibitors are not entirely clear.

Overview of the MAPK Pathway
The MAPK signal transduction pathway, EGFR-RAS-RAF-MEK-ERK cascade or ERK1/2 pathway, plays an important role of regulating cell proliferation, migration, gene transcription, and cell survival (37). Besides EGFR, ERK1/2 pathway can also be activated by other factors that include EGF, PDGF, GPCRs, PDGF, osmotic stress and cytokines (38,39). Activated ERK1/2 can phosphorylate more than 160 substrates from cytoplasm, nucleus to cell membrane (40). By phosphorylating these substrates, ERK1/2 can regulate cell functions.

Mutational activation of the components in this cascade has been reported in several kinds of cancer, such as lung cancer, melanoma, pancreatic cancer and colon cancer (41). One-third of all types of cancers have been detected with Ras mutations, and these mutations keep Ras constantly binding to GTP and continuously activated (41). 70% melanoma cases harbor active mutational Raf, and this is critical for malignant transformation and tumorigenesis (42,43). Moreover, in several cancer types including lung cancer, EGFR harbors mutational activation (44-46). These active mutations make EGFR, Ras and Raf suitable targets for inhibitor development and therapeutic treatment (47-54), although intrinsic and acquired resistance to these inhibitors is a critical challenge in the field that must be overcome (55,56).

ERK1/2 are the main moderators in the downstream portion of the MAPK pathway, so these two kinases are obtaining interesting as alternative targets for cancer treatment, especially the cases resistant to the inhibitors of the upstream components (57). Thus far, there are several ERK specific inhibitors that have been developed and used to
overcome the resistance to EGFR, Raf or MEK inhibitors (58-61). So far, more than 160 ERK substrates have been discovered, several of which have important roles in cancer progression, including c-Myc and ELK1 (40). c-Myc controls cell cycle progression and is considered to be a proto-oncogene. ERK phosphorylates serine 62 of c-Myc and controls c-Myc stability (62). ELK1 is one member of ETS transcription factor family, and serine 383 of ELK1 is phosphorylated by ERK. The phosphorylation status of ELK1 S383 is critical for its transcriptional activation (63).

**Post-Translational Modification of the MAPK Pathway**

Post-translational modification (PTM) is a crucial method to quickly modulate protein functions. The PTMs of histones manipulate gene transcription, and PTMs are strongly critical in perpetuating a signaling cascade. Histone acetylation is the well-known example of acetylation status regulating protein functions; however, there are many cytoplasmic proteins that can also be acetylated (64-66). Beside the indispensable phosphorylation, reports have shown that acetylation also plays a significant role in EGFR-RAS-RAF-MEK-ERK signaling. Three members in this pathway have been identified as acetylated proteins. HDAC6 controls EGFR acetylation and further regulates its intracellular trafficking, stability and degradation (67). The acetylation level of Ras is manipulated by HDAC6 and SIRT2, and they further influence the oncogenic activities of Ras (24). In addition, mitogen-activated protein kinase kinase-1 (MEK1) has been reported to be acetylated by SIRT1 and SIRT2. Both Sirtuin family deacetylases negatively regulate MEK1 activity (68).
PTM of ERK1/2

Although ERK1 phosphorylation, especially the Thr202 and Tyr204 sites (Thr185/Tyr187 in ERK2) of the Thr-Xxx-Tyr motif, has been well studied, other post-translational modifications (PTMs) of ERK1 such as acetylation, methylation and ubiquitination are not well characterized. Recently, it has been discovered that ERK1/2 can be both methylated and unubiquitinated. Two ERK1 C-terminal sites, Lys302 and Lys361, are revealed being tri-methylated, and these methylations enhance ERK1’s phosphorylation (69). Arg309 of ERK1 has also been reported as a methylation site via proteome-wide analysis, but the function of this methylation has yet to be clarified (70). Ubiquitination of ERK1 and ERK2 has also been reported, but the impact of ubiquitination on ERK1/2 is uncharacterized (71-73). One ERK1 acetylation site has been identified by SILAC assays, but the function and the effect of this acetylation site still remains unknown (74). Several different reports showed that when cell lines, including A549, MB361, BT474, MV4-11, PC-3, SKBR-3, HN-9 and SQ20B, are treated with HDAC6 inhibitors, the level of phospho-ERK1/2 decreases (75-78). However, the manner by which HDAC6 inhibition regulates ERK1/2 phosphorylation is still not very clear.

Overview of MAPKs

Mitogen-activated protein kinases (MAPKs), also named extracellular signal-regulated kinases (ERKs), are a conserved group of serine/threonine protein kinases associated with various essential cellular processes. So far in human, 14
different MAPKs are identified, and these MAPKs are grouped to seven different classes. ERK 1/2, P38 α/β/ρ/δ, c-Jun N-terminal kinases (JNK) 1/2/3, and ERK5 are classified as conventional MAPKs, and the other three MAPKs, Nemo-like kinase (NLK), ERK3/4 and ERK7 are classified to atypical MAPKs. Among these MAPKs, members of conventional group are well studied, but the atypical MAPKs are still not understood clearly (79). All MAPKs are regulated by their upstream kinases that are activated by multiple extracellular signals. Upon initiation of the cascade by extracellular stimulus, first tier MAPKKKs are activated, which stimulate MAPKKs to phosphorylate MAPKs. These MAPK pathways have their own primary kinases in different tiers, but they also share some minor kinases (79,80). All MAPKs have been found to contain a conserved Thr-Xxx-Tyr motif in their kinase domain, except ERK3/4 and NLK. The phosphorylation of this motif is a critical step in their activation (79).

p38

There are four p38 kinases: p38α, p38β, p38γ and p38δ all have a Thr-Xxx-Tyr motif. In the p38 family, this motif is a Thr-Gly-Tyr (TGY) motif. These kinases gain their activation through phosphorylation of threonine and tyrosine in this motif by MKK3 and MKK6 (81-83). The Tyr323 site of p38 can be phosphorylated by lymphocyte-specific protein tyrosine kinase (Lck) in normal T cells after antigenic stimulation, and then induce p38 autoprophosphorylation of Thr180 and Tyr182, thus activating p38 (84). One paper published in 2006 showed that phosphorylation of Thr123 that is located in docking groove (D domain) by GRK2 inactivates p38 by blocking the binding between p38 and its substrates (85).
Through several different global kinomic or proteomic surveys, some new phosphorylation sites of p38 were distinguished, they are: Ser2, Thr16, Thr175 and Thr263 of p38α (86-88), Ser243 of p38β (89) and, Thr265 and Ser250 of p38δ (90). More work is necessary to discern the function of these sites. In p38γ, one new phosphorylation site, Ser3 on the N-terminus was found besides Thr183 and Tyr185, which are equivalent to the threonine and tyrosine of TGY motif. Phosphorylations of Thr183 and Tyr185 activate p38γ and result Ser3 phosphorylation, but the phosphorylation level of Ser3 does not affect the kinase activity of p38γ (91).

In p38, so far there are two acetylation sites, Lys 53 and Lys152, and each has a specific effect on p38 activity. Lys53 of p38 is located in the ATP-binding pocket, and acetylation of Lys53 residue promotes ATP binding and enhances p38 phosphorylation of its substrates (92). Lys152 of p38 is located in a substrate binding domain, nonacetylation mimic mutation or alanine mutation both decrease phosphorylation level of p38 substrates but do not affect the binding p38 and its substrates (92,93). Two reports utilizing comprehensively proteomic survey reveal some ubiquitination sites of p38. In p38α, Lys15, Lys139 and Lys165 were found. Lys226 of p38δ was also found to be a ubiquitination site (94,95). Like most of the other high-throughput surveys that have been conducted, these sites still need further works to validate their functions.

**JNK**

There are three different JNKs currently classified: JNK1, JNK2 and JNK3. As other MAPKs, all of these JNKs have their own conserved Thr-Xxx-Tyr motif, Thr-Pro-Tyr (TPY) motif and, phosphorylation of threonine and tyrosine of this motif is needed for
JNK activation. Through a new PTMScan direct method, several phosphorylation sites have been found in JNK1 (Ser179, Thr183, Tyr185 and Thr188), JNK2 (Thr188) and JNK3 (Thr216, Ser217, Thr221, Tyr223 and Thr226), but with the exception of the threonine and tyrosine in TPY motif, the function of these phosphorylation sites still remains unclear (87). Another proteomic analysis of protein kinases revealed that Thr178 of JNK1 can be phosphorylated, but further analysis is required to know the effect of phosphorylation on this site (96).

In 2000, a report revealed that Thr404 and Ser407 of JNK2a2 can be phosphorylated by either MMK7 or CDK2, but the phosphorylation status of these two residues does not affect JNK2a2 activity directly. The phosphorylation status of these two residues may play other roles related to JNK2a2’s partner binding, stability or subcellular localization (97). For ubiquitination, by proteomic survey, Lys300 of JNK1 and Lys250 and Lys353 of JNK2 are found be ubiquitinated (94,95). The Lys250 of JNK2 can be acetylated as well (98), but the functions or effects of the modifications of these sites have not been explained.

**ERK5**

ERK5 was found to contain a conserved Thr-Asp-Tyr (TDY) motif in 1995 and, Thr219 and Tyr221 are phosphorylated to activate ERK5 (99,100). These two sites are phosphorylated by MEK5, the upstream kinase of ERK5, to activate the kinase activity of EKR5. Recombinant GST-ERK5 has also found to autophosphorylate these two sites in TDY motif (99). Finally, Thr28, Ser421, Ser433, Ser496, Ser731 and Thr733 can be phosphorylated by ERK5 itself when activated (101). The autophosphorylation on EKR5
C-terminal region can activate its transcriptional activity (102).

The ERK5 phosphorylation does not only occur in TDY motif and the aforementioned sites; it has been found that ERK5 can be phosphorylated during mitosis without Thr219 and Tyr221 phosphorylation (103). There are more than 16 new phosphorylation sites the have been found in ERK5 during cell mitosis, and most of them are in C-terminal region (104). This phosphorylation happens during mitosis and is regulated by CDK1 when Thr219 and Tyr221 are not phosphorylated (105). Besides phosphorylation, ERK5 are also found be sumoylated. Unlike other MAPKs, ERK5 also serves as transcription factor in nucleus. The sumoylation of Lys6 and Lys22 of ERK5 diminish its transcriptional activity but not kinase activity (106,107). ERK5 Lys35 was found acetylated by a high-throughput analysis but the function has yet to be determined (108).

MAPK Pathways and Cancer

These MAPK pathways have been reported to play roles in cancer cell maintenance, proliferation, invasion and metastasis (37,109-111), and the members of these pathways have the potential to serve as prediction or prognostic markers (112-116). Among all four conventional MAPK pathways, the RAS-RAF-MEK-ERK pathway has been strongly linked to cancer development.

RAF

Of the three Raf isoforms, BRAF is highly important due to its ability to activate its
downstream target MEK, then ARAF and Raf-1/CRAF, although sometimes alternate isoforms may show compensation to each other when one of their expression levels is low (117-119). A study from Hoeflich et al., showed that BRAF is a critical figure in tumor cell growth and maintenance in melanoma cell lines as well as in mouse model (43). Several BRAF mutations can be found across different tumor types, and in malignant melanoma, 66% of all cases contain missense mutations. These mutations are located in the kinase domain, and 80% of which are glutamic acid substitution of valine at position 599 (120). In melanoma, BRAF also shows the ability to induce transformation (42), tumorigenesis and the development of vessels (121). BRAF can also contribute to melanoma invasion (122). Besides melanoma, mutant BRAF was observed in other cancers. In low-grade ovarian serous carcinoma, the V599E mutant BRAF has been found together with codon 12 and 13 mutated KRAS (123), and 36% of low-grade serous ovarian neoplasm cases, have the V599E mutation (124). The V600E mutation and other mutations on BRAF exon 11 were identified in pediatric glioma (125). Moreover, BRAF mutations are observed in colon cancer and papillary thyroid cancer, and in each 10% and 50% contain a mutation in BRAF, respectively (126,127). Different BRAF mutations were screened and recognized in different types of lung cancer (128). Overall, BRAF mutations are mainly identified in melanoma, colon cancer and lung cancer, and observed in some other cancers such as ovarian cancer, glioma cancer and breast cancer (120,127).

**MEK**

MEK has been reported to be related to cancer development (129). Although there is no report thus far that directly links cancer occurrence to MEK mutation, some
researches showed that MEK mutations provide resistance of cancer cells to MEK specific inhibitors. Emery et al. revealed that melanoma relapsed patients treated with allosteric MEK inhibitor AZD6244 may develop a resistant cancer. Several mutated residues of MEK1 have been found on its drug-binding pocket or N-terminal negative regulatory helix (helix A) (130). In terms of MEK1 Q56P mutation, its homologous residue on MEK2 position 60 has also been found to be replaced from Glutamine to Proline when melanoma was chronically exposed to trametinib, and this mutation imparts on MEK2 the ability of resisting Trametinib (131). Overexpressed MEK can stimulate neuronal differentiation of PC12 cells, as well as NIH 3T3 cell transformation. Interfering mutants of MAPKK1 inhibited growth factor-stimulated differentiation, proliferation of PC12 cells and reverted v-src and ras transformed cells (132).

**ERK1/2**

Similar to MEK1/2, no ERK1/2 mutation cases have been reported, but through a random mutagenesis screen numerous point mutations conferring resistance to ERK or RAF/MEK inhibitors in ERK1 and ERK2 (133). One report showed that breast cancer patients with phosphorylated ERK1/2 have shortened duration, poor quality of anti-hormone response, and lowered survival time from therapy starting (134). However, another study has indicated that high levels of phospho-EKR1 significantly correlates with decreased recurrence frequency, lower death rate, suggesting that activated ERK1/2 could be unnecessary in advanced mammary carcinomas (135).

In bladder cancer, activation of ERK1/2 has been shown to increase migration and invasion ability of 5637 bladder cancer cell when stimulated with growth factor
proepithelin (136). A screening of NSCLC patients shows connections between the levels of nuclear and cytoplasmic phosphorylated ERK1/2, higher tumor stage, and lymph node metastasis (137). Activated ERK1/2 caused by cannabinoid receptor agonist WIN-55,212,2 in LNCaP human prostate cancer cells brings G1 cell cycle arrest through induction of cyclin kinase inhibitor p27KIP1 (138). Furthermore, endometrial cancer bears similarity to NSCLC patients with higher phospho-EKR1/2 expression in that they have better prognosis, especially when the signaling is independent from KRAS and BRAF (139). In the ERK pathway, the signal from overexpression of RAS, RAF, and MEK is highly related to cancer occurrence and happening of other cancer features like invasion and metastasis. However, EKR1/2 seems to play different roles across different cancer types, so this phenomenon still requires further study to determine its function in cancer.

p38

Although p38 is not targeted in cancer treatment as often as ERK1/2 pathway, many reports show the importance of p38 in cancer. In breast cancer, activation of p38 by heregulin-beta1 enhances expression and secretion of VEGF, which leads to endothelial cell migration (140). Another report in 2003 showed that in HER2 negative breast cancer, phosphorylated p38 reduces progression-free survival (141). p38 was also described as continuously activated in NSCLC (142), and the isoforms p38α and p38δ both lead tumor cell growth and invasion in head and neck squamous carcinoma cells (143).

In hepatocellular carcinoma, decreased activities of p38 and its upstream kinase MKK6 allow human HCC to grow unrestrictedly and gain resistance to apoptosis (144).
Similar circumstances have been found in human pancreatic cancer cell line PANC-1. Blockade of p38 by its specific inhibitor SB203580 dramatically increases proliferation of PANC-1, but this phenomena is diminished when the activity of ERK cascade is inhibited (145). One research article showed that inactivation of p38 promotes human cancers development through p53 activation (146). Down-regulation of phospho-p38 also leads to follicular lymphoma progression (147).

The isoform p38α was detected at highly expression levels and active in different type of thyroid tumoural tissues and mainly located in cytoplasm (148). However, another article reported a contrasting result, that overexpression of one p38 phosphatase DUSP26 inhibits p38-mediated apoptosis and promotes thyroid cancer growth (149). The role p38α plays in tumorigenesis may be regulated by oncogene-inducing ROS (150). Activated p38 and its upstream regulator MMK3 also correlated with progression of glioma (151).

**JNK**

Besides ERK1/2 and p38, reports about the role of JNKs and ERK5 in cancer have been published. Activation of JNK1 but not JNK2 has been shown to be related to proliferation of human and mouse liver tumors (152), and increased JNK1 activation was also detected in more than half of HCC samples, which was not seen in JNK2 (153). Another research article also showed JNK1 is more important in hepatocarcinogenesis than JNK2 (154). JNK is critical in proliferation and mobility of EPHA2-dependent NSCLC (155), and in pancreatic cancer activation of JNK1 is needed for the onset of cell migration (156). However, a later report revealed that JNK can function in opposing
manners in nonparenchymal cells; JNK promotes HCC development and JNK inhibit HCC development in hepatocytes (157). Otherwise, MKK4, an upstream regulator of both JNK and p38, acts as tumor suppressor in several different cancers (158).

**ERK5**

In breast cancer cells expressing ErbB2, ERK5 is up-regulated by Neuregulin and leads to cell proliferation (159). ERK5 has been found expressed in most breast cancer patients and overexpressed in 20% of cases, and overexpression of ERK5 correlates to a worse, disease-free survival rate (160). Activation of ERK5 is also linked to Osteosarcoma invasion through MMP9 induction (161) and metastases of oral squamous cell carcinoma (OSCC) (162). Overexpression of ERK5 and its upstream activator MEK5 triggers cell proliferation, invasion, MMP9 expression and other phenotype related to invasion like invadopodia formation in prostate cancer cell (163,164).

**Inhibitors of MAPKs**

There are several kinds of kinase inhibitors have been developed to target kinases in MAPK pathways and most of them are intentionally used to treat cancer.

For Raf, most inhibitors are designed to target B-RafV600E, which is an activation mutant that has constitutive kinase activity against downstream effectors and has already been detected in cancer types like melanoma, ovarian cancer, and colorectal carcinoma (127). Vemurafenib (PLX4032 or RG7204) is one of the selective
B-RafV600E inhibitors that prohibits melanoma cell and breast cancer cell that contain V600E mutation (51). GDC-0879 and PLX4720 are both have good inhibitory activity against tumors containing oncogenic B-RafV600E (50,165). Sorafenib, a pan-Raf inhibitors, was also developed to treat cancer. It can inhibit tumor growth and angiogenesis not only through inhibiting C-Raf, but also by blocking receptor tyrosine kinase platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor 2 (VEGFR2) (166). Besides utilizing these drugs in tumor cell proliferation inhibition, inhibitors like GW5074, which can inhibits c-Raf kinase activity in vitro, have the potential to be used against neurodegenerative diseases by increasing the activity of B-Rad in neuron (167).

MEK inhibitors have also been developed for targeting cancer, especially when the resistance to RAF inhibitors arises in cancer patients. PD098059 is an example of a drug developed to show MEK kinase inhibition and block stimulated cell growth and Ras-transformed phenotype in BALB 3t3 mouse fibroblast cells (168). In addition to wild type MEK, PD098059 also inhibits constitutively active MEK mutants (S217E and S221E) (169). U0126 is another inhibitor that is capable of inhibiting MEK (169). It does not compete with PD098059 due to each inhibitor’s unique mechanism of targeting MEK, but PD098059 has a higher IC50 than U0126 (170). Trametinib (JTP-74057) shows highly specific and potent MEK1/2 inhibition ability in xenograft models of colorectal cancer cell lines COLO25 and HT-29, and Trametinib can increase cell sensitivity to apoptosis if treated together with Akt inhibitor API-2 (171). Selumetinib (AZD6244 or ARRY-142886) is an uncompetitive inhibitor of MEK1/2, and at present, it is in clinical trials. Selumetinib displays good growth inhibition in breast and non-small cell lung cancer (NSCLC) cell
lines, and the breast cell lines containing RAF mutations and NSCLC cell lines with RAS mutation are particularly sensitive to Selumetinib (172).

To overcome the increasing cases of resistance to RAF and MEK inhibitors, a few ERK1/2 inhibitors have been developed (58-60). ERKi, a selective inhibitor of ERK1/2 has the ability to decrease cell viability in three KRAS mutant and MEK inhibitor resistant cell lines (59). The selective ERK1/2 inhibitor SCH772984 works at nanomolar concentrations and is able to induce tumor regression in xenograft models, and inhibits cell growth in B-Raf or MEK inhibitor-resistant tumor cells (60).

In other MAPK pathways, a few inhibitors have been developed. VX-745 is a pan-p38 inhibitor that can target p38α, p38β and p38γ, and also serves as a suppressor of anti-inflammatory responses (173). For each p38 isoforms, inhibitors are available to target them specifically. BIRB796 is a p38α specific inhibitor, and has high affinity for p38 at picomolar level (174). Another specific inhibitor is SB202190, which has the ability to induce apoptosis by inhibiting p38β (175).

In JNK pathway, small molecule SP600125 was developed to inhibit phosphorylation of JNK target c-Jun and decreases the expression of pro-inflammatory genes like TNF-α and IFN-γ (176). In 2012, Zhang et al. also developed a series of covalent inhibitors, which showed good inhibition of JNK’s ability to phosphorylate c-Jun. Among these inhibitors, some of them are more specific to JNK3 (177). BIX02188 and BIX02189 are MEK5 inhibitors, blocking MEK5’s catalytic function and preventing the phosphorylation of ERK5 (178).
Interaction of HDACs and MAPKs

Although HDACs and MAPKs are all important in maintaining control of many cell functions, the study of the interaction between HDACs and MAPKs are not well studied. A MAP3K, MEKK2, can be deacetylated by HDAC4, and this acetylation is negatively related to MEKK2 activity (179). The MAP2K of the ERK1/2 pathway, MEK1, has been shown to be an acetylated kinase, and its acetylation level is controlled by p300, SIRT1 and SIRT2. Two acetylation lysine residues, K175 and K362, have been identified, and acetylation of these two lysine residues increases MEK1 activity (180). Interactions between HDACs and other MAPKs were also reported. In 2011, a study revealed that HDAC3 and PCAF/p300 reversely regulate the acetylation level of p38, and acetylation of K53 residue can increase p38 activity (92). Furthermore, in our lab’s previous study, we found that ERK1 phosphorylates HDAC6 on its S1035 and increase its enzymatic activity (181).

Combination Treatment of HDAC Inhibitors and MAPK Pathway Inhibitors

Several combination treatments of MAPK pathway inhibitors and HDACi in cancer cell lines have been reported. Usage of HDACi SAHA against BCR/ABL-positive human leukemia cells together with MEK inhibitor PD184352 showed a synergistic effect (182). In 2008, cotreatment of HDACi benzamide MS-275 and MAPK pathway inhibitor AZD6244 in different human AML also showed synergy in induction of cell apoptosis (183). Another study used VPA and farnesylthiosalicylic acid (FTA, Salirasib) together to
treat NSCLC, colon carcinoma and thyroid carcinoma cell lines, and in all cell lines, this cotreatment reduced cell proliferation synergistically (184).

Cotreatment with HDACi panobinostat (LBH589) and EGFR/HER2 inhibitor Lapatinib was reported in colorectal cancer. This combined treatment showed a synergistic effect in inhibiting cell proliferation in six different CRC cell lines and worked synergistically in decreasing tumor growth in KRAS mutant xenograft models (185). Panobinostat (LBH589) worked synergistically with other TKI, such as Dasatinib, in different thyroid cancer cell lines (186). In melanoma cells, combination usage of SAHA and BRAF inhibitor PLX4720 can kill BRAF^{V600E} cells synergistically through necrosis, an observation that was repeated successfully in mouse xenografts (187). Cotreatment of SAHA with AZD6244 also showed similar result (188). A research also showed that combination treatment of ACY-1215 and RAF inhibitor can induce cell death in A375 melanoma cells (189).
Chapter 2

Materials and Methods

Antibodies

Anti-acetylated lysine mouse mAb (Ac-K-103) (#9681), anti-acetyl-p53 (Lys382) (2525), anti-p44/42 (ERK1/2) (#9102), anti-phospho-p44/42 (ERK1/2) (Thr202/Tyr204) (#9101) anti-ELK-1 (#9182), anti-α-tubulin (#2125), anti-β-tubulin (#2146), and anti-β-actin (#4967) antibodies were purchased from Cell Signaling Technology, Inc. The anti-Flag M2 antibody (F1804) and anti-Myc antibody (C3956) were purchased from Sigma. Anti-p53 (sc-126), anti-p-ELK-1(B4) (sc-8406) and anti-HDAC6 (sc-11420) antibodies were purchased from Santa Cruz Biotechnology. The Anti-HA antibody (16B12) was purchased from Covance.

Chemicals and Reagents

Q5® High-Fidelity DNA Polymerase (#M0491S), BamHI (#R0136S), NotI (#R0189S), XhoI (#R0146S), Sall (#R0138S), and SpeI (#R0133S) restriction enzymes were purchased from New England Biolabs. Protease inhibitor cocktail (#11836170001) was purchased from Roche. Protein G agarose (#15920-010) and Western blotting substrates (#32106) were purchased from ThermoFisher Scientific. Glutathione agarose (#G4510) and Trichostatin A (#T8552) were purchased from Sigma. Ni²⁺-NTA agarose (#635659) was purchased from Clontech. ELK-1 fusion protein (#9184) was purchased from Cell Signaling Technology. Recombinant CBP protein (#BML-SE452-0100) was
purchased from Enzo Life Sciences. ACY-1215 was purchased from Selleckchem.

**Plasmids Construction**

pGEX-4T-1-HDAC6 was generated by PCR using HA-HDAC6-F (190) as the template and the following primers: GST-HD6-F 5'-CCCGTGCAGACTCATGACCTCAACCGGCCAGGA-3' (Sall) and GST-HD6-R 5'-TGCGGCGCTTATAGTGGGTTGGGGCATATC-3' (NotI). The PCR product was inserted into the Sall and NotI site of the pGEX-4T-1 vector to generate pGEX-4T-1-HDAC6. pLEX-GST-ERK1 was generated from the pGEX-ERK1 plasmids described in Williams et al.(191). Briefly, ERK1 cDNA was isolated from the pDONR223-MAPK3 (Addgene Plasmid 23509) vector by PCR using the following primers: ERK1-F 5'-CCCGGATCCATGGCGGCGGCGGCTCAG-3' (BamHI) and ERK1-R 5'-GGGCTCGAGCTAGGGGGCCTCCAGCCTCCAGC-3' (Xhol). The PCR product was then inserted into the BamH1 and Xho I sites into the pGEX-4T-1 vector to generate pGEX-ERK1. GST-ERK1 cDNA was isolated by PCR using pGEX-ERK1 as the template and the following primers: GST-SpeI 5'-CCCACGTAGTATGCCCTTACTAGGTTATTG-3' (SpeI) and ERK1-R 5'-GGGCTCGAGCTAGGGGGCCTCCAGCCTCCAGC-3' (Xhol). The PCR product was then inserted into the SpeI and XhoI sites of the pLEX-MCS vector to generate pLEX-GST-ERK1. The pLEX-GST-DN-ERK1 plasmid was described in William et al. (191). The mammalian expression GST-tagged ERK2 construct pLEX-GST-ERK2 was generated as follows. Human wild-type ERK2 cDNA was amplified from pDONR223-MAPK1 (Addgene plasmid 23498) by PCR and the PCR product was inserted into pGEX-4T-1 between Sall and NotI sites to construct
pGEX-4T-1-GST-ERK2. The primers used were 5'-GCGGTCGACTTATGGCGGCGGCGGCGGCGGGGC-3' and 5'-CGCGCGGCGGCTCAAGATCTGTATCCTGGCTGGAATC-3'.
pGEX-4T-1-GST-ERK2 was then used as a template for PCR using the following primers: 5’-CCCACTAGTATGTCCCCCTATACTAGTTATTG-3’ (SpeI) and 5’-CGCGCGGCGGCTCAAGATCTGTATCCTGGCTGGAATC-3’ to generate GST-ERK2 cDNA which was further subcloned into the pLEX-MCS vector (Thermo Scientific, Catalog # OHS4735) between SpeI and NotI sites to generate pLEX-GST-ERK2.

Then we generated the mammalian expression K72Q and K72R mutants of ERK1 in the pLEX-MCS vector as described below. The GST-ERK1 cDNA was generated by PCR using pGEX-4T-1-ERK1 as a template and the following primers: 5’-CCCACTAGTATGTCCCCCTATACTAGTTATTG-3’ and 5’-GGGCTCGAGCTAGGGGGCCTCCAGCACTCC-3’. Then the PCR product was inserted between SpeI and XhoI sites to generate pcDNA3.1/Hygro-GST-ERK1. The pcDNA3.1/Hygro-GST-ERK1 (K72Q) and pcDNA3.1/Hygro-GST-ERK1 (K72R) plasmids were made by site-directed mutagenesis using the following primers: for K72Q, 5’-GTGGGCCATCAAGCGATCAGCCCTTC-3’ and 5’-GATGGGCCACGCGAGTCTTGCGCA-3’; for K72R, 5’-GTGGGCCATCAAGAGGAGTCTTGCGCA-3’ and 5’-GATGGGCCACGCGAGTCTTGCGCA-3’. The PCR cycle for site-directed mutagenesis was as follows: 95 °C 5 min, 95 °C 3 min, 55 °C 1 min, 72 °C 6 min for 16 cycles; and finally 72°C 10 min. Then the pcDNA3.1/Hygro-GST-ERK1 (K72Q) and pcDNA3.1/Hygro-GST-ERK1 (K72R) plasmids were cut with SpeI and XhoI to isolate the the cDNA fragments of GST-ERK1(K72Q) and GST-ERK1(K72R) which were subcloned
into SpeI and XhoI sites of the pLEX-MCS vector to generate pLEX-GST-ERK1(K72Q) and pLEX-GST-ERK1(K72R), respectively. To generate the 6xHis-tagged ERK1, pET-ERK1, the cDNA of ERK1 was transferred from pGEX-4T-1-ERK1(191) to the pET28a vector by digesting with BamHI and XhoI to generate pET28a-ERK1. To generate pET28a-ERK2, the cDNA of ERK2 was excised from the pGEX-4T-1-GST-ERK2 vector as described above. Then the insert was subcloned into the pET28a vector via SalI and NotI sites.

Cell Culture and Cell Lines

HEK293T cells were grown in Dulbecco’s modified Eagles’s medium (DMEM) with 10% bovine calf serum. HDAC6 wild-type and HDAC6 knockout mouse embryonic fibroblasts (MEFs) were cultured in DMEM with 10% fetal bovine serum (FBS). HDAC6 control and HDAC6 knockdown A549 cells were cultured in RPMI-1640 with 10% FBS. All cell lines were cultured in the medium with penicillin (100 U/ml), and streptomycin (100 g/ml) and in the incubators with 5% CO₂ at 37 °C.

Establishment of HDAC6 Stable Knock-Down A459 Cells

A549 scramble and HDAC6 knockdown stable cell lines were clonally selected by 0.5 µg/ml puromycin. First, A549 cells were transiently transfected with control vector pRS (Cat.# TR20003) or shRNA vector against HDAC6 (recognize sequence 5-AGGTCTACTGTGGTCGTACATCAATGGC-3', tube ID:T1349960, ORIGENE). Twenty-four hours after transfection, cells were split to duplicate plates of 1:20 in RPMI1640 medium containing 0.5 µg/ml puromycin. Puromycin was replenished every 2 days to maintain sufficient level of selection pressure. The well-isolated single clones
were transferred into 24-well plates. The knockdown effect was verified by Western Blotting analysis using anti-HDAC6 antibodies.

**Establishment of ERK1 Stable Knock-Down H292 and A459 Cells**

H292 and A549 scramble and HDAC6 knockdown stable cell lines were clonally selected by 0.5 µg/ml puromycin. First, H292 cells were transiently transfected with control vector pRS (Cat.# TR20003) or shRNA vector against ERK1 (TRCN0000006150, Sigma). Twenty-four hours after transfection, cells were split to duplicate plates of 1∶20 in RPMI1640 medium containing 0.5 µg/ml puromycin. Puromycin was replenished every 2 days to maintain sufficient level of selection pressure. The knockdown effect was verified by Western Blotting analysis using anti-ERK1/2 antibodies. For ERK2 knockdown, shRNA vector against ERK2 (TRCN0000010040, Sigma) was used. For A549, same control vector and shRNA vectors were used.

**Immunoprecipitation and Immunoblotting**

For immunoprecipitation assays, cells were lysed in lysis buffer (25 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol and protease inhibitor cocktail (11836170001, ROCHE)). Then the lysates were first pre-cleared with protein G agarose for 30 minutes at 4 °C with rotation, then incubated with interested antibodies overnight at 4 °C on a rocker, and followed by protein G agarose incubation for 4 hours at 4 °C with rotation. The samples were washed by washing buffer (TBS with 0.5% Triton X-100) 4 times and subject to further analyses. For immunoblotting, the samples were resolved on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked by 5% non-fat milk in TBST (0.1% Tween-20 in TBS (20 mM Tris, pH 7.5,
150 mM NaCl)) for 1 hour at room temperature. Then the membranes were incubated with a first antibody overnight at 4 °C and washed with TBST three times. Membranes were further incubated with an appropriate secondary antibody that was conjugated with horseradish peroxidase for 2-4 hours at room temperature and washed with TBST three times. Proteins on the membranes were detected by Western blotting substrates and exposed to the X-ray films. To re-blot the same membrane with a different antibody, the membrane would be stripped with stripping buffer (2% SDS, 62.5 mM Tris-HCl pH6.8, and 0.8% β-mercaptoethanol) for 45 minutes at 50°C. The membrane would then be re-blocked by 5% non-fat milk in TBST and subject to the above immunoblotting procedures.

**Purification of GST-tagged ERK Proteins from 293T Cells**

PLEX-GST-ERK1, PLEX-GST-ERK2, PLEX-GST-ERK1(K72Q), or PLEX-GST-ERK1 (K72R) was transfected into 293T cells. Cells were lysed in lysis buffer (25 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol) followed by incubation with glutathione agarose at 4°C overnight. The agarose beads were spun down and washed four times with cold wash buffer (TBS with 0.5% Triton X-100). The agarose-bound GST proteins were subject to next assays.

**Purification of His-ERK1/2 Proteins**

BL21 harboring His-ERK1 or His-ERK2 were grown log phase and induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 4 hr. The cell pellets were lysed in bacteria lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, 1.5% N-lauroylsarcosine and 1% Triton X-100). The mixtures were further sonicated in
appropriate conditions until the mixtures were clear. The mixtures were centrifuged at 10,000xg at 4°C for 5 minutes, and the supernatants were incubated with Ni²⁺-NTA agarose (#635659, Clontech) at 4°C overnight. The agarose was washed four times with cold washing buffer (TBS with 0.5% Triton X-100) after incubation. His-ERK1 and His-ERK2 were further eluted by 250 mM imidazole from Ni²⁺-NTA agarose for next assays.

**GST Pull-Down Assay**

BL21 harboring GST or GST-HDAC6 were grown log phase and induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 4 hr. The cell pellets were re-suspended in bacteria lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, 1.5% N-lauroylsarcosine and 1% Triton X-100) and sonicated in appropriate conditions until the mixtures were clear. The mixtures were centrifuged at 10,000xg at 4°C for 5 minutes, and the supernatants were incubated with glutathione-agarose to isolate beads-bound GST or GST-HDAC6. The purified His-ERK1 or His-ERK2, described in the “Experimental Procedures” was incubated with either glutathione agarose-bound GST or GST-HDAC6 for 30 minutes at 4°C on a rotator. After incubation, the glutathione-agarose beads were spun down and washed four times with cold washing buffer (TBS with 0.5% Triton X-100). The samples were then subject to Western-blotting analyses.

**Radioactive In Vitro Kinase Assay-**

GST-ERK1 wild type, K65Q, K65R, or dominant-negative mutant proteins were overexpressed in 293T cells, and the cells were lysed in lysis buffer. These GST fusion
proteins were purified by glutathione agarose. All purified proteins were incubated with myelin basic protein (MBP)(M1891, Sigma), 10mM of ATP, 5 µCi of [r-32p]ATP in 1x kinase buffer (10mM Tris, pH7.4, 150mM NaCl, 10mM MgCl₂, and 0.5mM Dithiothreitol(DTT)) for 30 minutes at 30 °C. The reactions were stopped by adding 5x SDS sample loading buffer and heating for 5 minutes at 100 °C. The samples were subjected to SDS-PAGE, and the MBP phosphorylation status was visualized by autoradiography.

**Non-Radioactive In Vitro Kinase Assay**

Wild-type, dominant-negative, K72Q or K72R mutant ERK1 proteins were overexpressed in 293T cells, and the cells were lysed in lysis buffer (25 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol and protease inhibitor cocktail (11836170001, ROCHE)). These GST fusion ERK1 proteins were purified as described in “Experimental Procedures.” GST fusion proteins were washed one time by 1x kinase buffer (5 mM Tris [pH 7.5], 5 mM β-Glycerolphosphate, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂) before reaction. All glutathione agarose-bound proteins were incubated with 250 ng ELK-1 (9184, Cell Signaling), 200 µM ATP and 1x kinase assay buffer for 30 minutes at 30°C. The reactions were stopped by adding 5x SDS sample loading buffer and heating for 5 minutes at 100°C. The samples were subject to Western blotting analyses, and the anti-phospho-Ser383-ELK1 antibody was used for phospho-ELK1 detection.

**In Vitro Acetylation Assay**

Bacterial GST-ERK1 or GST-ERK2 protein was purified by glutathione agarose as
described in GST pull-down assay. The glutathione agarose-bound GST-ERK1 or GST-ERK2 protein was mixed with 2 µg recombinant CBP proteins (BML-SE452-0100, Enzo Life Sciences), 100 nM acetyl-CoA in 1x acetylation buffer (50 mM Tris-HCl, pH8.0, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 10 mM sodium butyrate, and protease inhibitor cocktail), and the mixtures were incubated at 30°C for 60 minutes. Then the agarose beads were washed with washing buffer (TBS with 0.5% Triton X-100) three times and the reactions were terminated by adding 5x SDS sample loading buffer and boiling at 100°C for 5 minutes. The samples were then subject to Western blotting analyses.
ERK1/2 Interacts with HDAC6 Directly

In our previous studies, we have discovered that both ERK1 and ERK2 interact with HDAC6 \textit{in vivo}, and these interaction happen in both nucleus and cytoplasm (191). However, how these proteins interact with each other was not clear. To determine whether ERK1/2 interacts with HDAC6 directly or by the assistance of other associated proteins, we performed \textit{in vitro} GST pull-down assay. As shown in figure 1A, GST-HDAC6 can pull-down His-ERK1, but GST did not show the ability. The interaction between GST-HDAC6 and His-ERK2 also showed similar consequence (Figure 1B). These results showed that ERK1/2 physically interact with HDAC6.

ERK1/2 has two major structural parts, N-lobe and C-lobe. N-lobe has the ability of ATP binding, and C-lobe, which contains the active loop, binds to its substrates (37). To identify which part of ERK1/2 interact to HDAC6, we have executed the pull-down assay by using N-terminal GST-ERK1/2 and N-terminal GST-ERK1/2. As shown in Figure 2A, both ERK1 N-terminus and C-terminus can pull down HDAC6 as full-length ERK1 did. But N-terminus showed stronger binding ability to HDAC6 than C-terminus and full-length protein of ERK1. Similar to ERK1, both N-terminus and C-terminus of ERK2 interact with HDAC6, and the strength is same as full-length ERK2 (Figure 2B).
FIGURE 1. ERK1/2 interacts with HDAC6 physically.
A, His-ERK1 binds to GST-HDAC6. The GST pull-down assays were performed with indicated proteins as described in the “Materials and methods.” The proteins pulled down by GST agarose were resolved on SDS-PAGE followed by Western blotting analyses using the anti-ERK1/2 antibody (upper panel). Bacterially purified proteins, GST, GST-HDAC6, and His-ERK1 were stained by coomassie blue (middle and lower panels). B, His-ERK2 binds to GST-HDAC6. The GST pull-down assays and coomassie blue staining were conducted as A.

FIGURE 2. HDAC6 interacts with both N-terminus and C-terminus of ERK1/2
A, both N-terminal and C-terminal ERK1 interacts with HDAC6. The GST pull-down assays were performed with indicated proteins as described in the “Materials and methods.” The proteins pulled down by GST agarose were resolved on SDS-PAGE followed by Western blotting analyses using the anti-HDAC6 antibody (upper panel). Bacterially purified proteins, GST, full-length GST-ERK1, N-terminal GST-ERK1, and C-terminal GST-ERK1 were stained by coomassie blue (lower panels). B, both N-terminal and C-terminal ERK2 interacts with HDAC6. The GST pull-down assays and coomassie blue staining were conducted as A.
Inhibition or Depletion of HDAC6 Increases ERK1/2 Acetylation

Although in our previous study, we have shown that HDAC6 is a substrate of ERK1 (191), whether ERK1/2 is substrate of HDAC6 and whether ERK1/2 is acetylation proteins were not defined. To determine whether ERK1 is acetylated, GST-ERK1 was expressed in HEK293T cells. Before cells were harvested, the cells were treated with different concentrations pan-HDAC inhibitor Trichostatin A (TSA) for 12 hours or with 600ng/ml TSA for different amount of time. With higher dose or longer time of treatment, GST-ERK1 showed higher acetylation level (Figure 3A). Moreover, to narrow down which HDAC could be the deacetylase of ERK1, we applied clinical trial used HDAC6-specific inhibitor ACY-1215 for further treatment. We overexpressed GST-ERK1 in HEK293T cells and treated the cells with different concentrations of ACY-1215 for 12 hours. The acetylation status became stronger with higher concentrations of ACY-1215 treatment (Figure 3C). The similar results were also observed in GST-ERK2 when treated with TSA or ACY-1215 (Figure 3B and 3D).
FIGURE 3. Inhibition of HDAC6 increases ERK1/2 acetylation.
A, TSA increases ERK1 acetylation in a dose- and time-dependent manner. Left panels: HEK293T cells were transfected with GST-ERK1 followed by treatment with TSA for 12 hours at indicated concentrations. GST-pull-down assays were performed as described in “Materials and Methods.” Western blotting analyses were performed with the indicated antibodies. Right panels: HEK293T cells were transfected with GST-ERK1 followed by treatment with 600 ng/ml TSA at indicated time points. GST pull-down assays were performed similarly as described in “Materials and Methods.” Western blotting analyses were performed with the indicated antibodies. The acetylated GST-ERK1 bands were quantified by densitometry. The untreated band was designated as 1, and the density of other bands was indicated as a fold change relative to the untreated band. B, TSA increases ERK2 acetylation in a dose- and time-dependent manner. The experiments were performed the same as described in A. C, ACY-1215 increases ERK1 acetylation in a dose-dependent manner. The experiments were performed the same as described in A. D, ACY-1215 increases ERK2 acetylation in a dose-dependent manner. The experiments were performed the same as described in A.
To further confirm that whether class III HDACs work as the deacetylases of ERK1/2, we also executed similar experiments but treated the cell with class III HDAC inhibitor nicotinamide. When HEK293T cells were treated with different concentration of nicotinamide for 12 hours, the acetylation level of overexpressed GST-ERK1 did not increase with higher concentration treatment (Figure 4A). In ERK2, similar result was observed, although a slight difference was seen between non-treatment and 50 µM treatment (Figure 4B). The results suggest that class III HDACs may be not the deacetylase of ERK1/2.

To test whether endogenous ERK1/2 is acetylated, we treated 293T cells with TSA or ACY-1215 and analyzed the acetylation level of endogenous ERK1/2. Both treatments raised the acetylation strength of endogenous ERK1/2 (Figure 5A and 5B). To confirm
that HDAC6 controls the deacetylation of ERK1/2, we tested the acetylation of endogenous ERK1/2 in wild type and HDAC6 knockout 293T cells via immunoprecipitation. The acetylation of endogenous ERK1/2 rose when HDAC6 was depleted (Figure 5C). Similar results were also observed in HDAC6 knockdown A549 and HDAC6 knockout MEFs (Figure 5D and 5E). These results showed that without HDAC6, when HDAC6 level was low, or when HDAC6 activity was impeded, the endogenous ERK1/2 acetylation level raised in lung cancer cells, and mouse cells.
FIGURE 5. Depletion or knockdown of HDAC6 increases ERK1/2 acetylation. A, TSA increases endogenous ERK1/2 acetylation. HEK293T cells were treated with a vehicle or 600 ng/ml TSA for 24 hours. The anti-AcK antibody was used to immunoprecipitate acetylated ERK1/2. The immunoprecipitates were resolved on SDS-PAGE and the anti-ERK1/2 Western blot analysis was performed. 0.5% of whole cell lysate was used as the input. Western blotting analyses were performed using anti-ERK1/2, anti-acetyl-α-tubulin, or α-tubulin antibodies as indicated. B, ACY-1215 increases endogenous ERK1/2 acetylation. The experiments were performed as A but the cell was treated with ACY-1215. C, Knockout of HDAC6 increases ERK1/2 acetylation in HEK293T. The anti-AcK antibody was used to immunoprecipitate acetylated ERK1/2 in HDAC6 wild-type or HDAC6 knockout HEK293T. The other experiments were performed as A. Anti-HDAC6 antibody was used to indicate the HDAC6 knockout efficiency. D, Knockout of HDAC6 increases ERK1/2 acetylation in MEFs. The anti-AcK antibody was used to immunoprecipitate acetylated ERK1/2 in HDAC6 wild-type or HDAC6 knockout MEFs. The other experiments were performed as A. Anti-HDAC6 antibody was used to indicate the HDAC6 knockout efficiency. E, HDAC6 knockdown increases ERK1/2 acetylation HDAC6 knockdown A549 cells. The experiments were performed as described in A. Anti-HDAC6 antibody was used to indicate the HDAC6 knockdown efficiency.
Chapter 4

ERK1/2 Acetylation is Regulated by CBP/p300 and HDAC6

ERK1/2 Acetylation is Controlled by CBP/p300 In Vivo

To identify the HAT of ERK1/2, we tested five general HATs that cover major HAT categories (192,193). These HATs have been co-expressed with GST-ERK1 in 293T cells. When GST-ERK1 was co-expressed with CBP, the acetylation level of GST-ERK1 was much higher than that with empty vector and other HATs. p300 also showed a minor ability to acetylate ERK1 (Figure 6A). To further confirm the result, we co-expressed different amounts of CBP or p300 with GST-ERK1. The results showed that with higher amounts of CBP or p300, GST-ERK1 showed higher acetylation levels (Figure 6B and 6C). To further confirm the effect on endogenous ERK1, we overexpressed p300 in HEK293T cells and test the acetylation level change of ERK1. When the p300 presented, endogenous ERK1 acetylation level increased (Figure 6D). GST-ERK2 also displayed similar property of being acetylated by CBP or p300 (Figure 7 A-C). Furthermore, the endogenous ERK2 also exhibited increase on acetylation level (Figure 6D).

ERK1/2 Acetylation is Controlled by CBP/p300 In Vitro

In order to decrease the effects of other proteins that could also modify the acetylation of ERK1 in the cells, we executed in vitro acetylation assay to confirm the acetylation capability of CBP on ERK1. The results showed that CBP could acetylate GST-ERK1 in vitro. (Figure 8A). Same as GST-ERK1, CBP also showed the ability to
acetylate GST-ERK2 \textit{in vitro} (Figure 8B). Furthermore, as the result observed in the co-expression experiments, p300 showed weaker capability to acetylate GST-ERK1 than CBP in this \textit{in vitro} assay (Figure 8A). However, p300 did not display the ability to acetylate GST-ERK2 in vitro (Figure 8B).

**HDAC6 Deacetylates ERK1**

Because of low basal acetylation level of ERK1, to confirm whether HDAC6 deacetylates ERK1; we co-expressed CBP to increase ERK1 acetylation level. In figure 9A, when co-expressed only with CBP, GST-ERK1 acetylation level increased. But when HDAC6 was co-expressed, the acetylation level of GST-ERK1 decreased. Similar results could be seen when GST-ERK1 co-expressed with p300, but the overall acetylation level was lower than with CBP (Figure 9B).
FIGURE 6. ERK1 is acetylated by CBP and p300.

A, CBP and p300 acetylate ERK1. GST-ERK1 was co-transfected with each of indicated HAT plasmids into HEK293T cells. GST-ERK1 was pulled-down by glutathione-agarose, then the beads-bound proteins were resolved on SDS-PAGE followed by Western blotting analyses with anti-acetyl-lysine antibodies. The membrane was then stripped and rebotted with anti-ERK1/2 antibody. The input was subject to Western blotting analyses with indicated antibodies. B, CBP acetylates ERK1 in a dosage-dependent manner. GST-ERK1 was co-transfected with an increasing amount of HA-CBP plasmids as indicated into HEK293T cells. GST-ERK1 was further pulled-down by glutathione agarose, then the beads-bound GST-ERK1 was subject to the anti-acetyl-lysing Western blotting analysis. The membrane was then stripped and reprobed with anti-ERK1/2 antibody. The input was subject to Western blotting analyses with indicated antibodies. C, p300 acetylates ERK1. HA-p300 was co-transfected with an increasing amount of HA-p300 plasmids. The experiments were performed as described in B. D, p300 acetylates endogenous ERK1/2. HEK239T cells were transfected with empty vector or HA-p300. The immunoprecipitation assays were carried out with anti-AcK antibodies as described in the “Materials and methods.” The immunoprecipitates were subject to the anti-AcK Western blotting analysis. 0.5% of whole cell lysate was used as input which was subject to Western blotting analyses with indicated antibodies. For panels A-C, the untreated band was designated as 1, and the density of other bands was indicated as a fold change relative to the untreated band. For panel D, the ERK1/2 bands in the empty vector-transfected sample were designated as 1, and density of the ones in the HA-p300-transfect sample was indicated as a fold change relative to 1.
FIGURE 7. ERK2 is acetylated by CBP and p300.
A, CBP and p300 acetylate ERK2. GST-ERK2 was co-transfected with each of indicated HAT plasmids into HEK293T cells. GST-ERK2 was pulled-down by glutathione-agarose, then the beads-bound proteins were resolved on SDS-PAGE followed by Western blotting analyses with anti-acetyl-lysine antibodies. The membrane was then stripped and reblotted with anti-ERK1/2 antibodies. The input was subject to Western blotting analyses with indicated antibodies.

B, CBP acetylates ERK2 in a dosage-dependent manner. GST-ERK2 was further pulled-down by glutathione agarose, then the beads-bound ERK2 was subject to anti-acetyl-lysing Western blotting analysis. The membrane was then stripped and reprobed with anti-ERK1/2 antibodies. The input was subject to Western blotting analyses with indicated antibodies.

C, p300 acetylates ERK2. HA-p300 was co-transfected with an increasing amount of HA-CBP plasmids in HEK293T cells. The experiments were performed as described in B.
FIGURE 8. **CBP acetylates ERK1 and ERK2 in vitro.**

A, Recombinant CBP and recombinant p300 acetylate ERK1 in vitro. Bacterially expressed GST-ERK1 and catalytic domain of CBP or catalytic domain of p300 were subject to in vitro acetylation assay as described in “Materials and methods.” Equal amount of GST-ERK1 was incubated with or without 2 mg recombinant CBP catalytic domain or p300 catalytic domain, and the reactions were analyzed by the anti-AcK Western blotting analysis (upper panel). After transfer, the membrane was stained with Ponceau S to confirm the amount and purity of GST-ERK1 (lower panel). B, Recombinant CBP acetylates ERK2 in vitro. Bacterial version GST-ERK2 was subjected to in vitro acetylation as described in A.
FIGURE 9. HDAC6 deacylates ERK1.
A, HDAC6 reduces CBP-induced ERK1 acetylation. GST-ERK1 was transfected into HEK293T cells alone, with CBP, or with CBP and HDAC6. GST pull-down was performed with the transfected cells followed by the anti-AcK Western blotting analysis. The membrane was then stripped and then reprobed with anti-ERK1/2 antibody. The input was subject to Western blotting analyses with indicated antibodies. B, HDAC6 reduces p300-induced ERK1 acetylation. The experiments were performed as described in A, except that HA-p300 was used instead of HA-CBP.
Chapter 5

Acetylation of ERK1/2 Affects Its Kinase Activity

Acetylation Sites are Identified in ERK1 and ERK2

To locate the lysine acetylation sites in ERK1 and ERK2, we have prepared different samples for mass-spectrometry. Six different treatments have been used to prepare different batch of GST-ERK1 for mass spectrometry. In the first method, GST-ERK1 was overexpressed in HEK239T cell, and cells were treated with 600 ng/ml TSA for 24 hours before harvest. In the second method, GST-ERK1 was overexpressed in HEK293T cell, and cells were treated with 600 ng/ml TSA and 20mM nicotinamide for 24 hours before harvest. In the third and fourth methods, GST-ERK1 was co-expressed in 293T cell with CBP only or with both CBP and p300, respectively. GST-ERK1 from these four methods are further pulled-down by glutathione agarose and analyzed SDS-PAGE. The specific coomassie blue-stained bands were excised for sending to mass-spectrometry.

The fifth and sixth methods are in vitro acetylation assay. N-terminal of GST-ERK1 was overexpressed in E. coli and purified by glutathione agarose. Agarose bound GST-ERK1 N-terminus was further subject to in vitro acetylation. In fifth method, only recombinant CBP was used, but in sixth method, both recombinant CBP and recombinant p300 were used. In ERK2, only one method has been used to prepare samples. GST-ERK2 was overexpressed in HEK293T cell, and cells were treated with 600 ng/ml TSA for 24 hours before harvest. GST-ERK2 was further pulled down by
glutathione agarose and analyzed by SDS-PAGE. SDS-PAGE was further stained by coomassie blue, and the particular bands were excised for mass-spectrometry.

There are 7 acetylation sites were identified in ERK1. They are Lys32, Lys71, Lys72, Lys155, lys287, Lys294, and Lys 361 (Figure 10, 11, 12, 13 and Table 1). In ERK2, there are three acetylation sites detected. They are Lys48, Lys203, and Lys344 (Figure 10 and Table 1). From the website PhosphoSitePlus®, which provides the database of protein modification; we have found that Lys181 of ERK1 is an acetylation site (74).

In order to identify more acetylation sites in ERK1 and ERK2, and because ERK1 and ERK2 share 84% identity of their amino acid sequence (37), we align the amino acid sequences of these two kinases. According to the result, we have identified more assumed acetylation sites in each protein. In ERK1, Lys65 and Lys220 are discovery as two new assumed acetylation sites, and in ERK2, Lys138, Lys164, and Lys270 are the novel ones (Figure 10).

**Acetylation Mimetic of Part of EKR1 Lysine Residues Decrease ERK1 Kinase Activity on ELK1**

To exam the acetylation effect on ERK1 and ERK2, we have mutated all the identified acetylation lysine residues to Glutamine (Q) or Arginine (R) to mimic the acetylation and deacetylation, respectively. All the single mutant of ERK1 and ERK2 did not make strong change of acetylation status comparing to wild-type (Figure 14). Only the mutations of two residues in ERK1 change ERK1’s phosphorylation status. The acetylation-mimic of Lys65 (K65Q) decrease the acetylation of ERK1 and totally
abolishes the phosphorylation of ERK1, but deacetylation-mimic of Lys65 (K65R) did not make any change on both acetylation and phosphorylation (Figure 14A and 15A).

**FIGURE 10. Acetylation sites of ERK1 and ERK2.** The acetylation sites identified by mass-spectrometry are indicated with black numbers. Site found from website (www.phosphosite.org) is shown in blue numbers. Assumed sites discovered by sequences alignment are displayed in green numbers. The critical phosphorylation sites of threonine and tyrosine for enzymatic activation are marked with blue circles.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Site</th>
<th>Facility</th>
<th>Digest</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK1</td>
<td>K32</td>
<td>Moffitt</td>
<td>Trypsin/ endoproteinase GluC</td>
<td>GST-ERK1/ TSA/ 293T</td>
</tr>
<tr>
<td></td>
<td>K71</td>
<td>Moffitt</td>
<td>Chymotrypsin/ Lys-C</td>
<td>GST-ERK1 N-terl/ CBP/ in vitro/ E. coli</td>
</tr>
<tr>
<td></td>
<td>K72</td>
<td>Moffitt</td>
<td>Chymotrypsin/ Lys-C</td>
<td>GST-ERK1/ CBP/ 293T</td>
</tr>
<tr>
<td></td>
<td>K155</td>
<td>Moffitt</td>
<td>Trypsin/ endoproteinase GluC</td>
<td>GST-ERK1/ TSA/ 293T</td>
</tr>
<tr>
<td></td>
<td>K287</td>
<td>Moffitt</td>
<td>Trypsin/ endoproteinase GluC</td>
<td>GST-ERK1/ TSA/ 293T</td>
</tr>
<tr>
<td></td>
<td>K361</td>
<td>Moffitt</td>
<td>Trypsin/ endoproteinase GluC</td>
<td>GST-ERK1/ TSA/ 293T</td>
</tr>
<tr>
<td></td>
<td>K287</td>
<td>U Chicago</td>
<td>Chymotrypsin</td>
<td>GST-ERK1/ CBP/p300/ 293T</td>
</tr>
<tr>
<td></td>
<td>K294</td>
<td>U Chicago</td>
<td>Chymotrypsin</td>
<td>GST-ERK1/ TSA/ Nio/ 293T</td>
</tr>
<tr>
<td></td>
<td>K71</td>
<td>Karmanos</td>
<td>Chymotrypsin</td>
<td>GST-ERK1 N-terl/ CBP/ p300/ in vitro/ E. coli</td>
</tr>
<tr>
<td>ERK2</td>
<td>K48</td>
<td>Moffitt</td>
<td>Trypsin/ endoproteinase GluC</td>
<td>GST-ERK2/ TSA/ 293T</td>
</tr>
<tr>
<td></td>
<td>K203</td>
<td>Moffitt</td>
<td>Trypsin/ endoproteinase GluC</td>
<td>GST-ERK2/ TSA/ 293T</td>
</tr>
<tr>
<td></td>
<td>K344</td>
<td>Moffitt</td>
<td>Trypsin/ endoproteinase GluC</td>
<td>GST-ERK2/ TSA/ 293T</td>
</tr>
</tbody>
</table>

Table 1. The acetylation sites detected by mass-spectrometry
FIGURE 11. The Lysine 72 site of ERK1 is acetylated. The Lys72 site of ERK1 is acetylated. The doubly charged peptide was detected with mass-to-charge ratio 646.3179, which represents an error of -3.8 ppm. The tandem mass spectrum matched the following sequence, KISPFEHQTY indicating that the Lys72 (highlighted in red) was acetylated; the detection of b2 is consistent with this localization. The assignment was made with Sequest with Xcorr score 2.1 and ΔCN score 0.19.

FIGURE 12. The Lysine 287 site of ERK1 is acetylated. Lysine 287 acetylation was detected with a mass-to-charge ratio of 700.4008, which represents an error of 0 ppm. The tandem mass spectrum matched the following sequence, LQSLPSKTVAW indicating that the second lysine was acetylated; the detection of y6 is consistent with this localization. The assignment was made with Mascot with Score 40.
FIGURE 13. The Lysine 294 site of ERK1 is acetylated.
Lysine 294 acetylation was detected with mass-to-charge ratio of 449.5946, which represents an error of 1.11 ppm. The tandem mass spectrum matched the following sequence, AK_{ac}LPKSDSKAL indicating that the first lysine was acetylated; the detection of b2 is consistent with this localization. The assignment was made with Mascot with Score 45.
FIGURE 14. Acetylation-mimetic mutations change the phosphorylation level in ERK1 and ERK2.
A, Wild-type and different mutation of GST-ERK1 were overexpressed in HEK293T cell. Pulled-down GST-ERK1 proteins are analyzed by Western blot with anti-acetyl-lysine antibody. Same membrane was stripped then rebotted with anti-phospho-ERK1/2 antibodies to see the level change of phospho-ERK1. Same membrane was stripped again and rebotted with anti-ERK1/2 antibody to confirm the protein level. B, Wild-type and different mutation of GST-ERK2 were overexpressed in HEK293T cell. The Western blot analyses were done as described in A.
FIGURE 15. **The acetylation-mimicking mutant of ERK1(K65Q) decreases the ERK1 kinase activity**

**A**, the acetylation-mimicking mutant of ERK1(K65Q) decreases the level of ERK1 acetylation and phosphorylation in HEK293T cells. The GST-tagged dominant-negative, wild-type, K65Q, or K65R ERK1 was transfected into HEK293T cells, and GST pull-down assays were performed as described in the "Materials and Methods." Glutathione-agarose bound proteins were analyzed by anti-phospho-ERK1/2 Western Blotting analysis. The membrane was then stripped and reprobed with anti-ERK1/2 antibody.

**B**, The K65Q mutant of ERK1 exhibits a decreased kinase activity toward MBP. Dominant-negative, wild-type, K65Q, or K65R GST-ERK1 was transfected into HEK293T cells. GST pull-down assays were performed as described in the "Materials and Methods." The glutathione agarose bound proteins were subject to in vitro kinase assays using recombinant MBP as a substrate. The MBP phosphorylation status was visualized by autoradiography. Active recombinant ERK1 was used as positive control.
FIGURE 16. The acetylation-mimicking mutant of ERK1(K72Q) decreases the ERK1 kinase activity. 
A, the acetylation-mimicking mutant of ERK1(K72Q) decrease the level of ERK1 phosphorylation in HEK293T cells. The GST-tagged dominant-negative, wild-type, K72Q, or K72R ERK1 was transfected into HEK293T cells, and GST pull-down assays were performed as described in the "Materials and Methods." Glutathione-agarose bound proteins were analyzed by anti-phospho-ERK1/2 Western Blotting analysis. The membrane was then stripped and reprobed with anti-ERK1/2 antibody. B, the K72Q mutant of ERK1 exhibits a decreased kinase activity toward ELK1. Dominant-negative, wild-type, K72Q, or K72R GST-ERK1 was transfected into HEK293T cells. GST pull-down assays were performed as described in the "Materials and Methods." The glutathione agarose bound proteins were subject to non-radioactive in vitro kinase assays using recombinant ELK1 as a substrate. The phosphorylation of ELK1 was measured by anti-phospho-ELK1 (Ser383) Western blotting analysis. The same membrane was stripped and the re-blotted with anti-ELK1 antibodies. The anti-ERK1/2 Western blotting analysis was also performed.
K181Q of ERK1 and K203Q of ERK2 both did not change the acetylation grade of ERK1 and ERK2, but both slightly decrease the phosphorylation level of ERK1 and ERK2, respectively. Moreover, ERK1 K72Q has decreased the phosphorylation of ERK1 dramatically comparing to wild-type and K72R, and both mutation did not alter the acetylation level of ERK1 (Figure 16).
Because of the change of ERK1 phosphorylation status, next we further examined the enzymatic activity of ERK1 by using *in vitro* kinase assay. As the alteration of phosphorylation level, the enzymatic activity of K65Q intensely decreased about 80% comparing to wild-type. The K65R's activity decline a bit more than 50 % although the phosphorylation of K65R did not very too much (Figure 15B).

ELK1 is one member of Ets transcription factor family, and several serine and threonine sites of ELK1 are phosphorylated by ERKs. Among all these sites, phosphorylation status of ELK1 Ser383 alone is much more pivotal for ELK1 transcriptional activation (194). Due to the mentioned reason, we used ELK1 as the substrate to execute nonradioactive kinase assay. The acetylation mimetic protein, ERK1 K72Q showed lower kinase activity on ELK1, but the non-acetyl mimetic protein, ERK1 K72R performed as wild type (Figure 16B).

In order to determine the way of Lys72 in regulating kinase activity of ERK1, we examined ERK1 crystal structure. According to the structure, Lys72 located on β3-strand of ERK1 N-lobe and is very close to glycine-rich loop, and Lys72 formed a salt bridge with Asp117 and links to Tyr119 with a hydrogen bond. Acetylation mimetic of Lys72 would break the contacts to Asp117 and Tyr119, and changed the confirmation of ATP binding site. This would further influence the binding of ATP and the ATP binding site of ERK1 (Figure 17).

To show the importance and conservation of Lys72 and Lys65 in ERK1, ERK1 sequences of different species from human to nematode were included for Tcoffee
alignment. The alignment results showed that ERK1 Lys72 was highly conserved among mammals and even in Zebrafish (*Danio rerio*), *Drosophila*, and *C. elegans* (Figure 18). Lys65 also showed high conservation among all vertebrates but did not include *Drosophila*, and *C. elegans* (Figure 18).

![Alignment](image)

**ERK1 Knock-Down Decreases Colony Formation and Cell Migration in NSCLC H292**

To further confirm how does ERK1 activity change cell’s phenotype, stable ERK1 knockdown cells have been prepared. Two NSCLC cell lines are used for ERK1 knockdown. Meanwhile, ERK2 knockdown cells were also prepared. As shown in Figure 19, in A549 cells, both ERK1 and ERK2 were knockdown efficiently, but in H292, only ERK2 have been knockdown well. ERK1 knockdown H292 and A549 have both been...
further subject to colony formation assay and wound healing assay. A549 displayed better ERK1 knockdown efficiency, however, these cells did not show large difference from control cell in both colony formation and wound healing. On the other hand, ERK1 knockdown H292 exhibit stronger effect on decreasing colony formation and wound healing ability (Figure 20).

**FIGURE 19.** ERK1 knockdown in H292 and A549
Control and stable ERK1 or ERK2 knockdown cells were prepared by using scramble vector and shRNA vector against ERK1 or ERK2. Cells were further selected by 0.5 µg/ml puromycin. Same amount of cell lysates of these established stable cells were subject to Western blot analysis. Anti-ERK1/2 antibody is used to detect the knockdown efficiency of ERK1 and ERK2. α-tubulin is the loading control.
FIGURE 20. **Knockdown of ERK1 in H292 weakens its ability of colony formation and migration**

A, ERK1 knockdown lowered the ability of colony formation in H292. 2000 scramble or ERK1 knockdown H292 cells were seeded in the 6-well plate and cultured for 7 days. Cells then were fixed and stained in the staining solution (6% glutaraldehyde and 0.5% crystal violet). B, ERK1 knockdown significantly downregulated the ability of colony formation in H292. Colonies were counted by ImageJ. N=6. *, p<0.001. C, ERK1 knockdown lowered the ability of cell migration in H292. Scramble or ERK1 knockdown H292 cells were cultured in 60mm dish until the 100% confluence. Yellow tip was used to scratch a wound area. The photo of wound areas is taken under the microscope right after the scratch. After 16 hours incubation, the photo of same areas was taken again. D, ERK1 knockdown meaningfully deregulated cell migration in H292. Wound area were analyzed by ImageJ. N=5. 8, p<0.001.
Chapter 6
Discussion and Future Directions

In this study, we have demonstrated that acetylation/deacetylation status of ERK1 regulates its enzymatic activity. For the first time, we have indicated that ERK1 and ERK2 are acetylated proteins and are new substrates of HDAC6. We have also revealed 9 lysine residues of ERK1 and 6 residues of ERK2 as novel acetylation sites, and 6 sites of ERK1 and 3 sites of ERK2 are detected by mass-spectrometry. Furthermore, we have shown that ERK1 Lys72 acetylation-mimic mutant displayed reduced kinase activity comparing with wild type and deacetylation-mimic mutant. ERK1 Lys65 also showed similar consequence. Overall, our results suggest that HDAC6 governs ERK1 kinase activity via deacetylating Lys72 in ERK1.

In our previous study, we have shown the endogenous interaction of ERK1/2 and HDAC6. As shown in figure one, we further determined that ERK1/2 and HDAC6 directly bind to each other without the support of other cofactors. We have also shown HDAC6 is a novel substrate of ERK1 in our previous study, however, we did not locate any canonical sequence of D-site and F-site, both are usually found in ERK1/2 substrates (37), in HDAC6. Dozens of HDAC6 substrates have been identified, and the specificity of HDAC6’s substrates has been studied (195). But the conserved sequence of a designated motif or domain of these HDAC6 substrates is still not clear.
By inhibiting the enzymatic activity of HDAC6 with pan-HDAC or HDAC6-specific inhibitors, the acetylation level of endogenous and exogenous ERK1/2 increased (Figure 2). Although the results showed HDAC6 should be one of the major HDACs deacetylating ERK1/2, we cannot exclude other class I/II HDACs due to TSA treatment. Specific inhibitors treatment of other HDACs or knockdown of particular HDACs would further be executed to find out any other possible HDAC of ERK1/2. The HDACs of class III are excluded from being ERK1/2 deacetylase, because nicotinamide treatment did not show obvious change of ERK1 acetylation level. This suggests that class III HDACs could not regulate ERK1/2.

ERK1/2 acetylation has been shown enhanced in HDAC6 null MEFs cells and HDAC6 knockdown A549 cells compared to wild type cells in this study (Figure 3). This reveals that ERK1/2 acetylation may generally exist in human lung cancer cells and mice, and this can benefit the future study of ERK1/2 acetylation in mice or lung cancer cell lines. Both CBP and p300 show the function of acetylating exogenous ERK1 and ERK2, and CBP shows better acetylation than p300 does on both ERK1 and ERK2. This outcome was also observed in the in vitro acetylation assay (Figure 4). This shows CBP can acetylate ERK1/2 without forming a complex with other protein factors.

Here, we have identified that Lys72 is a novel acetylation site in ERK1, and the acetylation status of Lys72 controlled ERK1’s enzymatic activity on ELK1 (Figure 7&8). Our results showed that the acetylation-mimic mutation abolished ERK1 kinase activity, however, the deacetylation-mimic mutation still kept similar activity strength as wild type.
More than two decades ago, it was demonstrated that Lys71 within subdomain II is critical for ATP binding (196). Substitution of Lys to Arg at this site therefore abolishes ERK1 kinase activity (197). Interestingly, we found that Lys71 can be acetylated by mass spectrometry analysis (data not shown). It was expected that replacement of Lys with any other amino acid would ablate ERK1 kinase activity. Because of this reason, Lys to Arg (deacetylation mimetic mutation) or Lys to Glu (acetylation mimetic mutation) substitution of Lys71 would not tell us how deacetylation/acetylation regulates ATP binding and ERK1 enzymatic activity. Future studies using a special t-RNA synthetase capable of binding Nε-acetyl lysine to synthesize ERK1 with acetylated Lys72 may shed light to elucidate the role of acetylation of this site in ERK1 function.

Here, we have identified a conserved lysine Lys72 adjacent to Lys71 as a novel acetylation site in ERK1, and the acetylation status of Lys72 significantly decreases ERK1’s enzymatic activity toward a well-known ERK1’s substrate ELK1. According to the structural analysis, the acetylation mimetic mutant of Lys72 (K72Q) would block the formation of the salt bridges to Asp117 and Tyr119, leading to decreased stability of the β3-strand, diminished ATP binding, and reduced ERK1 kinase activity. In contrast, acetylation of Lys53, a homologous site of ERK1 Lys71, in p38 augments p38’s kinase activity (198). Moreover, Lys52 in ERK2 and Lys55 in JNK1 and JNK2 are also homologous to ERK1 Lys71 (198,199), but whether these sites are acetylated remains to be determined. It is attempting to hypothesize that acetylation/deacetylation of the conserved lysines which bind to ATP or form salt bridges and affect ATP binding is an approach for HATs and HDACs to fine-tune the enzymatic activities of MAPKs.
Although Lys72 single mutant displays significant effect on ERK1 enzymatic activity, further study involving single mutant or multiple mutants of other lysine are still needed to illustrate the effect of acetylation/deacetylation on ERK1’s other cellular functions such as substrate binding, subcellular localization, dimer formation, phosphorylation status or kinase activity.

ELK1 has been shown playing important role in cancers including NSCLC (200-204). ELK1 is critical transcriptional regulator that controls the expression of several oncogenes such as c-Fos via MAPK pathways (194,205-207). ERK1 is one of the major regulators phosphorylating ELK1 Ser383 and several other important active sites while ELK1 is induced by growth factors. Since acetylation of ERK1 negatively regulates its enzymatic activity, and non-acetylated ERK1 keeps its kinase activity, this shows that HDAC6 plays an oncogenic role in the cell.

As the main moderator in the downstream of the pathway, ERK1/2 are emerging as an alternative to be aimed, especially in the cases which are resistant to the inhibitors of the upstream components in this pathway (208). Thus far, there are several ERK1/2-specific inhibitors being used to overcome the resistance to EGFR, Raf or MEK inhibitors, in clinical trials. Meanwhile, HDAC6-specific inhibitors are also developed and applied in clinical trials. Most of these trials were conduct with other anti-cancer drugs (209-218). Several instances of the combination usage of HDACs inhibitor and ERK pathway inhibitors are reported, and these combination regimens really show the capability of synergistic cell killing (219-223). Because we now show that inhibition of HDAC6 down-regulates ERK1’s enzymatic activity, combination of HDAC6 inhibitors and
ERK1/2 inhibitors may be a promising strategy to overcome the resistance to EGFR, Raf or MEK inhibitors.
Reference

with histone deacetylase 6 and represses the p21(CIP1/WAF1) promoter. Mol Cell Biol 22, 7982-7992.


91. Fox, T., Fitzgibbon, M. J., Fleming, M. A., Hsiao, H. M., Brummel, C. L., and Su, M.


1090-1099


Involvement of the p38 mitogen-activated protein kinase cascade in hepatocellular carcinoma. Cancer 97, 3017-3026


209. Ricolinostat, Gemcitabine Hydrochloride, and Cisplatin in Treating Patients With
Unresectable or Metastatic Cholangiocarcinoma.

210. ACY-1215 in Combination With BCR Pathway Inhibitors in Relapsed CLL.
211. A Phase 1b Study of Paclitaxel And Ricolinostat For The Treatment Of Gynecological Cancer.
212. ACY-1215 + Nab-paclitaxel in Metastatic Breast Cancer.
213. Phase 1b Study Evaluating ACY-1215 (Ricolinostat) in Combination With Pomalidomide and Dexamethasone in Relapsed or Relapsed-and-Refractory Multiple Myeloma.
214. ACY-1215 for Relapsed/Refractory Lymphoid Malignancies.
215. Alternative 10 mg/mL Liquid Formulation of ACY 1215 (Ricolinostat) in Healthy Subjects.
216. ACY-1215 (Ricolinostat) in Combination With Pomalidomide and Low-dose Dex in Relapsed-and-Refractory Multiple Myeloma.
217. Study of ACY-1215 in Combination With Lenalidomide, and Dexamethasone in Multiple Myeloma.
218. Study of ACY-1215 Alone and in Combination With Bortezomib and Dexamethasone in Multiple Myeloma.


