Histone deacetylase 6 (HDAC6) deacetylates extracellular signal-regulated kinase 1 (ERK1) and thereby stimulates ERK1 activity *

Jheng-Yu Wu a,b, Shengyan Xiang b, Mu Zhang c, Bin Fang c, He Huang d, Oh Kwang Kwon d, Yingming Zhao a, Zhe Yang c, Wenlong Bai b, Gerold Bepler a, Xiaohong Mary Zhang a,1

From the aDepartment of Oncology, Molecular Therapeutics Program, Karmanos Cancer Institute, 4100 John R. St., Detroit, Michigan 48201, bDepartment of Pathology and Cell Biology, Morsani College of Medicine, University of South Florida, 12901 Bruce B. Downs Blvd. Tampa, FL 33612, cThe Proteomics Core, H. Lee Moffitt Cancer Center and Research Institute, 12902 USF Magnolia Dr. Tampa, FL 33612, dBen May Department of Cancer Research, The University of Chicago, 929 East 57th St. W410, Chicago, Illinois 60637, eDepartment of Microbiology, Immunology & Biochemistry, Wayne State University School of Medicine, 540E Canfield Avenue, Detroit, MI 48201.

*Running Title: HDAC6 deacetylates ERK1

1To whom correspondence should be addressed: Xiaohong Mary Zhang, Karmanos Cancer Institute, 4100 John R. St., Detroit, MI 48201, US, Tel.: (313)576-8672; Fax: (313)576-8928; E-mail: zhangx@karmanos.org.

Key words: extracellular signal-regulated kinase1/2 (ERK1/2); histone deacetylases (HDACs); acetylation; deacetylation; mitogen-activated protein kinases (MAPKs)

ABSTRACT

Histone deacetylase 6 (HDAC6), a class IIb HDAC, plays an important role in many biological and pathological processes. Previously, we found that ERK1, a downstream kinase in the MAPK signaling pathway, phosphorylates HDAC6, thereby increasing HDAC6-mediated deacetylation of α-tubulin. However, whether HDAC6 reciprocally modulates ERK1 activity is unknown. Here, we report that both ERK1 and 2 are acetylated and that HDAC6 promotes ERK1 activity via deacetylation. Briefly, we found that both ERK1 and 2 physically interact with HDAC6. Endogenous ERK1/2 acetylation levels increased upon treatment with a pan-HDAC inhibitor, an HDAC6-specific inhibitor or depletion of HDAC6, suggesting that HDAC6 deacetylates ERK1/2. We also noted that the acetyltransferases CBP and p300 both can acetylate ERK1/2. Acetylated ERK1 exhibits reduced enzymatic activity toward the transcription factor ELK1, a well-known ERK1 substrate. Furthermore, mass spectrometry analysis indicated Lys-72 as an acetylation site in the ERK1 N-terminus, adjacent to Lys-71, which binds to ATP, suggesting that acetylation status of Lys-72 may affect ERK1 ATP binding. Interestingly, an acetylation-mimicking ERK1 mutant (K72Q) exhibited less phosphorylation than the WT enzyme and a deacetylation-mimicking mutant (K72R). Of note, the K72Q mutant displayed decreased enzymatic activity in an in vitro kinase assay and in a cellular luciferase assay compared with the WT and K72R mutant. Taken together, our findings suggest that HDAC6 stimulates ERK1 activity. Along with our previous report that ERK1 promotes HDAC6 activity, we propose that HDAC6 and ERK1 may form a positive feed-forward loop, which might play a role in cancer.

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are the enzymes that regulate core histones and non-histone proteins by deacetylation and acetylation, respectively (1). HATs acetylate proteins via adding acetyl groups to lysine residues, while HDACs catalyze a reverse reaction by removing the acetyl group from lysine residues. Up to now, a total of 18 HDACs are identified in humans and grouped
into four classes based on their sequence similarity to yeast orthologs (2). Class I HDACs are homologous to yeast reduced potassium dependency 3 (Rpd3) and include HDACs 1, 2, 3 and 8. Class II HDACs are homologous to yeast histone deacetylase 1 (HdaI) and are further divided into class IIA and class IIB. Class IIA contains HDACs 4, 5, 7 and 9, and class IIB includes HDACs 6 and 10. HDAC11 is the only member in class IV. The deacetylase activity of HDAC classes I, II and IV is zinc-dependent. Class III HDACs, also known as sirtuins, are homologous to yeast silent information regulator 2 (Sir2), and the deacetylase activity of this class is oxidized nicotinamide adenine dinucleotide (NAD⁺)-dependent.

HDAC6 belongs to class IIB HDACs. Its structure is quite unique among all HDACs, in that it contains two functional deacetylase domains in tandem and a zinc finger domain in the C-terminus (2,3). HDAC6 participates in numerous biological and pathologic processes, such as cell migration, DNA damage response and oncogenesis, through modulating its substrates (4-7). For example, HDAC6 deacetylates cytoskeleton proteins and their associated proteins, such as α-tubulin and cortactin, to regulate cell mobility (4,5). HDAC6 also deacetylates and ubiquitinates the DNA mismatch repair protein MSH2, in order to regulate MutSα homeostasis, DNA mismatch repair, and DNA damage response (6). In addition, HDAC6 deacetylates cell signaling regulators K-Ras and β-catenin, leading to altered oncogenic activity and nuclear localization, respectively (8,9).

Mitogen-activated protein kinases (MAPKs) are a conserved family of serine/threonine protein kinases connected to various essential cellular processes (10). To date, a total of 14 MAPKs have been isolated in humans, all of which fall into the seven classes that follow: the extracellular signal-regulated kinase class, p38 class, c-Jun N-terminal kinase (JNKs) class, and ERK5 class belong to the conventional group of MAPKs, all of which have been studied extensively, whereas the Nemo-like kinase (NLK) class, ERK3/4 class, and ERK7 class belong to the atypical group of MAPKs, all of which have been studied inadequately (10,11). In general, the MAPK pathway contains at least three tiers: a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK. These MAPK pathways participate in transducing signals from the surface to the interior of the cell. Being triggered by extracellular stimulus, first tier MAP3Ks are activated to phosphorylate MAP2Ks, which subsequently phosphorylate MAPKs. These MAPK pathways have their own unique primary kinases in different tiers, but they also share some minor activators (10,11). All MAPKs, except ERK3/4 and NLK, contain a conserved Thr-X-Tyr motif in their kinase domain. Phosphorylation of both Thr and Tyr residues in this motif is a critical step for MAPK activation (10).

Human ERK1 (also known as MAPK3 or p44MAPK) and ERK2 (also known as MAPK1 or p42MAPK) are 84% identical in sequence; they share many functions (12). Thus they are often referred to as ERK1/2. Among these MAPKs, ERK1/2 are associated with cell proliferation, cell growth, cell mobility, and cell survival (13), and the Ras-Raf (MAP3K)-MEK (MAP2K)-ERK1/2 (MAPK) signal transduction cascade can be activated by growth factors, osmotic stress, and cytokines (11,14,15). To date, more than 160 substrates of ERK1/2 have been discovered from the nucleus and cytosol to the cell membrane (16).

Post-translational modifications (PTMs) have long been documented as a critical means of regulating ERK1/2 activity. Compared with the decades of studies of ERK1/2 phosphorylation, especially at the Thr202/Tyr204 sites in ERK1 and Thr185/Tyr187 in ERK2 of the Thr-X-Tyr motif, the studies of other PTMs of ERK1/2, including acetylation, methylation, and ubiquitination, are just emerging. Recently, two lysine sites at the ERK1 C-terminus, Lys302 and Lys361, have been revealed to be tri-methylated, and methylation of ERK1 enhances its phosphorylation (17). Arg309 of ERK1 has also been reported as a methylation site via a proteome-wide analysis, yet the function of this site is not clear (18). Ubiquitination of ERK1/2 has been reported from three different proteome-wide analyses, but the role of ubiquitination in ERK1/2 still remains elusive (19-21). Likewise, one ERK1 acetylation site has been identified by SILAC assays, but the function of this site still remains to be determined (22). Lately, several
reports have shown that when cell lines including A549, MB361, BT474, MV4-11, PC-3, SKBR-3, HN-9 and SQ20B were treated with HDAC6 inhibitors, the level of phosphorylated ERK1/2 decreased (23-26), suggesting that acetylation of ERK1/2 compromises their activities and that HDAC6 inhibition may down-regulate ERK1/2 activities. However, the mechanisms underlying this observation are not clear.

Our previous study showed that ERK1 phosphorylates HDAC6 at its Ser1035 site, and phosphorylation of this site increases HDAC6’s activity toward α-tubulin and stimulates cell migration (27). In this study, we have determined that ERK1/2 are acetylated proteins, and that ERK1/2 are novel substrates of HDAC6. Both ERK1/2 show the ability to physically interact with HDAC6 in vitro. Also, both CBP and p300 acetylate ERK1/2. One novel acetylation site, Lys72, was identified in ERK1 via mass-spectrometry analysis, and the acetylation-mimicking mutant of ERK1 exhibits reduced kinase activity, suggesting that the acetylation status of ERK1 plays an important role in regulating ERK1 enzymatic activity.

RESULTS

ERK1/2 interact with HDAC6 directly – We previously showed that ERK1/2 interact with HDAC6 endogenously (27). However, how these proteins interact with each other was unknown. To determine whether ERK1/2 interact with HDAC6 directly or through other proteins, we performed in vitro GST pull-down assays with bacterially-purified HDAC6 and ERK1/2. As shown in Figure 1A, GST-HDAC6, but not GST, efficiently pulled down His-ERK1. Similarly, as shown in Figure 1B, GST-HDAC6 also pulled down His-ERK2. Therefore, ERK1/2 physically interact with HDAC6.

Inhibition or depletion of HDAC6 increases ERK1/2 acetylation – Previously, we demonstrated that ERK1 phosphorylates HDAC6 at the Ser1035 site (27). Given the fact that HDAC6 is a deacetylase, we interrogated HDAC6 to see if it deacetylates ERK1, in other words, whether ERK1 is a substrate of HDAC6. To this end, we set out to determine whether ERK1 is acetylated. Before using the anti-acetylated lysine (AcK) antibodies to examine ERK1 acetylation, we tested two commercial anti-AcK antibodies from Cell Signaling Technology. As shown in Figure 2, both antibodies specifically recognized acetylated BSA but not no-acetylated BSA. We then used these two antibodies in the following experiments. To determine whether ERK1 is acetylated, mammalian expression vector GST-ERK1 was transfected into HEK293T cells. Then the transfected cells were treated with 0, 50, 100, 200, 400, or 600 ng/ml of pan-HDAC inhibitor, Trichostatin A (TSA) 12 hours prior to harvest. As shown in Figure 3A, after normalizing with the total ERK1, the level of acetylated GST-ERK1 was increased as the dosage of TSA was increased. 600 ng/ml TSA increased the level of acetylated GST-ERK1 by four-fold as compared to a vehicle. We then treated the GST-ERK1-transfected HEK293T cells with 600 ng/ml TSA at 0, 2, 4, 8, 12 and 24 hours. As shown in Figure 3B, the level of acetylated GST-ERK1 increased as the TSA treatment time increased. At the 24-hour time point, the longest time point, the level of acetylated GST-ERK1 is nearly two-fold as compared to that at the 0-hour time point. To ensure that TSA is functional (TSA inhibits class I, II, and IV HDACs including HDAC6) we also detected the level of acetylated α-tubulin, which is a well-known HDAC6 substrate (4). Using a similar approach, we have shown that ERK2 is acetylated upon TSA treatment (Figure 3C and 3D). Taken together, TSA increases ERK1/2 acetylation in a dose- and time-dependent manner.

Next, we set out to determine whether specific inhibition of HDAC6 would increase ERK1/2 acetylation. We treated GST-ERK1- or GST-ERK2-transfected HEK293T cells with an HDAC6-specific inhibitor, ACY-1215, at the concentrations of, 0, 0.5, 1, 2, 4, and 6 μg/ml, for 12 hours. As shown in Figures 4, ACY-1215 increased the level of acetylated GST-ERK1 and GST-ERK2 by 9-fold and 4-fold, respectively.

To determine whether endogenous ERK1/2 are acetylated, we treated 293T cells with the pharmacological inhibitor TSA or ACY-1215 and examined the level of acetylation of ERK1/2. As shown in Figure 5, both inhibitors increased the acetylation levels of endogenous ERK1/2, suggesting that inhibition of HDAC6 increases the acetylation of endogenous ERK1/2. To further confirm the role of HDAC6 in regulating ERK1/2 deacetylation, we compared the...
acetylation level of endogenous ERK1/2 in HDAC6 wild-type and HDAC6 knockout MEFs. As shown in Figure 6A, the acetylation of endogenous ERK1/2 increased significantly in HDAC6 knockout MEFs as compared with HDAC6 wild-type MEFs. Likewise, the level of endogenous ERK1/2 increased in HDAC6 knockout A549 cells as compared with that of control A549 cells (Figure 6B). These results validate that inhibition or depletion of HDAC6’s enzymatic activity is responsible for the increase of ERK1/2 acetylation.

**CBP and p300 acetylate ERK1/2 in vivo and in vitro** – To determine which histone acetyltransferase (HAT) acetylates ERK1/2, we tested five HATs, which belong to the following three families: Gen5-related N-acetyltransferase (GNAT) family (PCAF), MYST family (TIP60 and HBO1), and p300/ CREB-binding protein (CBP) family (p300 and CBP) (28-30). These HATs were 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 (Figure 7A). p300 also weakly acetylated ERK1 (Figure 7A). To further confirm the results, we co-expressed the increasing amounts of CBP or p300 with GST-ERK1. As shown in Figures 7B and 7C, CBP and p300 acetylated GST-ERK1 in a dose-dependent manner. To further confirm CBP and p300’s effect on endogenous ERK1, we overexpressed p300 in HEK293T 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 (Figure 7A). p300 also weakly acetylated ERK1 (Figure 7A). To further confirm the results, we co-expressed the increasing amounts of CBP or p300 with GST-ERK1. As shown in Figures 7B and 7C, CBP and p300 acetylated GST-ERK1 in a dose-dependent manner. To further confirm CBP and p300’s effect on endogenous ERK1, we overexpressed p300 in HEK293T cells and tested the acetylation level of endogenous ERK1. As shown in Figure 7D, exogenous p300 increased the acetylation of endogenous ERK1. To eliminate the potential influence of endogenous HATs and HDACs in the cells on ERK1 acetylation, we executed in vitro acetylation assays to confirm that CBP is an ERK1 acetyltransferase. As shown in Figure 7E, recombinant CBP indeed acetylated bacterially purified GST-ERK. Similarly, we have shown that both CBP and p300 promote ERK2 acetylation in cells and that CBP acetylated ERK2 in vitro. (Figure 8A-D).

**HDAC6 deacetylates ERK1 in vivo and in vitro** – Because of the low basal acetylation level of ERK1, to confirm whether HDAC6 deacetylates ERK1, we co-expressed CBP to increase the ERK1 acetylation level. As shown in Figure 9A and 9B, as expected, both CBP and p300 increased acetylation of GST-ERK1, whereas overexpression of HDAC6 reduced CBP- or p300-mediated ERK1 acetylation. Then we tested whether HDAC6 is able to deacetylate in vitro acetylated GST-ERK1 purified from bacteria. As shown in Figure 9C, HDAC6 purified from 293T cells could efficiently deacetylate acetylated ERK1 in vitro, suggesting that ERK1 is a bona fide substrate of HDAC6.

**Acetylation of ERK1 reduces ERK1’s enzymatic activity** - We next examined whether acetylation of ERK1 affects its enzymatic activity. Because HDAC6 deacetylates ERK1, we then hypothesize that in the absence of HDAC6, ERK1 acetylation would be increased which may alter ERK1’s enzymatic activity. To test this hypothesis, the ERK1 plasmid was transfected into wild-type 293T cells or HDAC6KO 293T cells. Then ERK1 was isolated from these two type of cells and ERK1’s kinase activity was examined using recombinant ELK1 as a substrate. ELK1 is a member of Ets transcription factor family, and several serine and threonine sites of ELK1 can be phosphorylated by ERKs (31). Among these sites, phosphorylation status of ELK1 Ser383 is pivotal for ELK1 transcriptional activation (32). Because of this reason, we used ELK1 as the substrate to execute non-radioactive kinase assays to measure the ability of ERK1 to phosphorylate the Ser383 site in ELK1. As shown in Figure 10A, ERK1 purified from HDAC6KO cells displayed significantly lower activity than that from wild-type cells. Then we also directly acetylated ERK1 using CBP and investigated the enzymatic activity of vehicle-incubated ERK1 versus CBP-incubated ERK1. As shown in Figure 10B, CBP-acetylated ERK1 harbored lower enzymatic activity. In summary, we concluded that acetylation of ERK1 decreases its enzymatic activity.

**ERK1 Lysine 72 is acetylated** – To detect the acetylation site of ERK1, we prepared samples for mass spectrometry analyses. GST-ERK1 and CBP were co-expressed in HEK293T cells for 36 hours. Cells were harvested and lysed in lysis buffer. GST-ERK1 was then pulled-down by glutathione-agarose and resolved on SDS-PAGE. The SDS-PAGE gel was stained by coomassie blue, and the specific bands were excised. Samples were further digested with chymotrypsin and Lys-C endoproteinase.
sequentially and subjected for LC-tandem mass spectrometry analysis. Lys72 was identified as a novel acetylation site of ERK1 (Figure 11A). Lys72 is located on β3-strand of ERK1’s N-lobe and is very close to the glycine-rich loop (Figure 11B). To show the conservation of Lys72 in ERK1, ERK1 sequences from human to nematode were compared by the T-Coffee alignment program. The alignment results showed that this mass spectrometry-identified ERK1 Lys72 was highly conserved among mammals and even in Zebrafish (Danio rerio), Drosophila, and C. elegans (Figure 11C), indicating that Lys72 plays an important role in ERK1 function.

**Acetylation mimetic mutant of ERK1 abolishes ERK1 kinase activity toward ELK1** – To test whether acetylation status of Lys72 in ERK1 affects its kinase activity, Lys72 was mutated to glutamine (an acetylation mimetic mutant, K72Q) or arginine (a deacetylation mimetic mutant, K72R), and the resulting mutants were tested by their phosphorylation status in cells followed by kinase assays. As shown in Figure 12A, K72Q, but not K72R, displayed a reduced level of phosphorylation as compared with wild-type ERK1, implying that the K72Q mutant exhibits a diminished enzymatic activity. To confirm this notion, the kinase assay using the most thoroughly studied ERK1 substrate, ELK1, was performed. As shown in Figure 12B, the acetylation mimetic mutant, ERK1 (K72Q) displayed a significantly reduced kinase activity toward ELK1 as compared with the deacetylation mimetic mutant, ERK1 (K72R) and the wild-type of ERK1.

To further demonstrate the impact of ERK1-K72 acetylation in vivo, we monitored activity of ELK1 in a reporter assay. The luciferase construct, (ELK1)2-TATA-Luc, being used in this assay was a kind gift from Dr. Manohar Ratnam. In this construct, the cis-element preferred by ELK1 was placed as two tandem repeat elements upstream of a minimal TATA-dependent Firefly luciferase promoter (33). Wild-type, K72Q and K72R of ERK1 were examined by their ability to activate ELK1-mediated transcription by luciferase assays in HeLa cells. The pRL plasmid encoding Renilla luciferase was also transfected in HeLa cells together with the above constructs. The Renilla reading was then used to normalize the Firefly luciferase reading. As shown in Figure 12C, the ELK1-dependent promoter activity is significantly lower in acetylation mimetic mutant ERK1(K72Q) transfected cells than in wild-type or deacetylation mimetic mutant ERK1(K72R) transfected cells, indicating that acetylation at K72 site reduces ERK1’s activity to activate ELK1-mediated transcription.

In order to determine how acetylation/deacetylation of Lys72 regulates ERK1 kinase activity, we examined ERK1’s crystal structure. Lys72 is located near the ATP binding site and stabilizes one wall of the ATP binding site via intramolecular contacts. In particular, Lys72 forms a salt bridge with Asp117 and links to Tyr119 with a hydrogen bond. Acetylation mimetic Lys72 would break the contacts to both Asp117 and Tyr119, and might change the conformation of the ATP binding site leading to reduced enzymatic activity of ERK1 (Figures 8C and 8D).

**DISCUSSION**

In this study, we have demonstrated that the acetylation/deacetylation status of Lys72 in ERK1 regulates its enzymatic activity. For the first time, we have revealed that ERK1 and ERK2 are acetylated proteins and are novel substrates of the deacetylase HDAC6 and the acetyltransferases CBP and p300. We have also discovered that Lys72 of ERK1 is a novel acetylation site. Furthermore, we have shown that the ERK1 Lys72 acetylation-mimicking mutant (K72Q) displayed reduced kinase activity as compared with the wild-type and deacetylation-mimicking mutant (K72R). Overall, our results suggest that HDAC6/CBP and p300 govern ERK1’s kinase activity via deacetylation/acetylation of Lys72.

Although we have provided strong evidence that HDAC6 deacetylates ERK1/2, we cannot rule out the possibility that other Class I, II and IV HDACs can deacetylate ERK1/2. In addition, we tested whether Class III HDACs, also called sirtuins, can deacetylate ERK1/2. We found that the Sirtuin inhibitor, nicotinamide, increased ERK2, but not ERK1, acetylation (data not shown), suggesting that one or multiple sirtuins may deacetylate ERK2. Further investigations are...
warranted to study whether other HDACs and sirtuins regulate ERK1/2.

In addition, we have identified a conserved lysine, Lys72, which is adjacent to a critical ATP-binding site, 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 substrate, ELK1. According to the structural analysis, the acetylation mimetic mutant of ERK1(K72Q), but not the deacetylation mimetic mutant ERK1(K72R), 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. Our study is the first to report that the acetylation/deacetylation of a conserved lysine in subdomain II of ERK1 could influence its enzymatic activity. It would be interesting to speculate whether the acetylation/deacetylation of Lys53 in ERK2, which is equivalent to Lys72 in ERK1, regulates ERK2’s kinase activity, although Lys53 has not been identified as an acetylation site yet.

More than two decades ago, it was demonstrated that Lys71 within subdomain II is critical for ATP binding (34,35). Substitution of Lys to Arg at this site therefore abolishes ERK1 kinase activity (35). Interestingly, we found that Lys71 can be acetylated by mass spectrometry analysis (data not shown). It was expected that the replacement of Lys with any other amino acid would ablate ERK1 kinase activity. Because of this reason, Lys to Arg (the deacetylation mimetic mutation) or Lys to Glu (the 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 elucidate the role of the acetylation of this site in ERK1 function.

However, it is intriguing that acetylation of Lys53, a homologous site of ERK1’s Lys71, in p38 augments p38’s kinase activity (36). Moreover, Lys52 in ERK2 and Lys55 in JNK1 and JNK2 are also homologous to ERK1’s Lys71 (36,37), but whether these sites are acetylated remains to be determined. It is tempting to hypothesize that the acetylation/deacetylation of the two conserved lysines in subdomain II (in the case of ERK1, K71 and K72), which either bind to ATP or form salt bridges to affect ATP binding, is a strategy employed by HATs and HDACs to fine-tune the enzymatic activities of MAPKs.

As the main moderator in the downstream of the MAPK pathway, ERK1/2 are emerging as alternative targets, especially when inhibitors of the upstream kinases become resistant to patients (38). There are several ERK1/2-specific inhibitors being used to combat the resistance to EGFR, Raf or MEK inhibitors in clinical trials. HDAC6-specific inhibitors are also being tested in clinical trials. Most of these trials were conducted with other anti-cancer drugs (33,39-47). The combination of HDAC inhibitors and ERK1/2 pathway inhibitors have shown synergistic cell killing (48-52). Here we show that inhibition of HDAC6 down-regulates ERK1’s enzymatic activity, suggesting that the combination of HDAC6 inhibitors and ERK1/2 inhibitors may be a promising strategy to overcome the resistance to EGFR, Raf or MEK inhibitors.

**EXPERIMENTAL PROCEDURES**

**Antibodies**- Anti-acetylated lysine mouse mAb (Ac-K-103)(#9681), anti-acetylated lysine rabbit polyclonal antibody (#9441), 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-actin (#4967) antibodies were purchased from Cell Signaling Technology, Inc. The anti-Flag M2 antibody (F1804), anti-Flag® M2 affinity gel (A2220) 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), SalI (#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...
HDAC6 deacetylates ERK1

GCTGGAATC-3' (NotI).
pGEX-4T-1-GST-ERK2 was then used as a template for PCR using the following primers: 5'-CCCACTAGTATGTCCTCCTATACTAGGTGTA TTG-3' (SpeI) and 5'-CGCGCGCCGCTAGATCTGATATC CTG-3' (NotI) 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'-CCCACTAGTATGTCCTCCTATACTAGGTGTA TTG-3' (SpeI) and 5'-GCGCTGAGCTAGGGGCTCCAGACT CC-3' (XhoI). Then the PCR product was inserted between SpeI and XhoI sites to generate 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'-CAGCTGAGGCAGTTTCTTTGGAACAT-3' and 5'-GATGGCCACGCGAGTCTTGCGC-3'; for K72R, 5'-CAGCTGAGGCAGTTTCTTTGGAACAT-3' and 5'-GATGGCCACGCGAGTCTTGCGC-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 cDNA fragments of GST-ERK1(K72Q) and GST-ERK1(K72R), respectively. To generate the 6xHis-tagged ERK1, pET-ERK1, the cDNA of ERK1 was transferred from pGEX-4T-1-ERK1(27) 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

Plasmids construction-pGEX-4T-1-HDAC6 was generated by PCR using HA-HDAC6-F (5) as the template and the following primers: GST-HD6-F 5'-CCCGTCGACTCATGACCTCAACCGGCCAGGA-3' (SalI) and GST-HD6-R 5'-TGCGGCCGCTTAGTGTGGGTGGGGCATA TC-3' (NotI). The PCR product was inserted into the SalI 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.(27). Briefly, ERK1 cDNA was isolated from the pDONR223-MAPK3 (Addgene Plasmid 23509) vector by PCR using the following primers: ERK1-F 5'-CCCGGATCCATGGCGGCGGCGGGCTC AG-3' (BamHI) and ERK1-R 5'-GGGCTCGAGCTAGGGGGCCTCCAGCAC CC-3' (XhoI). The PCR product was then inserted into the BamHI and XhoI sites into the pGEX-4T-1 vector to generate pGEX-ERK1. GST-ERK1 cDNA was isolated by PCR using pGEX-4T-1-ERK1 as a template and the following primers: GST-SpeI 5'-CCCACTAGTATGTCCTCCTATACTAGGTGTA TTG-3' (SpeI) and ERK1-R 5'-GGGCTGAGCTAGGGGCTCCAGACCT CC-3' (XhoI). The PCR product was then inserted into the SpeI and XhoI sites 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'-CCCACTAGTATGTCCTCCTATACTAGGTGTA TTG-3' (SpeI) and 5'-GCGCTGAGCTAGGGGCTCCAGACT CC-3' (XhoI). 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'-CAGCTGAGGCAGTTTCTTTGGAACAT-3' and 5'-GATGGCCACGCGAGTCTTGCGC-3'; for K72R, 5'-CAGCTGAGGCAGTTTCTTTGGAACAT-3' and 5'-GATGGCCACGCGAGTCTTGCGC-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 cDNA fragments of GST-ERK1(K72Q) and GST-ERK1(K72R), respectively. To generate the 6xHis-tagged ERK1, pET-ERK1, the cDNA of ERK1 was transferred from pGEX-4T-1-ERK1(27) 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

Plasmids construction-pGEX-4T-1-HDAC6 was generated by PCR using HA-HDAC6-F (5) as the template and the following primers: GST-HD6-F 5'-CCCGTCGACTCATGACCTCAACCGGCCAGGA-3' (SalI) and GST-HD6-R 5'-TGCGGCCGCTTAGTGTGGGTGGGGCATA TC-3' (NotI). The PCR product was inserted into the SalI 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.(27). Briefly, ERK1 cDNA was isolated from the pDONR223-MAPK3 (Addgene Plasmid 23509) vector by PCR using the following primers: ERK1-F 5'-CCCGGATCCATGGCGGCGGCGGCTC AG-3' (BamHI) and ERK1-R 5'-GGGCTCGAGCTAGGGGGCCTCCAGCAC CC-3' (XhoI). The PCR product was then inserted into the BamHI and XhoI sites into the pGEX-4T-1 vector to generate pGEX-ERK1. GST-ERK1 cDNA was isolated by PCR using pGEX-4T-1-ERK1 as a template and the following primers: GST-SpeI 5'-CCCACTAGTATGTCCTCCTATACTAGGTGTA TTG-3' (SpeI) and ERK1-R 5'-GGGCTGAGCTAGGGGCTCCAGACCT CC-3' (XhoI). The PCR product was then inserted into the SpeI and XhoI sites into the pLEX-MCS vector (Thermo Scientific, Catalog # OHS4735) between SpeI and NotI sites to generate pLEX-GST-ERK2.
H D A C 6 deacetylases ERK1

pET28a vector via Sall and NotI sites. pCMV-ELK1 purchased from Origene (SC116858) contains untagged human ELK1 (member of ETS oncogene family (ELK1), transcript variant 2, NM_005229.2). The construct was made by inserting the cDNA of ELK1 into the Not I site of the pCMV6-XL4 vector.

**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 A549 cells and HDAC6 knock-out 293T 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′-AGGTCTACTGTGGTCGTTACATCAATGGC -3′, tube ID:TI349960, 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. HDAC6 knockout 293T cells were created using CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats) method. Briefly, the guide RNA targeting HDAC6 exon 5 (5′-GAAAGGACACGCAGCAGTCT-3′) was selected and inserted into the LentiCRISPRv2 vector (Addgene plasmid 52961) to generate the HDAC6KO vector which also express the codon-optimized Cas9 protein as well as puromycin resistance gene. The 293T cells infected with the HDAC6-KO virus were selected for stable clones using puromycin at 1 µg/ml. The HDAC6-Knockout clones were screened by anti-HDAC6 (H-300) Western blot analysis.

**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 TBS-T (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 pH 7.4, 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 assays that follow.

**Purification of His-ERK1/2 proteins**-BL21 bacteria harboring His-ERK1 or His-ERK2 were grown in the log phase and induced with Isopropyl β-D-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 in the 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.

**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 pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol and protease inhibitor cocktail (11836170001, ROCHE)). These GST fusion ERK1 proteins were washed one time by 1x the kinase buffer (5 mM Tris pH 7.5, 5 mM β-Glycerol phosphate, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂) before the 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 the 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 pH 8.0, 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.

**Samples preparation for Mass-spectrometry** - GST-ERK1 and HA-CBP were co-expressed in 293T cells for 36 hours. Cells were harvested and lysed in lysis buffer. GST-ERK1 was then pulled-down by glutathione agarose and analyzed by SDS-PAGE. The gel was stained by coomassie blue, and the specific bands were excised. The excised gels were further digested with chymotrypsin and Lys-C endoproteinase sequentially and subject for mass spectrometry analysis.

**LC-MS/MS Analysis** - A nanoflow ultra high performance liquid chromatograph (RSLC, Dionex, Sunnyvale, CA) coupled to an electrospray bench top orbitrap mass spectrometer (Q-Exactive plus, Thermo, San Jose, CA) was used for tandem mass spectrometry peptide sequencing experiments. The sample was first loaded onto a pre-column (2 cm x 100 µm ID packed with C18 reversed-phase resin, 5µm, 100Å) and washed for 8 minutes with aqueous 2% acetonitrile and 0.04% trifluoroacetic acid. The trapped peptides were eluted onto the analytical column, (C18, 75 µm ID x 50 cm, 2 µm, 100Å, Dionex, Sunnyvale, CA). The 90-minute gradient was programmed as: 95% solvent A (2% acetonitrile + 0.1% formic acid) for 8 minutes, solvent B (90% acetonitrile +
0.1% formic acid) from 5% to 38.5% in 60 minutes, then solvent B from 50% to 90% B in 7 minutes and held at 90% for 5 minutes, followed by solvent B from 90% to 5% in 1 minute and re-equilibrate for 10 minutes. The flow rate on analytical column was 300 nl/min. Sixteen tandem mass spectra were collected in a data-dependent manner following each survey scan. Both MS and MS/MS scans were performed in Orbitrap to obtain accurate mass measurement using 60 second exclusion for previously sampled peptide peaks.

**Data Analysis**-Sequest (53) and Mascot (54) searches were performed against the Swiss-Prot human database. Two trypsin missed cleavages were allowed, the precursor mass tolerance was 10 ppm. MS/MS mass tolerance was 0.05 Da. Dynamic modifications included carbamidomethylation (Cys), oxidation (Met) and acetylation (Lys). Both MASCOT and SEQUEST search results were summarized in Scaffold 4.4.

**Luciferase Reporter Assay**- Briefly, HeLa cells were seeded in 24-well plates overnight prior to transfection. The cells were transfected with plasmids of (ELK1)2-TATA-Luc (33), pCMV-ELK1 (Origene), HA-MEK(S218/222D), GST-ERK1-WT, GST-ERK1-K72Q, or GST-ERK1-K72R as shown in Figure 12C. The pRL plasmid encoding Renilla luciferase gene was also transfected. 24 hours post transfection, cells were lysed with 100 μl 1X lysis buffer (Dual-luciferase reporter, E1910/Promega). 10 μl of lysates was taken out to mix with 50 μl LARI and 50 μl “STOP & Glo” for firefly and renilla reading, sequentially, in a white opaque 96-well plate using a BioTek Synergy 2 Multi-Mode reader.

**In vitro deacetylation assay**- F-HDAC6 was overexpressed in 293T cells and purified with ANTI-FLAG® M2 affinity gel by following vendor’s instruction. Purified F-HDAC6 was resolved on SDS-PAGE and stained by coomassie blue to verify the amount and purity. Purified GST-ERK1 was first acetylated by CBP *in vitro*, then acetylated GST-ERK1 was incubated with or without F-HDAC6 in the 1x deacetylation buffer (20 mM Tris [pH 8.0], 150 mM NaCl, and 10% glycerol) at 30°C for 2 hr. The reaction was stopped by adding 5x SDS-PAGE sample buffer and boiled in 95°C for 10 minutes.

**ACKNOWLEDGEMENTS**

We thank Victoria Izumi at the H. Lee Moffitt Cancer Center Proteomics facility and Dr. Paul Stemmer at the Wayne State University Proteomics Core Facility for the mass spectrometry analysis, Dr. Edward Seto for the HBO1 plasmid, Dr. Tso-Pang Yao for the CBP construct, Dr. Manohar Ratnam, Dr. Rayna Rosati for the plasmids of pCMV-ELK1 and (ELK1)2-TATA-Luc, and assistance of the luciferase reporter assays, Dr. Jie Wu for HA-MEK(S218/222D) plasmid and discussion, and Mr. Joshua Haakenson for proofreading the manuscript.

**CONFLICT OF INTEREST**

We have no conflict of interest to declare.

**AUTHOR CONTRIBUTIONS**

J-Y W performed most of the experiments. SX helped make some constructs. MZ did the luciferase reporter assays. BF, HH, OKK, and YZ did the mass spectrometry analyses. ZY did the structural analysis of ERK1. WB and GB provided the critical reagents and helped design the experiments. J-Y W and XZ wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

**REFERENCES**

1. Yang, X. J., and Seto, E. (2007) HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. *Oncogene* 26, 5310-5318
2. Yang, X. J., and Seto, E. (2008) The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. *Nat Rev Mol Cell Biol* 9, 206-218
3. Seto, E., and Yoshida, M. (2014) Erasers of histone acetylation: the histone deacetylase enzymes. *Cold Spring Harb Perspect Biol* 6, a018713
4. Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Nixon, A., Yoshida, M., Wang, X. F., and Yao, T. P. (2002) HDAC6 is a microtubule-associated deacetylase. *Nature* 417, 455-458
5. Zhang, X., Yuan, Z., Zhang, Y., Yong, S., Salas-Burgos, A., Koomen, J., Olashaw, N., Parsons, J. T., Yang, X. J., Dent, S. R., Yao, T. P., Lane, W. S., and Seto, E. (2007) HDAC6 modulates cell motility by altering the acetylation level of cortactin. *Mol Cell* 27, 197-213
6. Zhang, M., Xiang, S., Joo, H. Y., Wang, L., Williams, K. A., Liu, W., Hu, C., Tong, D., Haakenson, J., Wang, C., Zhang, S., Pavlovic, R. E., Jones, A., Schmidt, K. H., Tang, J., Dong, H., Shan, B., Fang, B., Radhakrishnan, R., Glazer, P. M., Matthias, P., Koomen, J., Seto, E., Bepler, G., Nicosia, S. V., Chen, J., Li, C., Gu, L., Li, G. M., Bai, W., Wang, H., and Zhang, X. (2014) HDAC6 Deacetylates and Ubiquitinates MSH2 to Maintain Proper Levels of MutSalpha. *Mol Cell* 55, 31-46
7. Lee, Y. S., Lim, K. H., Guo, X., Kawaguchi, Y., Gao, Y., Barrientos, T., Ordentlich, P., Wang, X. F., Counter, C. M., and Yao, T. P. (2008) The cytoplasmic deacetylase HDAC6 is required for efficient oncogenic tumorigenesis. *Cancer Res* 68, 7561-7569
8. Yang, M. H., Laurent, G., Bause, A. S., Spang, R., German, N., Haigis, M. C., and Haigis, K. M. (2013) HDAC6 and SIRT2 regulate the acetylation state and oncogenic activity of mutant K-RAS. *Mol Cancer Res* 11, 1072-1077
9. Li, Y., Zhang, X., Polakiewicz, R. D., Yao, T. P., and Comb, M. J. (2008) HDAC6 is required for epidermal growth factor-induced beta-catenin nuclear localization. *J Biol Chem* 283, 12686-12690
10. Cargnello, M., and Roux, P. P. (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev* 75, 50-83
11. Johnson, G. L., and Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298, 1911-1912
12. Lloyd, A. C. (2006) Distinct functions for ERKs? *J Biol* 5, 13
13. Roskoski, R. (2012) ERK1/2 MAP kinases: Structure, function, and regulation. *Pharmacological Research* 66, 105-143
14. Raman, M., Chen, W., and Cobb, M. H. (2007) Differential regulation and properties of MAPKs. *Oncogene* 26, 3100-3112
15. Boulton, T. G., Yancopoulous, G. D., Gregory, J. S., Slaughter, C., Moomaw, C., Hsu, J., and Cobb, M. H. (1990) An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science* 249, 64-67
16. Yoon, S., and Seger, R. (2006) The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. *Growth Factors* 24, 21-44
17. Vougiouklakis, T., Sone, K., Saloura, V., Cho, H. S., Suzuki, T., Dohmae, N., Alachkar, H., Nakamura, Y., and Hamamoto, R. (2015) SUV420H1 enhances the phosphorylation and transcription of ERK1 in cancer cells. *Oncotarget* 6, 43162-43171
18. Larsen, S. C., Sylvestersen, K. B., Mund, A., Lyon, D., Mullari, M., Madsen, M. V., Daniel, J. A., Jensen, L. J., and Nielsen, M. L. (2016) Proteome-wide analysis of arginine monomethylation reveals widespread occurrence in human cells. *Science Signalig* 9, rs9
19. Kim, W., Bennett, E. J., Huttlin, E. L., Guo, A., Li, J., Posemmato, A., Sowa, M. E., Rad, R., Rush, J., Comb, M. J., Harper, J. W., and Gygi, S. P. (2011) Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell* 44, 325-340
20. Mertins, P., Qiao, J. W., Patel, J., Udeshi, N. D., Clauser, K. R., Mani, D. R., Burgess, M. W., Gillette, M. A., Jaffe, J. D., and Carr, S. A. (2013) Integrated proteomic analysis of post-translational modifications by serial enrichment. *Nat Methods* 10, 634-637
21. Wagner, S. A., Beli, P., Weinert, B. T., Nielsen, M. L., Cox, J., Mann, M., and Choudhary, C. (2011) A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. *Molecular & Cellular Proteomics* 10, M111 013284
22. Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res* 43, D512-520
23. Chuang, M. J., Wu, S. T., Tang, S. H., Lai, X. M., Lai, H. C., Hsu, K. H., Sun, K. H., Sun, G. H., Chang, S. Y., Yu, D. S., Hsiao, P. W., Huang, S. M., and Cha, T. L. (2013) The HDAC inhibitor LBH589 induces ERK-dependent prometaphase arrest in prostate cancer via HDAC6 inactivation and down-regulation. *Plos One* 8, e73401
24. Inks, E. S., Josey, B. J., Jesinkey, S. R., and Chou, C. J. (2012) A novel class of small molecule inhibitors
of HDAC6. *ACS Chem Biol* **7**, 331-339

25. Kim, I. A., No, M., Lee, J. M., Shin, J. H., Oh, J. S., Choi, E. J., Kim, I. H., Atadja, P., and Bernhard, E. J. (2009) Epigenetic modulation of radiation response in human cancer cells with activated EGFR or HER-2 signaling: potential role of histone deacetylase 6. *Radiother Oncol* **92**, 125-132

26. Tien, S. C., and Chang, Z. F. (2014) Oncogenic Shp2 disturbs microtubule regulation to cause HDAC6-dependent ERK hyperactivation. *Oncogene* **33**, 331-339

27. Williams, K. A., Zhang, M., Xiang, S., Hu, C., Wu, J. Y., Zhang, S., Ryan, M., Cox, A. D., Der, C. J., Fang, B., Koomen, J., Haura, E., Bepler, G., Nicosia, S. V., Matthias, P., Wang, C., Bai, W., and Zhang, X. (2013) Extracellular signal-regulated kinase (ERK) phosphorylates histone deacetylase 6 (HDAC6) at serine 1035 to stimulate cell migration. *J Biol Chem* **288**, 33156-33170

28. Marks, P., Rifkind, R. A., Richon, V. M., Breslow, R., Miller, T., and Kelly, W. K. (2001) Histone deacetylases and cancer: causes and therapies. *Nature reviews. Cancer* **1**, 194-202

29. Lee, K. K., and Workman, J. L. (2007) Histone acetyltransferase complexes: one size doesn't fit all. *Nature reviews. Molecular cell biology* **8**, 284-295

30. Roth, S. Y., Denu, J. M., and Allis, C. D. (2001) Histone acetyltransferases. *Annual review of biochemistry* **70**, 81-120

31. Gille, H., Kortenjann, M., Thomae, O., Moomaw, C., Slaughter, C., Cobb, M. H., and Shaw, P. E. (1995) ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J* **14**, 951-962

32. Marais, R., Wynne, J., and Treisman, R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73**, 381-393

33. Patki, M., Chari, V., Sivakumaran, S., Gonit, M., Trumbly, R., and Ratnam, M. (2013) The ETS domain transcription factor ELK1 directs a critical component of growth signaling by the androgen receptor in prostate cancer cells. *J Biol Chem* **288**, 11047-11065

34. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52

35. Robbins, D. J., Zhen, E., Owaki, H., Vanderbilt, C. A., Geppert, T. D., and Cobb, M. H. (1993) Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. *J Biol Chem* **268**, 5097-5106

36. Pillai, V. B., Sundaresan, N. R., Samant, S. A., Wolfgeher, D., Trivedi, C. M., and Gupta, M. P. (2011) Acetylation of a conserved lysine residue in the ATP binding pocket of p38 augments its kinase activity during hypertrophy of cardiomyocytes. *Mol Cell Biol* **31**, 2349-2363

37. Robinson, M. J., Harkins, P. C., Zhang, J., Baer, R., Haycock, J. W., Cobb, M. H., and Goldsmith, E. J. (1996) Mutation of position 52 in ERK2 creates a nonproductive binding mode for adenosine 5'-triphosphate. *Biochemistry* **35**, 5641-5646

38. Samatar, A. A., and Poulikakos, P. I. (2014) Targeting RAS-ERK signalling in cancer: promises and challenges. *Nature Reviews Drug Discovery* **13**, 928-942

39. Mevissen, T. E. T., and Komander, D. (2017) Mechanisms of Deubiquitinase Specificity and Regulation. *Annu Rev Biochem* **86**, 159-192

40. A Phase 1b Study of Paclitaxel And Ricolinostat For The Treatment Of Gynecological Cancer.

41. ACY-1215 + Nab-paclitaxel in Metastatic Breast Cancer.

42. Phase 1b Study Evaluating ACY-1215 (Ricolinostat) in Combination With Pomalidomide and Dexamethasone in Relapsed or Relapsed-and-Refractory Multiple Myeloma.

43. ACY-1215 for Relapsed/Refractory Lymphoid Malignancies.

44. Alternative 10 mg/mL Liquid Formulation of ACY 1215 (Ricolinostat) in Healthy Subjects.

45. ACY-1215 (Ricolinostat) in Combination With Pomalidomide and Low-dose Dex in Relapsed-and-Refractory Multiple Myeloma.

46. Study of ACY-1215 in Combination With Lenalidomide, and Dexamethasone in Multiple Myeloma.

47. Study of ACY-1215 Alone and in Combination With Bortezomib and Dexamethasone in Multiple Myeloma.

48. Zhang, L., Zhang, Y., Mehta, A., Boufraqech, M., Davis, S., Wang, J., Tian, Z., Yu, Z., Boxer, M. B., Kiefer, J. A., Copland, J. A., Smallridge, R. C., Li, Z., Shen, M., and Kebebew, E. (2015) Dual inhibition of HDAC and EGFR signaling with CUDC-101 induces potent suppression of tumor growth and metastasis in anaplastic thyroid cancer. *Oncotarget* **6**, 9073-9085

49. Carson, R., Celtikci, B., Fenning, C., Javadi, A., Crawford, N., Perez-Carbonell, L., Lawler, M., Longley, D. B., Johnston, P. G., and Van Schaeybroeck, S. (2015) HDAC Inhibition Overcomes Acute Resistance to
MEK Inhibition in BRAF-Mutant Colorectal Cancer by Downregulation of c-FLIPL. Clinical Cancer Research 21, 3230-3240
50. Ahn, M. Y., Ahn, J. W., Kim, H. S., Lee, J., and Yoon, J. H. (2015) Apicidin inhibits cell growth by downregulating IGF-1R in salivary mucoepidermoid carcinoma cells. Oncol Rep 33, 1899-1907
51. Yu, C., Friday, B. B., Lai, J. P., McCollum, A., Atadja, P., Roberts, L. R., and Adjei, A. A. (2007) Abrogation of MAPK and Akt signaling by AEE788 synergistically potentiates histone deacetylase inhibitor-induced apoptosis through reactive oxygen species generation. Clinical Cancer Research 13, 1140-1148
52. Bahr, J. C., Robey, R. W., Luchenko, V., Basseville, A., Chakraborty, A. R., Kozlowski, H., Pauly, G. T., Patel, P., Schneider, J. P., Gottesman, M. M., and Bates, S. E. (2016) Blocking downstream signaling pathways in the context of HDAC inhibition promotes apoptosis preferentially in cells harboring mutant Ras. Oncotarget 7, 69804-69815
53. Eng, J. K., McCormack, A. L., and Yates, J. R. (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J Am Soc Mass Spectrom 5, 976-989
54. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551-3567

FOOTNOTES
*This work was supported in part by an NIH grant, R01CA164147, and the Karmanos Cancer Institute start-up funds to X.Z. J-Y W was supported in part by the Department of Pathology & Cell Biology, USF Morsani College of Medicine.

ABBREVIATIONS
HDAC6, histone deacetylase 6; ERK1/2, extracellular signal-regulated kinase 1/2; MAPKs, mitogen-activated protein kinases; JNK, c-Jun N-terminal kinases; NLK, Nemo-like kinase; PTMs, post-translational modifications; MEFs, mouse embryonic fibroblasts; HATs, histone acetyltransferases; Rpd3, reduced potassium dependency 3; MAP3K, MAPK kinase kinase; MAP2K, MAPK kinase; TSA, Trichostatin A; NAD+, oxidized nicotinamide adenine dinucleotide; EGFR, epidermal growth factor receptor; GNAT, Gcn5-related N-acetyltransferase.

FIGURE LEGENDS
FIGURE 1. ERK1/2 interact with HDAC6 physically. A, His-ERK1 binds to GST-HDAC6. The GST pull-down assays were performed with indicated proteins as described in the “Experimental Procedures.” The proteins pulled down by glutathione 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 described in A.

FIGURE 2. Characterization of the anti-acetylated-lysine antibodies. Increasing doses of non-acetylated BSA and acetylated BSA were resolved on SDS-PAGE. Two commercial anti-acetylated lysine antibodies as indicated were used for Western blotting analyses. Both short exposures and long exposures were shown.

FIGURE 3. Inhibition of HDACs by TSA increases ERK1 and ERK2 acetylation. A, TSA increases ERK1 acetylation in a dose-dependent manner. Left panels: HEK293T cells were transfected with GST-ERK1 followed by treatment with TSA for 12 hours with indicated concentrations. GST-ERK1 proteins were then isolated from HEK293T cells as described in “Experimental Procedures.” Western blotting analyses were performed with the indicated antibodies. Right panel: The acetylated GST-ERK1 and total GST-ERK1 bands were quantified by densitometry using software Image Lab™ from Bio-Rad. The value
of untreated acetylated GST-ERK1 band normalized against the total GST-ERK1 band was designated as 1, and the value of other normalized treated acetylated GST-ERK1 bands were indicated as a fold change relative to the untreated one. A bar graph was used to show relative intensity of untreated and treated acetylated GST-ERK1. Four independent experiments were performed. B, TSA increases ERK1 acetylation in a time-dependent manner. Left panels: HEK293T cells were transfected with GST-ERK1 followed by treatment with 600 ng/ml TSA at indicated time points. GST-ERK1 proteins were then isolated from HEK293T cells as described in “Experimental Procedures.” Western blotting analyses were performed with the indicated antibodies. Right panel: A bar graph was drawn as described in A. Five independent experiments were performed. C and D, TSA increases ERK2 acetylation in a dose- and time-dependent manner. For C and D, the experiments were performed the same as described in A and B, respectively. For C, four independent experiments were performed. For D, three independent experiments were performed. Student’s t tests were performed with *, p<0.05, **, p<0.01. Error bars, S.D.

FIGURE 4. Inhibition of HDAC6 by ACY-1215 increases ERK1 and ERK2 acetylation. A, ACY-1215 increases ERK1 acetylation in a dose-dependent manner. The experiments were performed the same as described in 3A, except that ACY-1215 was used for the treatment instead of TSA. B, ACY-1215 increases ERK2 acetylation in a dose-dependent manner. The experiments were performed the same as described in A, except that GST-ERK2 was used for transfection instead of GST-ERK1. For A, four independent experiments were performed. For B, six independent experiments were performed. Student’s t tests were performed with *, p<0.05, **, p<0.01, ***. Error bars, S.D.

FIGURE 5. Inhibition of HDACs by TSA and inhibition of HDAC6 by ACY-1215 increase endogenous ERK1 and ERK2 acetylation. A, TSA increases endogenous ERK1/2 acetylation. Left panels: 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. Right panel: A bar graph was used to show relative intensity of endogenous ERK1 and ERK2. Three independent experiments were performed. B, ACY-1215 increases endogenous ERK1/2 acetylation. The experiments were performed as described in A except that ACY-1215 was used for the treatment instead of TSA. Four independent experiments were performed. Student’s t tests were performed with *, p<0.05, **, p<0.01, ***. Error bars, S.D.

FIGURE 6. Depletion or knockdown of HDAC6 increases endogenous ERK1/2 acetylation. A, Knockout of HDAC6 increases ERK1/2 acetylation. Left panels: The anti-AcK antibody was used to immunoprecipitate acetylated ERK1/2 in HDAC6 wild-type or HDAC6 knockout MEFs. The immunoprecipitates were resolved on SDS-PAGE and the anti-ERK1/2 Western blotting analysis was performed. 0.5% of whole cell lysate was used as the input. Western blotting analyses were performed using anti-ERK1/2, anti-HDAC6, anti-acetyl-α-tubulin, or α-tubulin antibodies as indicated. Right panel: A bar graph was used to show relative intensity of endogenous ERK1 and ERK2. Five independent experiments were performed. B, Knockdown of HDAC6 increases ERK1/2 acetylation. The experiments were conducted the same as A except that A549 control and A549 HDAC6-KD pair was used to replace the wild-type and HDAC6KO MEFs pair. Four independent experiments were performed. Student’s t tests were performed with *, p<0.05, **, p<0.01. Error bars, S.D.

FIGURE 7. ERK1 is acetylated by CBP and p300 in vivo and in vitro. A, CBP and p300 acylate ERK1. GST-ERK1 was co-transfected with each of the 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 reblotted with anti-ERK1/2 antibodies. 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 ERK1 was subject to the anti-acetyl-lysine 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 ERK1. GST-ERK1 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. HEK293T cells were transfected with empty vector or HA-p300. The immunoprecipitation assays were carried out with anti-AcK antibodies as described in the “Experimental Procedures.” The immunoprecipitates were subject to the anti-AcK Western blotting analysis. 0.5% of the whole cell lysate was used as input, which was subject to Western blotting analyses with indicated antibodies.

E, Recombinant CBP acetylates ERK1 \textit{in vitro}. Bacterially expressed GST-ERK1 and the catalytic domain of CBP were subject to \textit{in vitro} acetylation assays as described in “Experimental Procedures.” An equal amount of GST-ERK1 was incubated with or without 2 μg of the recombinant CBP 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). For panels A-C, the acetylated GST-ERK1 bands were quantified by the software Image Lab™ from Bio-Rad and normalized by the total GST-ERK1 bands. The values of the empty vector-transfected Ac-GST-ERK1 bands normalized by total GST-ERK1 were designated as 1, and the density of other HATs-transfected Ac-GST-ERK1 bands was indicated as a fold change relative to the corresponding empty vector transfected bands. For the panel D upper panel, the ERK1/2 bands were quantified by densitometry, and the empty vector-transfected ERK1/2 bands were designated as 1. The density of the HA-p300-transfected ERK1/2 bands were quantified relative to the empty vector-transfected ones.

FIGURE 8. \textit{ERK2 is acetylated by CBP and p300 \textit{in vivo} and \textit{in vitro}.} A, CBP and p300 acetylate ERK2. GST-ERK2 was co-transfected with each of the 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 rebotted 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 co-transfected with an increasing amount of HA-CBP plasmids as indicated into HEK293T cells. GST-ERK2 was further pulled down by glutathione agarose, then the beads-bound ERK2 was subject to anti-acetyl-lysine 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. GST-ERK2 was co-transfected with an increasing amount of HA-p300 plasmids in HEK293T cells. The experiments were performed as described in B. D, Recombinant CBP acetylates ERK2 \textit{in vitro}. Bacterially expressed GST-ERK2 and catalytic domain of CBP were subject to \textit{in vitro} acetylation assays as described in the “Experimental Procedures.” Equal amount of GST-ERK2 was incubated with or without 2 mg of the recombinant CBP catalytic domain, and the \textit{in vitro} acetylation reactions were analyzed by the anti-AcK Western blotting analysis (upper panel). For the above Western blotting analysis, after transfer, the membrane was stained with Ponceau S to confirm the amount and purity of GST-ERK2 (lower panel). For panels A-C, the acetylated GST-ERK2 bands were quantified as described in Figure 7.

FIGURE 9. \textit{HDAC6 deacetylates ERK1 \textit{in vivo} and \textit{in vitro}.} A, HDAC6 decreases CBP-induced ERK1 acetylation \textit{in vivo}. GST-ERK1 was transfected into HEK293T cells alone, with CBP, or with CBP and HDAC6. GST-ERK1 proteins were purified as described in “Experimental Procedures” followed by the anti-AcK Western blotting analysis. The membrane was then stripped and then reprobed with anti-ERK1/2 antibodies. The input was subject to Western blotting analyses with indicated antibodies. B, HDAC6 deacetylates p300-induced ERK1 acetylation \textit{in vivo}. The experiments were performed as described in A, except that HA-p300 was used to replace HA-CBP. C, HDAC6 deacetylates ERK1 \textit{in vitro}. Left panels: Acetylated bacterially purified GST-ERK1 protein was incubated with or without F-HDAC6 purified from 293T cells as described in “Experimental Procedures” followed by the anti-AcK Western blotting analysis.
The procedure for generating acetylated GST-ERK1 was described in “Experimental Procedures.” The membrane was stripped and rebotted with the anti-ERK1/2 antibody. Right panel: The relative intensity of acetylated GST-ERK1 band was quantified by densitometry against the total ERK1 band showing in a bar graph. This experiment was repeated three times. Student’s t tests were performed with ***, p<0.001. Error bars, S.D.

FIGURE 10. Acetylated ERK1 exhibits decreased enzymatic activity. A, GST-ERK1 exhibits lower enzymatic activity in 293T-HDAC6KO cells than in 293T wild-type cells. Left panels: GST-ERK1 was transfected into 293T wild-type cells and 293T-HDAC6KO cells. Then the GST-ERK1 protein was purified by GST pull-down followed by kinase assays using recombinant ELK1 as a substrate as described in “Experimental Procedures.” Anti-p-ELK(S383) Western blot was performed. The membrane was then stripped and reprobed with the anti-ELK1 antibody. Anti-ERK1/2, anti-HDAC6, anti-ac-α-tubulin, and anti-α-tubulin Western blots were also performed. Right panel: The pELK1 bands were quantified by densitometry against total ELK1 bands. The results were shown in a bar graph. The same experiments were repeated five times. B, Acetylated ERK1 exhibits decreased enzymatic activity towards ELK1. Left panels: In vitro kinase assays were performed with either vehicle incubated GST-ERK1 or CBP incubated GST-ERK1. GST-ERK1 protein was purified from 293T cells. Recombinant ELK1 fusion protein purified from E. Coli containing ELK1 residue 307-428 was used as a substrate. The kinase assays were performed as described in “Experimental Procedures.” Anti-pELK1(S383) Western blot was used as a readout for ERK1’s activity (upper panel). The membrane was stripped and reprobed with the anti-ELK1 antibody. The anti-AcK Western blot was performed to examine the acetylation status of ERK1 (third panel). The membrane was stripped and reprobed with the anti-ERK1/2 antibody. Right panel: The relative intensity of ELK1(S383) phosphorylation was plotted as a bar graph. The experiments were repeated five times. Student’s t tests were performed with **, p<0.01, ***, p<0.001. Error bars, S.D.

FIGURE 11. The Lysine 72 site of ERK1 is acetylated. A, The Lys72 site of ERK1 is acetylated. The doubly charged peptide was detected with a 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. B, A diagram of ERK1’s domain structure. The numbers showed the amino acid residues of ERK1. The lysine 72, detected by mass spectrometry as an acetylated site, is indicated in a red circle. Thr202 and Tyr204 of the Thr-X-Tyr motif, whose phosphorylation status is critical for ERK1 activity, are shown in blue triangles. The kinase domain is highlighted as light gray. Other important motifs and regions are indicated as well. C, A stretch of ERK1 amino acids shows the conservation of Lys72 in different species from mammals to nematode. The mass spectrometry-detected acetylation site Lys72 is highlighted in red.

FIGURE 12. The acetylation-mimicking mutant of ERK1(K72Q) decreases the ERK1 kinase activity. A, The acetylation-mimicking mutant of ERK1(K72Q) displays decreased ERK1 phosphorylation in HEK293T cells. The GST-tagged dominant-negative, wild-type, K72Q, or K72R ERK1 was transfected into HEK293T cells, and GST-tagged proteins were purified as described in the “Experimental Procedures.” Glutathione-agarose bound proteins were analyzed by the anti-phospho-ERK1/2 Western Blotting analysis. The membrane was then stripped and reprobed with anti-ERK1/2 antibodies. 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. The GST-tagged proteins were purified as described in the “Experimental Procedures.” 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 anti-ERK1/2 Western blotting analysis was also performed. C, HeLa cells were transfected with indicated plasmids. Luciferase reporter assays were performed with the Dual-luciferase reporter kit (Promega) as described in the “Experimental Procedures.” The expression of those plasmids was examined by anti-ELK1, anti-HA, anti-ERK1/2, and anti-β-actin
Western blotting analyses as indicated. This experiment was repeated three times. Student’s \( t \) tests were performed with \(*\), \( p<0.05\), \(*\ast\ast\ast\), \( p<0.001\), ns, not significant. Error bars, S.D. \( D \), Potential structural effects of K72 mutation and acetylation. Ribbon diagram of ERK1 structure (PDB code: 2ZOQ) and a close-up view of K72 interaction (right panel). K72 and its interacting residues (D117 and Y119) are depicted by sticks with their carbon atoms colored in gray and cyan, respectively. Residues T202 and phosphorylated Y204 indicate the location of the activation loop. The ATP binding site is indicated by binding of the inhibitor 5ID (5-Iodotubercidin). Hydrogen bonds are illustrated as red broken lines.
Wu et al. Figure 1

A

B

Coomassie blue Staining

GST pull-down IB anti-ERK1/2

His-ERK1

His-ERK2

GST-HDAC6

Coomassie blue Staining

GST

GST-HDAC6

His-ERK1

His-ERK2

GST

Mr (Kd)

5% input

GST

GST-HDAC6

His-ERK1

His-ERK2

GST

Mr (Kd)

250 - 150 - 75 - 50 - 37 - 25 - 100 - 250 - 150 - 75 - 50 - 37 - 25 - 100 -

250 - 150 - 75 - 50 - 37 - 25 - 100 - 250 - 150 - 75 - 50 - 37 - 25 - 100 -
Wu et al. Figure 2

Acetylated-lysine mouse mAb (Ac-K-103) #9681

Acetylated lysine antibody #9441

IB: anti-AcK103

IB: anti-AcK (9441)

IB: anti-AcK103 Long exposure

IB: anti-AcK (9441) Long exposure

IB: anti-AcK103 Short exposure

IB: anti-AcK (9441) Short exposure

IB: anti-AcK103 Long exposure

IB: anti-AcK (9441) Long exposure

Non-acetylated BSA

acetylated BSA

Mr (kd) 0.2 1.0 5.0 25 (ng)

Short exposure

Long exposure

Short exposure

Long exposure

Acetylated-lysine mouse mAb (Ac-K-103) #9681

Acetylated lysine antibody #9441

Wu et al.
**Wu et al. Figure 3**

**A**

TSA (12 hours) (ng/ml)

- GST Pull-down
  - IB anti-AcK
  - IB anti-ERK1/2
  - IB anti-Ac-α-tubulin
  - IB anti-α-tubulin

- Input
  - IB anti-AcK
  - IB anti-ERK1/2
  - IB anti-Ac-α-tubulin
  - IB anti-α-tubulin

**B**

TSA (600 ng/ml) (Hour)

- GST Pull-down
  - IB anti-AcK
  - IB anti-ERK1/2
  - IB anti-Ac-α-tubulin
  - IB anti-α-tubulin

- Input
  - IB anti-AcK
  - IB anti-ERK1/2
  - IB anti-Ac-α-tubulin
  - IB anti-α-tubulin

**C**

TSA (12 hours) (ng/ml)

- GST Pull-down
  - IB anti-AcK
  - IB anti-ERK1/2
  - IB anti-Ac-α-tubulin
  - IB anti-α-tubulin

- Input
  - IB anti-AcK
  - IB anti-ERK1/2
  - IB anti-Ac-α-tubulin
  - IB anti-α-tubulin

**D**

TSA (600 ng/ml) (Hour)

- GST Pull-down
  - IB anti-AcK
  - IB anti-ERK1/2
  - IB anti-Ac-α-tubulin
  - IB anti-α-tubulin

- Input
  - IB anti-AcK
  - IB anti-ERK1/2
  - IB anti-Ac-α-tubulin
  - IB anti-α-tubulin
Wu et al. Figure 4

A

ACY-1215 (12 hours)

GST Pull-down

IB anti-AcK

IB anti-ERK1/2

IB anti-Ac-α-tubulin

IB anti-α-tubulin

0 0.5 1 2 4 6 (µg/ml)

AC-GST-ERK1

GST-ERK1

Ac-α-tubulin

α-tubulin

Input

Relative intensity of Ac-GST-ERK1

Concentration (µg/ml) (n=4)

B

ACY-1215 (12 hours)

GST Pull-down

IB anti-AcK

IB anti-ERK1/2

IB anti-Ac-α-tubulin

IB anti-α-tubulin

0 0.5 1 2 4 6 (µg/ml)

Ac-GST-ERK2

GST-ERK2

Ac-α-tubulin

α-tubulin

Input

Relative intensity of Ac-GST-ERK2

Concentrations (µg/ml) (n=6)
Wu et al. Figure 5

(A) 

293T
IP anti-AcK 0.5% input
TSA (600 ng/ml) -
- +
IB: anti-ERK1/2
IB anti-Ac-α-tubulin
IB anti-α-tubulin

1 2 3 4
Input

(B) 

293T
IP anti-AcK 0.5% input
ACY-1215 (6 μg/ml) -
- +
IB: anti-ERK1/2
IB anti-Ac-α-tubulin
IB anti-α-tubulin

1 2 3 4
Input

Vehicle TSA (600 ng/ml) 
(n=3)

Vehicle ACY-1215 ( 6 μg/ml) 
(n=4)
Wu et al. Figure 6

A

Input

IB: anti-ERK1/2
IB anti-HDAC6
IB anti-Ac-α-tubulin
IB anti-α-tubulin

MEFs
IP anti-AcK 0.5% input
WT KO WT KO

IB anti-ERK1/2
IB anti-HDAC6
IB anti-Ac-α-tubulin
IB anti-α-tubulin

1 2 3 4

B

Input

IB: anti-ERK1/2
IB anti-HDAC6
IB anti-Ac-α-tubulin
IB anti-α-tubulin

A549
IP anti-AcK 0.5% input
Ctrl KD Ctrl KD

IB anti-ERK1/2
IB anti-HDAC6
IB anti-Ac-α-tubulin
IB anti-α-tubulin

1 2 3 4

Relative intensity of ERK1/2

0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5

ERK1 ERK2

MEFs WT MEFs HDAC6 KO

Ctrl KD Ctrl KD

A549 Ctrl A549 HDAC6-KD

(n=5)

(n=4)
Wu et al. Figure 8

A

GST pull-down

IB anti-AcK
IB anti-ERK1/2
IB anti-HA
IB anti-Flag
IB anti-Myc
IB anti-Ac-p53-K382
IB anti-p53
IB anti-β-tubulin

Input

1 2 3 4 5 6

GST-ERK2

1.00 6.32 10.3 2.02 1.79 0.68

IB anti-HA
IB anti-AcK
IB anti-ERK1/2
IB anti-β-tubulin

B

GST pull-down

IB anti-AcK
IB anti-ERK1/2
IB anti-HA
IB anti-Flag
IB anti-Myc
IB anti-Ac-p53-K382
IB anti-p53
IB anti-β-tubulin

Input

1 2 3 4

GST-ERK2

1.00 1.01 1.68 3.21

IB anti-HA
IB anti-AcK
IB anti-ERK1/2
IB anti-β-tubulin

C

GST pull-down

IB anti-AcK
IB anti-ERK1/2
IB anti-HA
IB anti-Flag
IB anti-Myc
IB anti-Ac-p53-K382
IB anti-p53
IB anti-β-tubulin

Input

1 2 3 4

GST-ERK2

1.00 2.16 3.16 3.69

IB anti-HA
IB anti-AcK
IB anti-ERK1/2
IB anti-β-tubulin

D

Mr (Kd)

IB Anti-AcK

Ponceau S staining

CBP

Ac-GST-ERK2

Auto-Ac-CBP

GST-ERK2
Wu et al. Figure 9

A

|           | GST-ERK1 | HA-CBP | HA-HDAC6 |
|-----------|----------|--------|----------|
| GST pull-down | +        | +      | +        |
| IB anti-AcK | Ac-GST-ERK1 |
| IB anti-ERK1/2 | GST-ERK1 |
| IB anti-HA | HA-CBP |
| IB anti-HA | HA-HDAC6 |
| IB anti-α-tubulin | α-tubulin |
| Input | 1 | 2 | 3 |

B

|           | GST-ERK1 | HA-p300 | HA-HDAC6 |
|-----------|----------|---------|----------|
| GST pull-down | +        | +      | +        |
| IB anti-AcK | Ac-GST-ERK1 |
| IB anti-ERK1/2 | GST-ERK1 |
| IB anti-HA | HA-p300 |
| IB anti-HA | HA-HDAC6 |
| IB anti-α-tubulin | α-tubulin |
| Input | 1 | 2 | 3 |

C

|                | GST-ERK1 |
|----------------|----------|
| Ctrl | F-HDAC6 |
| IB anti-AcK | Acetyl-GST-ERK1 |
| IB anti-ERK1/2 | GST-ERK1 |

Relative intensity of Ac-GST-ERK1

|                | Control | F-HDAC6 |
|----------------|---------|---------|
| (n=3)          |         |***      |
**A**

- GST-ERK1
- WT HDAC6 KO

- IB anti-pELK1^{Ser383}
- IB anti-ELK1
- IB anti-ERK1/2
- IB anti-HDAC6
- IB anti-Ac-α-tubulin
- IB anti-α-tubulin

Whole cell lysate

- In vitro kinase assay

**B**

- GST-ERK1
- Rec CBP - +

- IB anti-pELK1^{Ser383}
- IB anti-ELK1
- IB anti-AcK
- IB anti-ERK1/2

Whole cell lysate

- Acetyl-GST-ERK1

In vitro acetylation

**Graphs:**

- Relative intensity of ELK1 Ser383 phosphorylation
- WT HDAC6 KO (n=5)

- Control CBP (n=5)
A

B

C

H. sapiens  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  88
P. troglodytes  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  88
M. mulatta  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  74
B. taurus  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  71
S. scrofa  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  89
R. norvegicus  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  89
M. musculus  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  88
C. lupus  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  89
M. putorius  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  80
O. hannah  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  134
D. rerio  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  102
X. laevis  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  74
D. melanogaster  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  84
C. elegans  YGMVSSAYDHVRKTRVAIKK ISPFEHQTYCQRTLRE  74
cons  **** ** : . ********** ********
Histone deacetylase 6 (HDAC6) deacetylates extracellular signal-regulated kinase 1 (ERK1) and thereby stimulates ERK1 activity

Jheng-Yu Wu, Shengyan Xiang, Mu Zhang, Bin Fang, He Huang, Oh Kwang Kwon, Yingming Zhao, Zhe Yang, Wenlong Bai, Gerold Bepler and Xiaohong Mary Zhang

J. Biol. Chem. published online December 19, 2017

Access the most updated version of this article at doi: 10.1074/jbc.M117.795955

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts