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DEFINING THE ROLE OF EGFR ACETYLATION IN CELLULAR PROCESSES: CLINICAL IMPLICATIONS

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DEFINING THE ROLE OF EGFR ACETYLATION IN CELLULAR PROCESSES: CLINICAL IMPLICATIONS

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DEDICATION

I would like to dedicate this work and dissertation to my family: my wife, Fang Liu, my son, Tate, my father, Youming Song, and my mother, Zhilan Xu, for their unrelenting love, support and encouragement throughout all my life.

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DEFINING THE ROLE OF EGFR ACETYLATION IN CELLULAR PROCESSES: CLINICAL IMPLICATIONS

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Epidermal growth factor receptor (EGFR) is a cell membrane tyrosine kinase receptor and plays a pivotal role in regulating cell growth, differentiation, cell cycle, and tumorigenesis. Deregulation of EGFR causes many diseases including cancers. Intensive investigation of EGFR alteration in human cancers has led to profound progress in developing drugs to target EGFR-mediated cancers. While exploring possible synergistic enhancement of therapeutic efficacy by combining EGFR tyrosine kinase inhibitors (TKI) with other anti-cancer agents, we observed that suberoylanilide hydroxamic acid (SAHA, a deacetylase inhibitor) enhanced TKI-induced cancer cell death, which further led us to question whether SAHA-mediated sensitization to TKI was associated with EGFR acetylation. What we know so far is that SAHA can inhibit class I and II histone deacetylases (HDACs), which could possibly preserve acetylation of underlying HDAC-targeted proteins including both histone and non-histone proteins. In addition, it has been reported that an HDAC inhibitor, TSA, enhanced EGFR phosphorylation in ovarian cancer cells. EGFR acetylation has also been reported to play a role in the regulation of EGFR endocytosis recently. These observations indicate that there might be an intrinsic correlation between acetylation and phosphorylation of EGFR. In other words, the interplay between EGFR acetylation and phosphorylation may contribute to HDAC inhibitors (HDACi)-augmented EGFR phosphorylation.

In this investigation, we showed that CBP acetyltransferase acetylated EGFR *in vivo*. In response to EGF stimulation, CBP rapidly translocated from the nucleus to the cytoplasm. We also demonstrated protein-protein interaction between CBP and EGFR as well as the enhancement of EGFR acetylation by CBP. Moreover, EGFR acetylation enhanced EGFR tyrosine phosphorylation and augmented its association with Src kinase. Acetylation-deficient EGFR mutant (EGFR-K3R) significantly reduced the function and activity of EGFR. Furthermore, ectopic expression of EGFR-K3R mutant abrogated its ability to respond to EGF-induced cell proliferation, DNA synthesis, and anchorage-independent growth using cell-based assays and tumor growth in nude mice. In addition, we demonstrated that EGFR expression was associated with SAHA resistance in the treatment of cancer cells that overexpress EGFR. The knockdown of EGFR in MDA-MB-468 breast cancer cells could sensitize the cells to respond to SAHA. The overexpression of EGFR in SAHA-sensitive MDA-MB-453 breast cancer cells rendered the cells resistant to SAHA. Together, these findings suggest that EGFR plays an important role in SAHA resistance in breast carcinoma cells that we tested. The combination therapy of HDACi with TKI has been proposed for treating cancers with aberrant expression of EGFR. The evidence from pre-clinical or clinical trials demonstrated significant enhancement of therapeutic efficacy by using such a combination therapy. Our *in vivo* study also demonstrated that the combination of SAHA and TKI for the treatment of breast cancer significantly reduced tumor burden compared with either SAHA or TKI alone. The significance of our study elucidated another possible underlying molecular mechanism by which HDACi mediated sensitization to TKI. Our results unveiled a critical role of EGFR acetylation that regulates EGFR tyrosine phosphorylation and may further provide an experiment-based rationale for combinatorial targeted therapy.

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ABBREVIATIONS

CBP	**CREB-binding protein**
EGFR	**Epidermal growth factor receptor**
$EGFR^{Ac}$	**Acetylated EGFR**
wt-EGFR	**Wild type EGFR**
EGFR-K3R	**Acetylation-deficient EGFR mutant (K684R, K836R, and K843R)**
Erk	**Extracellular signal-regulated kinase**
HAT	**Histone acetyltransferase**
HDAC	**Histone deacetylase**
HDACi	**Histone deacetylase inhibitors**
MAPK	**Mitogen-activated protein kinase**
McAb	**Monoclonal antibody**
NSCLC	**Non small cell lung cancer**
OD	**Optical density**
p300	**Cellular 300-kD protein**
PCAF	**p300/CBP-associated factor**
PI	**Propidium iodide**
PTM	**Posttranslational modifications**
SAHA	**Suberoylanilide hydroxamic acid**
siRNA	**Small interfering RNA**
TKI	**Tyrosine kinase inhibitors**
TSA	**Trichostatin A**

CHAPTER 1 INTRODUCTION

1. 1 The biology of EGFR

Epidermal growth factor receptor is a membrane tyrosine kinase receptor that belongs to the EGF tyrosine kinase receptor family consisting of EGFR (ErbB1), Her2 (ErbB2), ErbB3, and ErbB4 (1, 2). The molecular weight of EGFR is about 165 kDa. EGFR monomer is composed of four domains (3), namely extracellular, transmembrane, intracellular (including kinase domain), and cytoplasmic tail (4, 5) (Figure1). Human EGFR gene is located on chromosome 7 within p13 and q22 region (6, 7). According to the literature, EGF or other grow factors can cause that EGFR changes its extracellular domain structure and then trigger dimerization of intracellular domain (8-15). In response to growth factors, EGFR forms either homodimer or heterodimer with other family members, therefore subsequently changes its protein structure and protein interaction with numerous intracellular proteins (16-19) (Figure2). Meanwhile, EGFR is subject to autophosphorylation or transphosphorylation that plays important roles in regulating several signaling pathways, namely MAPK, Akt, and Stat pathways (20-22). There are several critical tyrosine phosphorylation sites in the intracellular domain of EGFR, namely Y845, 992, 1045, 1068, 1086, and 1173. These differential phosphorylation sites correspond with different signal pathways and functions (21). Generally speaking, Y845 is associated with Stat signaling pathway. Y992, 1068, 1086, and 1173 are generally linked to MAPK and Akt signaling pathways (21), while Y1045 phosphorylation is associated with the interaction of Cbl that regulates EGFR ubiquitination and degradation (23). However, it is still unclear what determinates these phosphorylation sites and how an individual site is regulated. Environmental stimuli like ligands, IR, and UV have been reported to induce receptor tyrosine phosphorylation and/or ubiquitination (22, 24, 25). According to the literature,

Figure 1 Domain structure of EGFR

EGFR consists of extracellular domain (ECD), transmembrane region (TM), intracellular domain including kinase domain and cytoplasmic tail (CT). N: amino terminus, C: carboxyl terminus.

EGF induces EGFR phosphorylation including Src-mediated transphosphorylation and autophosphorylation. Phosphorylation of EGFR at Y1045 provides a docking site for Cbl, an E3 ubiquitin ligase, for binding to EGFR and inducing EGFR ubiquitination. This modification controls EGFR recycling, translocation, and degradation. Three types of EGFR ubiquitination including mono-, multi-, and poly-ubiquitination are also involved in this process, which play different roles in EGFR function (26-28). Although IR and UV are also able to induce EGFR phosphorylation and/or ubiquitination (22, 25, 29), however, these stimuli may not affect EGFR modification patterns in the same way. Among four family members, EGFR is one of the most critical and well-studied cell surface tyrosine kinase receptor that plays a pivotal role in controlling cell growth, differentiation, motility, and cell cycle (30). As a key member, EGFR is essential for cell growth or tumorigenesis (31-35).

The regulation of EGFR is through several mechanisms that include protein endocytosis (36-39), ubiquitin-proteasome dependent degradation (23, 40, 41), lysosomal degradation, and protein cytoplasmic and nuclear shuttling (42, 43). Provided that EGFR signaling is too strong, to maintain cellular balance, there are several processes that can be utilized by the affected cells. First of all, in order to alleviate cellular stimulation and maintain cellular balance, protein endocytosis generally down-regulates EGFR signaling by reducing surface EGFR number in response to cellular negative feedback signals (44, 45). Second, EGFR can be phosphorylated at Y1045 site that recruits Cbl E3 ligase and subsequently ubiquitinates EGFR (46). The ubiquitinated EGFR is then degradated by proteasome (47). Because of degradation function of Cbl-catalyzed protein ubiquitination, Cbl has been considered as a negative regulator in EGFR-regulated signal pathways (48-50). Ubiquitination of protein has been considered as a marker for protein degradation.

However, recent study demonstrated that not all EGFR ubiquitination was subject to degradation. In general, poly-ubiquitination leads to protein degradation, mono- or multi-ubiquitination is conferred to play a regulatory role with respect to EGFR function (26-28). Besides protein ubiquitination, there are several EGFR modifications similar to ubiquitination such as neddylation and sumoylation. Neddylation of EGFR is catalyzed by Cbl as well (51). The function of EGFR neddylation is very similar to ubiquitination. In summary, these Cbl-mediated EGFR modifications play an important role in negatively regulating EGFR signals in cell behavior and growth.

Another important negative regulation of EGFR phosphorylation is protein dephosphorylation by phosphatases. There are several identified phosphatases such as PTP1B (52-55), SHP-1 (56-58), and SHP-2 (59-62), which play very important roles in balancing EGFR phosphorylation and activation. These phosphatases act as negative regulators by removing phosphoryl group from relative tyrosine residues and mitigate EGFR activation and signal transduction. DEP-1 was recently reported as EGFR-regulating phosphatase by an unbiased screen approach (63). There are several additional phosphatases that have been reported to interact with EGFR (64, 65). Deregulation of phosphatases could cause various diseases including cancers (65).

EGFR shuttling is also regarded as an EGFR regulating pathway. As far as EGFR protein shuttling or trafficking and localization, it may be involved in many different functions depending on its differential modifications and interacting proteins as well as cellular locations (23, 66, 67). EGFR has been reported to translocate into the mitochondria that regulates cell growth and proliferation (68, 69). An increasing number of evidence has demonstrated that EGFR trafficking and cellular locations are associated with abnormal cell growth as well as resistance to conventional therapeutic regimens for EGFR-associated diseases, most frequently in cancers such as lung cancer

and glioblastoma multiforme (70, 71). For example, EGFR nuclear localization has been shown to associate with cancer resistance to antitumor agents like chemo-therapy and radiation therapy (70, 72-74). In addition, our laboratory has demonstrated that EGFR can shuttle from cell surface into the nucleus, which could participate in direct gene regulation (75).

A considerable body of evidence has demonstrated that the aberrant expression of EGFR results in many health problems and is a culprit in many life-threatening diseases such as cancers of the brain, head and neck, lung, colon, and skin (5, 76-83). A number of reports demonstrated that EGFR overexpression was highly correlated with non-small cell lung cancers (NSCLC) and pancreatic cancer in clinical studies (82, 84-86). Accordingly, 93% tissue samples from NSCLC patients and 69% pancreatic cancer tissue samples expressed high levels of EGFR (84, 86). Moreover, EGFR has been considered as a negative prognostic indicator in cancer treatment (87, 88). Clinical studies demonstrated that EGFR overexpression was significantly correlated with high tumor grade (89-91). A plausible of evidence showed that EGFR overexpression was correlated with shorter overall survival in the early stages of NSCLC and pancreatic cancer (89, 92, 93). Not only cancers, EGFR is also closely correlated with various biological abnormalities. Genetic study demonstrated that knockout of EGFR resulted in embryonic lethality (83, 94-96). EGFR is also essential for skin development and associated with epithelial tumor formation (79). In addition, EGFR is associated with controlling circadian clock (97, 98), eye development (99), renal development and disease (100), and liver regeneration (101). The defect of EGFR expression could cause neurodegeneration (102-105). Most recently, EGFR was reported to be inversely correlated with G-CSF-induced mobilization of hematopoietic stem and progenitor cells (HSPCs) from bone marrow into peripheral blood (106). Tyrosine kinase inhibitor,

erlotinib, could significantly augment G-CSF-induced mobilization of HSPCs. Traditionally, hematopoietic cells are considered as EGFR null and used for model cells to study EGFR function. The significance of this finding discloses insights into EGFR-regulated hematopoietic stem cells and indicates that the function of EGFR may be far beyond what we have known currently.

As far as clinical relevance of EGFR in cancer treatment, mutations of EGFR link with cancer sensitivity and resistance to conventional therapeutical regimens and tyrosine kinase inhibitors (1, 4, 21, 107-113). Since EGFR is such an important membrane molecule and involved in critical cellular regulations as well as many disease, a great deal of effort has been put into this area to understand underlying mechanisms that are associated with physiological and/or pathological cellular regulation. Therapeutic reagents that target EGFR are under development and promising for treating various diseases (114). For example, gefitinib exhibited significant efficacy in the treatment of lung cancer with several specific somatic mutations on EGFR (1, 4, 107). Humanized monoclonal antibodies have been developed to target EGFR-expressing cancers such as lung cancer, colon cancer, and breast cancer (115, 116). The further study that deciphers EGFR regulation, mutation, protein modification, trafficking, and localization will ultimately benefit for the treatment of EGFR-associated diseases. Moreover, based on our knowledge about this molecule, we can even develop personalized medicine and therapeutic regimens that effectively treat various diseases based on genetic background of patients (117-121).

1. 2 The biology of protein posttranslational modifications (PTM)

There are more than 200 different types of protein PTM (122). Generally accepted concept is that protein PTM provide surface recognition sites for protein interaction.

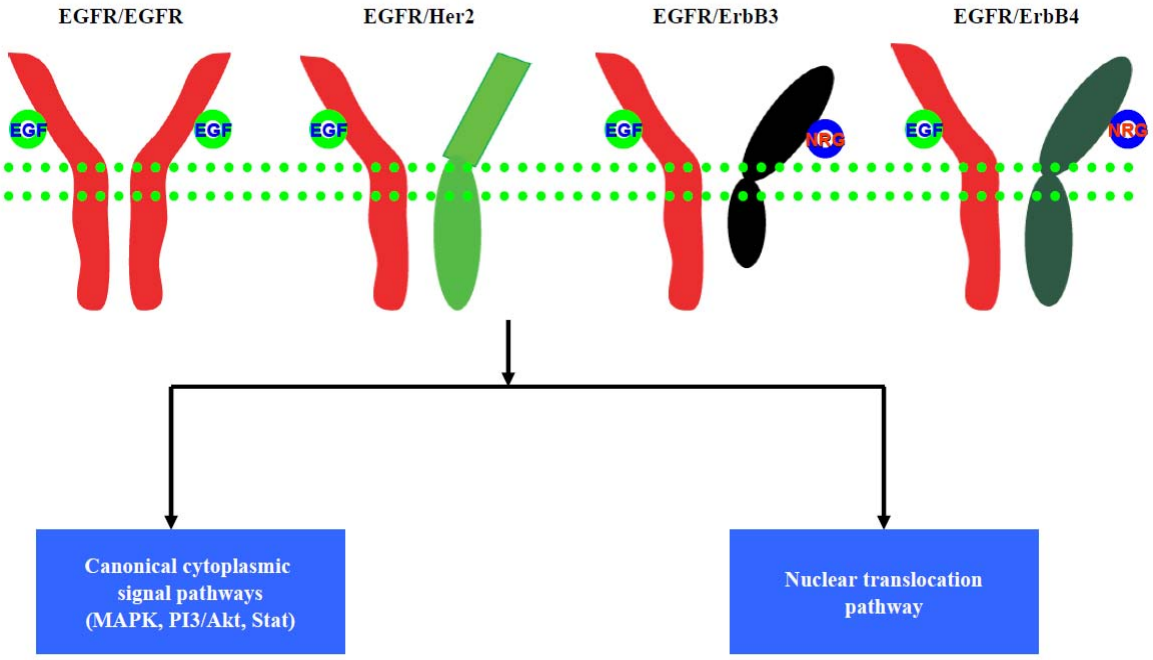


Figure 2 EGFR forms homodimer or heterodimer with Her2, ErbB3, and ErbB4

Two distinct signal pathways may be involved in the following functions upon the dimerization of EGFR with its partner, namely, canonical cytoplasmic pathways and nuclear translocation pathway.

Therefore, the protein code concept has been proposed to elucidate the function of protein PTM (123). Recently, a growing number of published papers demonstrated that a wide range of proteins had gone through extensive PTM and linked with different functions (124-129). EGFR is one of those identified proteins that is subject to PTM in order to perform its biological functions, such as glycosylation, phosphorylation, ubiquitination, neddylation, and sumoylation (129-133). Depending on the type and pattern of EGFR modifications, modified EGFR plays different roles in regulating proliferation, differentiation, cell cycle, and migration. In response to environmental challenges and stimulation, EGFR as a surface molecule senses and responds to such signals as well as triggers signal cascades all the way down to the nucleus in which dictates biological processes in the affected cells. In addition, modified EGFR itself can also translocate into the nucleus to perform regulatory function in the cells. Since phosphorylation and ubiquitination of EGFR are the most important PTM and have been intensively studied, in this dissertation, I will mainly review recent progress of phosphorylation and ubiquitination of EGFR in the combination of newly identified EGFR PTM.

With limited genome for encoding genes and translating proteins (134-136), an organism can still generate numerous functional molecules to respond to environmental challenges and/or hazards through a series of biological processes (122, 137-141). For example, alternative splicing, genetic, epigenetic, and protein modifications (126, 127, 142). Protein PTM such as phosphorylation, ubiquitination, sumoylation, methylation, and acetylation largely diversify protein function. Depending on modification type, pattern and subcellular location, modified proteins may act as potent activators or suppressors against their unmodified counterparts. Protein PTM are involved in a variety of protein families including histone and non-histone proteins (124, 126, 127,

143, 144). PTM expand array of biological molecules that make up for insufficient genetic expression and make an organism possible to respond to complicated biological processes with sufficient functional molecules. Protein PTM play profound biological roles in regulating cell growth and proliferation and maintaining homeostasis in an organism.

Up to now, protein phosphorylation is a well-studied area and mature field. Phosphorylation of protein includes tyrosine, serine, and threonine residues. Protein phosphorylation is involved in almost every aspect of biological processes (65, 145-151), For example, cell surface membrane protein activation, signal transduction, cell cycle, protein stability and shuttling, DNA repair, transcription, translation, and cellular regulation. To some extent, protein phosphorylation dictates entire cellular processes. Deregulation of protein phosphorylation is associated with many diseases including cancers and genetic diseases. It is also well understood that EGFR phosphorylation plays a key role in regulating its function and controls cell cycle and growth (149). Detailed mapping of phosphorylation sites in EGFR has been published as well (21, 152). Accordingly, tyrosine phosphorylation of EGFR is critical in controlling its function. In addition, EGFR-regulated downstream signal pathways are also controlled by phosphorylation cascades.

Protein ubiquitination became a hot spot recently. For a long period of time, scientists believed that proteins were not subject to degradation. However, since groundbreaking work on protein ubiquitination from Drs. Ciechanover and Hershko was accepted (153, 154), protein degradation has been widely accepted and extensively studied (155). Scientist and researchers gradually gain more and more insights about protein stability and degradation in biological processes. While a number of reports showed that protein ubiquitination was a marker for protein degradation, increasing

evidence demonstrated that not all protein ubiquitination is subject to degradation. Depending on the type of protein ubiquitination such as mono-, multi-, or poly ubiquitination, the modified proteins may have different fates but not all go through degradation (156). Currently, it has been proved that EGFR is also subject to these differential ubiquitination modifications (27). These suggested that protein PTM are indeed complicated biological processes.

With respect to protein acetylation and methylation, however, our previous knowledge only limits in a range of histone protein. Although, histone acetylation was reported in the early 1960s, little is known about its function and regulation in cellular process until the middle1990s (144, 157, 158). Currently, Discovery of acetylation of non-histone proteins has been accelerated and spans through almost all cellular compartments from membrane, cytoplasm, mitochondria, to the nucleus. These proteins include transcription factors E2F1, Stat3, β-catinin, NF-κB, MyoD, p53, cytoplasmic proteins Ku70, tubulin, IKK, MEK2, membrane proteins, and mitochondrial components (124, 126, 144, 159-163). Currently, the most intensively studied protein acetylation is N-acetylation including α-acetylation and ε-acetylation (144). Although, protein O-acetylation has recently been reported to play a role in regulating MEK2 and IKK function (144, 163, 164), in our study, since the most important signal-regulating domains of EGFR reside in the cytoplasmic domain, therefore we are focusing on lysine N^{ε}-acetylation that is anticipated to play a major role in EGFR function. We expect that N^{α}-acetylation of EGFR, if exists, may not be critical for EGFR function.

Protein lysine N^{ε}-acetylation is a reversible enzyme-mediated process that is involved in two groups of enzymes, namely histone acetylases (HATs) and histone deacetylases (HDACs). HATs consist of MYST, CBP/p300, and GCN5-related N-

acetylase families (165). MYST family includes Esa1, MOF, MOZ, Sas2, Tip60, and Ybf2. GCN5-related acetylase family includes Elp3, GCN5, and PCAF. CBP/p300 family is so called cAMP response element binding protein family and includes CBP and p300. So far as deacetylases, there are four classes of enzymes that are comprised of at least 18 histone deacetylases (HDACs) in human (166, 167). The class I is comprised of HDAC1, 2, 3, and 8. The class II includes class IIa consisting of HDAC4, 5, 7, and 9 (a and b isoforms) and class II b consisting of HDAC6 and HDAC10. HDAC11 belongs to the class IV. The class III includes SIRT1, 2, 3, 4, 5, 6, and 7. In the nucleus, neither HATs nor HDACs directly bind to DNA. The function of two groups of enzymes usually forms complexes with other nucleus proteins and regulates gene expression in an opposite fashion (166, 168). Recently, more and more non-nuclear proteins have been discovered as substrates of these enzymes. Moreover, more evidence demonstrated that the function of these enzymes is beyond the modulators of gene expression. The aberrant expression of either HATs or HDACs is associated with many human cancers (165). Therefore, the understanding of these groups of protein enzymes may help us to effectively treat a various types of human disease including cancers.

Protein acetylation plays an important role in the regulation of cellular function. Anti-HDAC therapy is emerging as an important field for anticancer therapy (166, 169-173). A growing number of anti-deacetylation therapies are undergoing clinical trials for the treatment of cancers. Despite these progresses, the potentials and functional mechanism(s) of protein acetylation remain largely unknown. The most frequently reported mechanism by which HDAC inhibitors (HDACi) acted through was increasing "expression of CDKN1A gene, which encodes the cyclin-dependent kinase inhibitor WAF1/p21" (169). Since the acetylation and deacetylation in histone play a critical role

in regulating gene expression, the agents that act through interaction with histone deacetylases are even more attractive in therapeutic field, especially in cancer therapy. As most studies showed that the regulation of histone acetylation and deacetylation was generally defective in various cancers (169, 174), therefore, correction of such deregulation of histone modification by corresponding agents may potentially be promising in cancer therapy. HDACi are under rapid development for treating various diseases, especially for cancer therapy (167, 172). Currently, more than 14 HDACi are under different phase of clinical trials. Among these HDACi, SAHA is most extensively investigated and the first HDACi that has been approved for the treatment of cutaneous T cell lymphoma by Food and Drug Administration in the US (171, 172, 175, 176). According to the literature, the mechanisms by which HDACi act through are involved in many different pathways including alteration of gene expression and cell cycle, activation of death pathway, interruption of HDAC function and others. The combination of HDACi with other antitumor therapy such as chemotherapy and radiation therapy was proposed and extensively investigated. Accordingly, HDACi demonstrated additive or synergistic effect in these combination therapies (172). Despite these enhanced therapeutic efficacy, some studies have also reported that the resistance to HDACi was developed during the treatment. Further studies identified that the mechanisms were involved in Bcl-2 antiapoptotic protein and multiple drug resistance protein (P-gp) (167). The understanding of the underlying molecular mechanisms by which tumor cells resist or respond to HDACi is obviously very important for developing therapeutic strategies to effectively target cancer cells.

An increasing number of reports demonstrated that EGFR was subject to various PTM that were associated with diversified EGFR function (27, 51, 177). Recent studies showed that EGFR was involved in mutations and extensive PTM over the course of

tumor development, for example, phosphorylation and ubiquitination. The gain-of-function of EGFR mutations renders high phosphorylation and activation to EGFR in NSCLC. This type of EGFR constitutive phosphorylation renders a subset of NSCLC patients bearing such mutations sensitive and/or responsive to a receptor tyrosine kinase inhibitor gefitinib (1, 5, 107) and ionizing radiation (22, 24). Other independent studies demonstrated that EGFR was ubiquitinated in three differentiate ways, namely mono-, multi-, and poly-ubiquitination. Ubiquitination of EGFR controlled its function, localization, and protein stability in cellular processes (22, 27). Clearly, on the one hand, phosphorylation and ubiquitination of EGFR confer EGFR to perform distinct function in response to different environmental stimuli. One the other hand, these modifications control protein localization, half-life, and turnover. During the time I was writing this dissertation, acetylation of EGFR was reported, in which demonstrated that acetylation of EGFR at lysine 1155, 1158, and 1164 was critical for recruiting AP-2 protein and regulating EGFR endocytosis (178). Together, the different EGFR modifications are reconciling to maintain cellular balance and homeostasis and participating in the regulation of cell behavior and physiological function. The loss of balance in EGFR modifications will cause pathological consequences. Although these advanced studies led scientists to develop therapeutic drugs such as humanized antibodies (Cetuximab or C225) and small molecular inhibitors (erlotinib, gefitinib, and lapatinib) to target various cancers with the altered EGFR, the underlying molecular mechanism(s) and signaling network modulation of EGFR modifications remain elusive in cellular and malignant development. There is still urgent in need of effective drugs and strategies to treat various cancers related to EGFR alteration. Recently, using HDACi to treat EGFR-expressing cancer was proposed. However, inconsistent results had also been reported from different research group. There is a report which

demonstrated that using HDACi to treat EGFR-expressing cancer cells increased EGFR phosphorylation (179). Considering HDACi worked as inhibitors of deacetylases that could be located in the cytoplasm such as HDAC6 (169), we postulated that not only histone proteins but some cytoplasmic proteins might be subject to the modifications of acetylation and deacetylation, which were involved in such enhanced EGFR phosphorylation. Therefore, EGFR might be a target for protein acetylation in this regard and subsequently participated in signaling networks and cellular processes. Based on this anticipation, we hypothesized that additional protein modifications likely participate in the regulation of EGFR function besides phosphorylation and ubiquitination. These undisclosed EGFR modifications may serve as a part of signaling network and cascades dominated by protein phosphorylation. The function of the modifications may enhance or reduce EGFR signaling and function in such a system. In addition, we can not rule out the possibility of independent signaling pathway regulated by protein acetylation and deacetylation. In a word, the complexity of EGFR-regulated cellular function and its metabolism is probably much more pronounced than previously thought.

1. 3 EGFR signaling pathways in tumorigenesis

EGFR is one of the most well studied receptor tyrosine kinases (RTK). Since this cell surface molecule plays an essential and fundamental role in dictating cell proliferation and differentiation, cell cycle control, biological development, tumorigenesis, and malignant development (180-182), it has been extensively investigated in all aspects of biomedical researches. EGFR regulates many signaling pathways including JAK-Stat3/5, PI3K-Akt, and MAPK kinase pathways (1, 5, 21, 22, 183, 184). As a traditionally accepted concept, upon ligand stimulation, EGFR forms

homodimer or heterodimer with one of other three family members in physiological condition (Figure 1), which subsequently results in phosphorylation of EGFR by either Src kinase or autophosphorylation. However, recent study demonstrated that high local EGFR concentration could activate EGFR and form dimers, which resulted in EGFR phosphorylation (16). The phosphorylated EGFR provides docking sites for binding downstream adaptor proteins and thereafter activates several downstream signaling pathways. Upon Src-induced phosphorylation of Y845 on EGFR, the phosphorylated Y845 serves as docking site to recruit Stat3/5 and subsequently phosphorylates Stat3 and/or Stat5, which form homo- or hetero-dimers. The dimerized Stat3 or Stat5 translocates into the nucleus and regulates cell proliferation, differentiation, cell cycle, and migration (Figure3). Src-activated signal pathway through Y845 is so called transphosphorylation activation pathway. Another EGFR activation pathway is autophosphorylation signal pathway. EGF ligand stimulation also causes autophosphorylation of EGFR. Several tyrosine residues in intracellular domain of EGFR such as Y992, 1068, 1086, and 1173, provide docking sites for adaptor proteins such as Shc, Grb2, and Gab and result in the activation of PI3K/Akt and Ras/MAPK signaling pathways (21). The activation of PI3K/Akt and /or Ras/MAPK pathways has been linked to various cancers (180, 185, 186). Depending on specific cell types or environment, one of these pathways may dominate or all of these pathways equally contribute to cellular processes. As far as the interrelationship between transphosphorylation and autophosphorylation of EGFR, different observations have been reported. One of these studies demonstrated that Src-regulated EGFR transphosphorylation acted independently of its autophosphorylation by using *in vitro* cell-based assays (187). Specifically, this study showed that although EGFR-Y845F

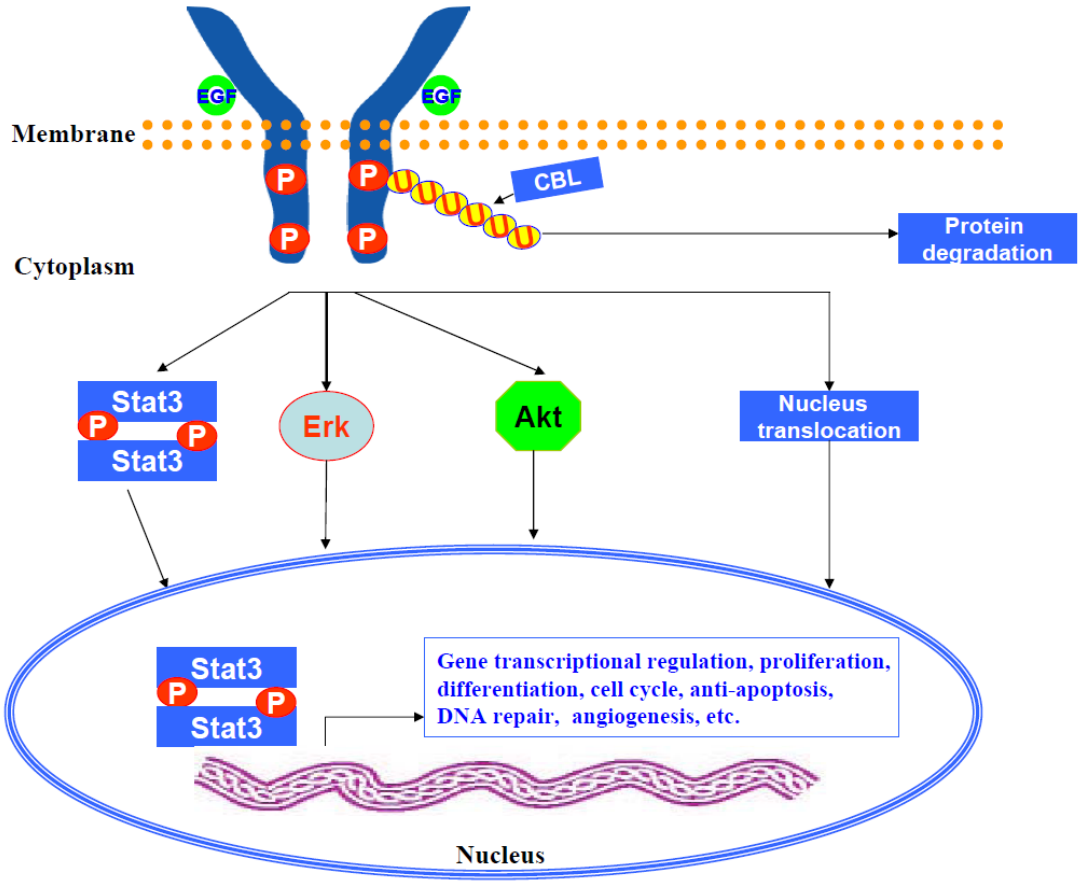


Figure 3 EGFR-regulated signaling pathways

Upon binding to EGF, EGFR is phosphorylated and activates three major downstream signal pathways, namely STAT, Akt, and MAPK pathways, which promote cell growth, cycle, and malignancy. Meanwhile, the phosphorylated EGFR is subject to ubiquitin-proteasome dependent degradation that attenuates EGFR signaling.

mutant resulted in the loss-of-function of EGFR-regulated cell growth and tumorigenesis, there was no alteration of EGFR kinase activity and MAPK activation, suggesting the independence of two types of EGFR phosphorylation activations. On the contrary, another study demonstrated that EGFR-Y845F mutant resulted in a decrease in EGFR phosphorylation at Y1068 which is an important tyrosine site of EGFR that regulates MAPK signal pathway (188). Using Src inhibitor, PP2, phosphorylation of Y1068 on EGFR was also reduced, suggesting that two types of EGFR activations are intrinsically correlated and interacted. However, there is no strong evidence that clearly demonstrates whether both phosphorylation activations affect and interact with each other. The interaction between transphosphorylation and autophosphorylation of EGFR is still ambiguous and controversial. A clear understanding of both phosphorylations on EGFR is essential for deciphering its function and targeting EGFR-expressing cancers such as lung cancer, breast cancer, and colorectal cancer.

Meanwhile, EGFR signaling pathways are also negatively regulated by phosphatases such as PTP1B and SHP1 and proteasome-dependent degradation (40, 189). Among these negative regulators, the phosphatases can effectively remove phosphoryl moiety from EGFR and downregulate EGFR activity. According to the literature, PTP1B regulates EGFR autophosphorylation mediated by phosphorylated Y992 (190, 191). SHP-1 attenuates EGFR activity mediated by phosphorylated Y1173 (56). Another negative regulation is protein ubiquitination that is Y1045 phosphorylation-dependent modification. Phosphorylation of EGFR at Y1045 triggers Cbl-mediated ubiquitination and induces EGFR ubiquitination and proteasome-dependent protein degradation (49, 177, 192). The regulation of EGFR phosphorylation and activation by both positive and negative processes together maintains the physiological function of EGFR and controls cell behavior.

Besides cytoplasmic signal transductional function, recent studies showed that full-length EGFR could directly translocate into the nucleus and form complexes with transcription factors such as Stat3, which regulated transcription and cellular function (75, 183). In addition, increasing evidence shows that EGFR nuclear translocation is involved in resistance to chemotherapy and radiation therapy (24, 72, 193-195). In summary, the function of both cytoplasmic and nuclear EGFR demonstrates a critical role in regulating cellular processes. Deregulation of EGFR signaling pathways causes severe healthy problems including cancers. The aberrant expression and activation of EGFR are frequently detected in cancers of the lung, breast, ovary, skin, brain, and head and neck (5, 196). In addition, Deregulation of EGFR function is also associated with diseases of the skin, brain, heart, eye and vision, and renal (79, 104). EGFR-mediated signaling pathways are also involved in many biological processes such as embryonic development, DNA repair, chromatin remodeling, anti-apoptosis, malignant development and metastasis (22, 83, 180-182).

In summary, there are two distinct portions of EGFR signaling pathways, namely cytoplasmic and nuclear pathways (Figure 2). In cytoplasmic signaling pathway or so called canonical pathway, EGFR is activated from cell membrane and sequentially phosphorylates downstream molecules which pass final growth signals into the nucleus and regulate gene expression. In nuclear pathway, however, EGFR could be directly shuttled into the nucleus with whole molecule and participated in the regulation of gene expression. The two pathways seem to have differential regulatory roles in this regard. The canonical cytoplasmic pathway most frequently regulates cell growth and cell cycle (183), while the nuclear signal pathway is often associated with resistance to therapeutic interventions for cancer therapy (70).

1. 4 EGFR is a common target for treating various cancers

Membrane receptor tyrosine kinase (RTK) family proteins are first layer of biological sensors which sense environmental changes in which a cell resides. Generally speaking, the current characterization of RTK function is predominantly focusing on phosphorylation and ubiquitination that correspond to the cellular events. Therefore, for cancer treatment, the agents that target protein phosphorylation and ubiquitination have become the mainstream medicine in therapeutic regimens. Since EGFR plays an essential and fundamental role in regulating cell proliferation, differentiation, cell cycle, and migration, it is one of the most attractive targets for developing anti-cancer drugs (40). Several common accepted approaches and strategies have been proposed in therapy and drug development. First, antagonizing EGFR binding ligands such as EGF and HB-EGF (197). However, this approach virtually requires to develop an individual ligand specific inhibitor that blocks each targeted ligand for binding to EGFR. In addition, off target events could result in severe side effects. These drawbacks limit the feasibility of this application in practice. Second, targeting ligand binding domain located at extracellular domain of EGFR (115, 198, 199). The successful case is that using EGFR humanized monoclonal antibody (McAb) competes to its ligand binding domain and blocks ligand to access to EGFR. The advantage of this approach is the need for only one specific antibody to block a group of ligands that share the same binding sites on EGFR, which makes this approach more attractive and cost effective. The third approach is to target the downstream signal molecules in EGFR-regulated pathways. In this approach, the core modulators in the pathway need to be identified before effective blockade of the EGFR pathways. Unfortunately, in reality the core modulators are difficult to identify. In addition to this, protein network and sequential activations are always involved in EGFR signaling

pathways. Therefore, combination therapy will be needed. Lastly, targeting intracellular domain of EGFR, usually tyrosine kinase domain. This approach generally uses small molecule inhibitors that can penetrate through cell membrane into the cytoplasm then perform their anti-EGFR function. Currently, these newly developed inhibitors generally bind to ATP binding sites and consequently inhibit EGFR activity. Since first small molecule inhibitor imatinib was developed to inhibit Abl kinase and was approved to treat chronic myelogenous leukemia (200, 201), this area is under rapid development and an increasing number of EGFR small molecule inhibitors have been developed and approved for treating various human cancers. Several clinical used drugs including humanized monoclonal antibody (Cetuximab or C225) and small molecular inhibitors (gefitinib, erlotinib, and lapatinib) exhibit their anti-EGFR function by blocking either its dimerization or ATP binding to kinase domain. Currently, humanized anti-EGFR monoclonal antibodies have been rapidly developed to treat EGFR-associated diseases including cancers of the lung, breast, brain, and colon. These antibodies demonstrated high effectiveness and efficacy to inhibit EGFR oncogenic function in the treatment of certain types of EGFR-expressing cancers. The working mechanisms act through either blocking ligands for binding to EGFR in extracellular portion and/or EGFR dimerization in intracellular domain. No matter what mechanisms these antibodies act through, these agents are potent to prevent EGFR activation.

Since first successful treatment of NSCLC by using small molecule receptor tyrosine kinase inhibitor gefitinib was reported (1), many anti-EGFR small molecule inhibitors have been developed recently. However, through unknown mechanism(s), EGFR-expressing cancer cells can still manage to escape the blockade by these agents and become resistant to these reagents. The possible reason, at least in part, is that EGFR has gone through genetic, epigenetic, and/or protein posttranslational changes in

response to stimuli, and then adapts to new environment and becomes tolerant to these therapeutical agents. For example, the EGFR variant III mutant (EGFR vIII) that does not require for ligand can constitutively form dimmer and activate its downstream survival signaling in various cancers. This mutant resists to C225 McAb treatment (202-205). As far as the effectiveness of targeting EGFR-associated cancer by TKI, studies have shown that somatic mutations and deletion in EGFR play a critical role in EGFR TKI sensitivity or resistance in the EGFR-expressing cancer cells (5). Accordingly, some somatic mutations of EGFR render certain types of cancer cells bearing such mutations sensitive to anti-EGFR inhibitors. For example, L858R point mutation or Del L747-P753 insS in EGFR rendered NSCLC sensitive to gefitinib (5, 21). Further studies suggested that only this subset of patients bearing these mutations responded well to tyrosine kinase inhibitors. In addition, genetic studies showed that people of Asian origin without smoking history often carried a high rate of mutation or deletion in EGFR in NSCLC (about 30%). However, there is a relative low mutation or deletion rate in EGFR in Caucasian group (about 10%), which explained low response rate to gefitinib treatment among Caucasian patients. Further studies on the molecular mechanism unveiled that these somatic mutations or deletions actually augmented EGFR phosphorylation at several critical tyrosine phosphorylation sites that elevated EGFR activity with unidentified reasons (5, 21). Of the EGFR-regulated signaling pathways, MAPK signaling pathway was mainly involved upon the treatment with TKI. However, depending on cell types, other pathways such as Stat and PI3/Akt might be also involved to respond to TKI. Although these TKI such gefitinib theoretically act through blocking the EGFR ATP binding site that consequently inhibits EGFR activity, these studies only demonstrated phosphorylation of EGFR was reduced but no evidence that showed whether EGFR kinase activity was altered in the presence of TKI. In

addition, very often, cancer relapses shortly after initial response due to acquiring second mutation T790M on EGFR, which results in resistance to these EGFR inhibitors (206). Therefore, clinical applications by using these inhibitors are limited and only effective to a specific subset of cancer patients with a limit period of time. We view these alterations of EGFR by genetic, epigenetic, and protein modifications as critical mechanisms by which EGFR-mediated cancer cells evolutionarily adapt to environmental pressure and challenges. This adaption provides survival advantages for EGFR-expressing cancer cells. Therefore, study for EGFR alterations in these cancer cells may possibly provide biomarkers for diagnosing cancer and predicting prognosis and will likely lead to more insights for developing more advanced and effective drugs that target EGFR-associated cancers.

In addition, the development of agents acting on EGFR ubiquitination is ongoing and exhibits potential for clinical applications (207). However, little is known about the role of whether or how EGFR ubiquitination plays in cancer development or cancer therapy. With respect to HDACi, recent studies demonstrated that HDACs such HDAC6 participated in EGFR regulation and turnover (208, 209). Therefore, targeting EGFR interacting HDAC by HDACi was proposed accordingly. Moreover, a variety of therapeutic approaches and agents that aim at EGFR associated adaptor proteins, signal proteins, and chaperon proteins are under development as well (210, 211). All in all, suggesting that EGFR is an attractive target in the treatment of various diseases.

1. 5 Development of combination therapy for targeting EGFR-associated cancers

Traditional approaches for treating cancers are including chemotherapy and radiation therapy. In the treatment of EGFR-expressing cancers, the resistance to these

conventional therapies is a major problem that needs to be tackled in the clinic. A number of studies demonstrated that several mechanisms could result in such resistance. Among those, EGFR alteration and localization played an important role in these therapies. EGFR mutation was reported to link with sensitivity or resistance to antitumor drugs, chemotherapy or radiation therapy (5, 206). For example, EGFR vIII was reportedly associated with resistance in the treatment of glioblastoma multiforme by chemotherapy or radiation therapy (212-214). An increasing number of reports demonstrated that EGFR shuttling and nuclear localization contributed to resistance to these conventional therapies as well (193). Therefore, the understanding of underlying mechanisms by which EGFR-expressing cancers develop resistance to these conventional therapies will obviously be critical in the treatment of relative cancers.

With respect to newly developed antitumor agents, anti-EGFR drugs including humanized antibodies and small molecule inhibitors that have been developed show to some extent efficacy for the inhibition of tumor growth and survival. However, cancer cells rapidly develop resistance to these drugs and relapse, typically within a year or so (215-217). A typical case is dramatic responsive to gefitinib in a subset of NSCLC patients. The population of approximately 10% Caucasian or 30% Asian women with non-smoking history showed dramatic response to gefitinib in the treatment of NSCLC. Further investigation demonstrated that these patients exclusively carried somatic mutations in EGFR, most significantly, L858R point mutation in exon 21 and/or deletion L747-P753 insS in exon 19 accounted for over 80% of responders among those patients. However, cancer relapsed after 6 months later that was mostly resulted from the acquisition of second T790M mutation so called “gate-keeper mutation”. Upon this mutation occurred, the patients became resistant to gefitinib treatment (206). A great deal of effort was engaged in developing new generation of irreversible TKI, which

attempt to overcome mutation-acquired resistance. It appears that cancer cells seem to have intrinsic mechanisms that adapt to environmental challenges and eventually alter their cell behavior that resists to various interventions. Several theories were proposed to explain cancer cell adaption. One of accepted theories is called oncogene addiction concept (218). In this theory, the cancer cells generally have more than one oncogene-regulated survival pathways that may be equally depended. The downregulation of one cancer cell survival pathway by antitumor agents will eventually lead to affected cells more dependent on another surrogated pathway to survive. This is so called acquired dependency. The blockade of such a surrogated pathway will lead to the lethality of the affected cancer cells. Such cancer cells become more vulnerable to antitumor agents that target this surrogate survival pathway, which is rationale for combination therapy to treat underlying cancers. Another theory is that heterogeneity of cancer cells that makes cancer cells not only more difficulty to treat with a single regimen but also rapid to develop resistance to these agents (210, 219, 220). In this regard, the combination for simultaneously targeting multiple cancer cell survival pathways could be more effective and least likely to develop resistance. No matter what theory can be adapted to explain this cancer resistance, a number of observations have demonstrated that the combination therapy seems more effective approach for cancer therapy and becomes trend for future cancer therapy. Moreover, due to the heterogeneity of cancer cells, the phenotype of an individual patient is ununified. This characteristics of cancer cells also makes the gene expression profile in an individual cancer patient different from each other. Therefore, personalized therapy was proposed in order to deal with such a complexity (120, 121).

A various combinations of antitumor agents have been proposed and applied in different phases of clinical trials (210, 221, 222). While developing new generation of

anti-cancer drug is a time- and money-consuming task, we consider that it is a feasible and economical way to combinatorially utilize current available anti-EGFR drugs to treat related cancers. Given sensitization effect of EGFR mutations in lung cancer to various inhibitors, we hypothesize that protein modifications of EGFR are the key events to target in the treatment of EGFR-expressing cancers. The investigation of EGFR additional modifications will provide insights and rationale for possible combinatory therapy. A growing number of proposed clinical trials for combined therapeutic regimens to treat various EGFR-associated cancers are under developing. These ongoing trials, to some extent, show potentials and benefits to cancer patients (126).

1. 6 Hypothesis, rationale, and significance

Hypothesis

1. EGFR is likely regulated in the early stage prior to ligand stimulation by protein PTM.
2. EGFR functioning may depend on its modifications through genetic, epigenetic, and protein modifications. Therefore, additional EGFR PTM may exist and play a role in the regulation of EGFR function.
3. EGFR PTM may be linked to therapeutical efficacy in the treatment of EGFR-associated diseases.

Rationale

We found EGFR lysine acetylation by using an anti-acetyl-lysine antibody. In A431 cells treated with a deacetylase inhibitor, EGFR acetylation and phosphorylation were enhanced simultaneously. Considering the fact that deacetylase inhibitors elevate

EGFR phosphorylation by our own observations as well as literature reports (179, 221), we speculate that EGFR acetylation may play an important role in cell survival and growth. However, due to oncogene addiction effect, HDACi-induced EGFR activation could lead cancer cells more susceptible to EGFR tyrosine kinase inhibitors such as erlotinib, gefitinib, and lapatinib. Therefore, combination of HDACi and EGFR inhibitor may benefit for the treatment of cancers associated with aberrant expression of EGFR.

Significance

This study provides experimental evidence and a molecular mechanism by which HDACi induce EGFR phosphorylation and activation. We found that EGFR was acetylated by CBP in the early stage of EGFR activation. Acetylation of EGFR could enhance its phosphorylation and activity. Meanwhile, HDACi-induced EGFR phosphorylation may provide cell growth advantage and contribute resistance to HDACi. Our study suggest that the combination of EGFR inhibitors and HDACi could benefit for the treatment of cancers associated with aberrant expression of EGFR.

CHAPTER 2 MATERIALS AND METHODS

2.1 Cell lines and chemicals

A431, A549, H3255, Hela, HEK293, MCF7, MDA-MB-453, MDA-MB-468, and T47D cells were purchased from ATCC and cultured according to ATCC's instructions. In general, the cells were cultured using DMEM containing 10% fetal bovine serum with 200 μg/ml penicillin and 200 μg/ml streptomycin in a 5% CO_2 incubator. HBE4 cells were cultured with serum-free medium. The medium was changed every 2-3 days. The cells were used for following experiments. All chemical reagents were purchased from Sigma or Fisher Scientific companies, respectively and are analytical grade except as otherwise noted (223).

2.2 Plasmids for expression of proteins

pcDNA6A-EGFR and pcDNA6A-EGFR-K3R (the Myc and His tagged vector) were constructed for expression of EGFR and EGFR-K3R mutant, respectively. The plasmids for expression of CBP, p300, and PCAF (CBP and PCAF are flag-tagged and p300 is HA-tagged) were generous gifts from Drs E.Y. Chin (Brown University) and T.P. Yao (Duke University). The plasmid for expression of Src was generously provided by Dr. G.E. Gallick (UT/MDACC) (223).

2.3 Antibodies

Antibodies were purchased from companies as follows: Polyclonal anti-acetyl-lysine and monoclonal anti-phospho-tyrosine (4G10) antibodies were purchased from Upstate Biotechnologies Incorporation, USA. Polyclonal anti-EFGR, anti-p300, and anti-CBP antibodies were purchased from Santa Cruz Biotechnologies Incorporation, USA. siRNA for knockdown of CBP, p300, and EGFR was obtained from Santa Cruz

company as well. Polyclonal anti-phosho-Erk, anti-Erk, anti-phospho-Akt, anti-Akt, anti-phospho-Stat3, and anti-Stat3 antibodies were purchased from Cell Signaling company, USA. Monoclonal anti-EGFR antibody (AB13) was obtained from NeoMarkers, USA. Anti-Src antibody (monoclonal Ab) was purchased from CalBioChem, USA (223).

To detect an individual EGFR acetylation, in collaboration with China Medical University, Taiwan, we generated an antibody against acetylated EGFR-K843 site. The peptide sequence for generating antibody was: RNVLVKTPQHVKITDFGLA KLLGAEE-**K**-EYHAEGGKVPIKWMALESILHR**.** Briefly, anti-acetyl-EGFR-K843 antibody was generated by injecting the acetylated peptide corresponding to the K843 site (synthesized by Quality Controlled Biochemicals, USA) into mice with boosting injection every 2 weeks. The sera were collected for Western blot analysis. For eliminating background, non-acetyl-peptide against K843 site (250 ng/ml) was added to block non-specific reaction. This antibody can be used for detecting endogeneous EGFR acetylation in various cell types.

2.4 Site-directed mutagenesis and PCR

Ultra-Blue site-directed mutagenesis kit was purchased from Stratagene, USA. The mutations were performed based on standard procedures and manufacturer's instructions. The mutation primers: 5'-CAGATTTTGGGCTGGCCAGACTGCTGGGT GCGGAAG, 5'-CTTCCGCACCCAGCAGTCTGGCCAGCCCAAAATCTG, 5'-CAG ATTTTGGGCTGGCCAGACTGCTGGGTGCGGAAG, 5'-CTTCCGCACCCAGCAG TCTGGCCAGCCCAAAATCTG, 5'-GCTCTCTTGAGGATCTTGAGGGAAACTGA ATTCAAAAAG, and 5'-CTTTTTGAATTCAGTTTCCCTCAAGATCCTCAAGAGA GC were synthesized by Sigma and used for generating EGFR-K3R mutant. The

EFGR-K3R mutant was confirmed by DNA sequencing at MD Anderson Cancer Center Sequencing Core Facilities (223).

2.5 Generation of stable clones for expression of EGFR or EGFR-K3R

MCF7 or HEK293 cells were seeded on 10 cm culture plates and cultured in DMEM with 10% FBS and appropriate antibiotics one day prior to transfection. On the second day, the cells reached to 90-95% confluence and were ready for transfection. The plasmid for expression of either EGFR or EGFR-K3R was pre-diluted in 0.5 ml Opti-MEM transfection medium at a final concentration of 16 μg/ml in a 15 ml tube. In a separate tube, 20 μl of liposome transfection reagent was pre-diluted in 0.5 ml Opti-MEM medium and incubated for 5 min at room temperature. Following to mix the plasmid with liposome and incubate the mixture for 25 min at room temperature. The cells were rinsed with Opti-MEM and added 4ml fresh Opti-MEM for transfection. The transfection mixture was added into the cells drop by drop with gentle shaking, then the cells were cultured for 6h. After 6h culture, removed transfection medium and added 10 ml fresh DMEM containing 10%FBS without antibiotics. After 2 days later, the cells were ready for antibiotics selection. The cells were splitted and selected with 10 μg/ml blasticidin in DMEM containing 10%FBS and appropriate antibiotics. After 3 weeks selection, individual clone was selected and continued culturing in DMEM containing 5 μg/ml blasticidin. The stable clones were tested for EGFR or mutant protein expression by Western blot. The final clones with appropriate protein expression were collected or stored for later analysis and study.

2.6 Cell lysate preparation

The cells were washed with pre-cold PBS containing appropriate protease inhibitors. After washing, the cells were scratched by a cell lifter in 3ml fresh PBS with protease inhibitors, then centrifuged for 5 min at 1000 rpm. Discarded the supernatant and the cell pallets were used for preparation of cell lysates. RIPA buffer containing 3 mM PMSF, 3 mM Na_3VO_4, 3 mM NaF, 3 μg/ml for each of aprotinin, leupeptin, pepstatin, 5 mM sodium butyrate, and 20 μM TSA. The cells were suspended and then briefly ultra-sonicated. Following to incubate for 15 min at 4ºC with gentle rotation and then centrifuged for 20 min at 10,000 rpm. the supernatant was transferred to a separate clear tube for immediate experiments or stored at -80ºC for later use.

For nucleus fractionation, the cells were lysed by using lysis buffer containing 20 mM, 10 mM KCl, 2 mM $MgCl_2$, 0.5% NP-40, 1 mM PMSF, and 2 ug/ml Aprotinin. After that, the cells were transferred into a Dounce homogenizer and homogenized with 30 strokes. Then, centrifuged for 5 min at 4000 rpm. The nucleus pellets were lysed with RIPA buffer as described above. The lysates can be used for studying nuclear portion of proteins (223).

2.7 Immunoprecipitation and immunoblot

For immunoprecipitation, a total of 500 μg of cell lysate was used and diluted in 500 μl of RIPA buffer containing 3 mM PMSF, 3 mM Na_3VO_4, 3 mM NaF, 5 mM sodium butyrate, and 20 μM TSA with corresponding antibodies. After adding 2μg either anti-EGFR antibody (mouse monoclonal, NeoMarkers) or anti-Myc antibody (Sigma), the mixture was incubated with gentle rotation for 2h at 4 ºC, then added 80 μl of protein-G beads and incubated for additional 2h. The beads were washed twice by

RIPA buffer and 50 μl 2x protein loading buffer was added into the beads. Then the beads were heated for 5 min at 100 ºC. After brief centrifuge, the supernatant was loaded on 8% SDS-polyacrylamide gel and proteins were separated under 100 voltage power.

For immunoblot, a total of 20 μg of cell lysate was used and heated by similar procedures as mentioned above, and following separated on 8% SDS-PAGE. Then, the proteins separated by SDS-PAGE were transferred to a PVDF membrane and probed with antibodies as indicated in Figures. The immunoblot and image capture were performed following standard procedures (223).

2.8 Nano-HPLC-MS/MS spectrophotometry

A431 cells were cultured in 10 cm plates in DMEM with 10% FBS and appropriate antibiotics. After reaching to 90% confluence, the cells were treated with 2 μM TSA and 5 mM sodium butyrate for 24h. On the second day, the cells were treated with 25 ng/ml EGF for 5 min prior to collection. The cells were lysed by RIPA buffer with 3 mM PMSF, 3 mM Na_3VO_4, 3 mM NaF, 3 μg/ml of aprotinin, leupeptin, and pepstatin, respectively, 5 mM sodium butyrate, 20 μM TSA, and 20 mM Nicotinamide and the procedures were followed as mentioned previously. Especially, for preserving EGFR acetylation, TSA and sodium butyrate are essential for inhibiting HDACs to deacetylate EGFR. The 5 mg of cell lysate extracted from pretreated A431 cells was immunoprecipitated with an anti-EGFR monoclonal antibody (NeoMarkers). After washing, the mixture was resolved on 8% SDS-polyacrylamide gel. The separated gel bands were excised and subject to in-gel trypsin digestion, then analyzed by nano-HPLC-MS/MS system (124, 223, 224).

2.9 Transfection of plasmid or siRNA into mammalian cells

wt-EGFR or EGFR-K3R was transfected by liposome, respectively. Co-transfection of EGFR or EGFR-K3R with either p300 or CBP or PCAF was also performed by using liposome followed procedures as mentioned previously. Briefly, eight μg plasmid for expression of target protein was transfected into the cells using liposome. After transfection, the cells were allowed to express for 48h prior to collecting the cells for protein expression analysis.

The transfection of siRNA for knockdown of p300, CBP, and EGFR was employed by using electroporation. The reagent for electroporation was purchased from Amaxa, Germany. The transfection was followed manufacturer's instructions. In brief, either A431 cells or MDA-MB-468 cells were trypsinized and washed in pre-cold PBS. The cells were re-suspended in 200μl transfection solution and 100 nM siRNA. Then transfection procedures were performed by employing an Amaxa electroporator (Nucleofector I, Germany). The cells then were cultured for 72h prior to assay. After 72h culture and expression, the cells were collected for protein expression analysis by Western blot.

2.10 Immunofluorescence assay

The cells for immunostaining were seeded in a chamber slide and cultured for 24h and subject to serum-starvation for additional 24h prior to assay. The cells were then treated with 25 ng/ml EGF for 30 min prior to immunostaining. After treatment, the cells were washed twice in pre-cold PBS. Then, the cells were fixed by 4% paraformaldehyde for overnight. On second day, discarded fixation solution and washed the cells twice in pre-cold PBS. After washing, added blocking buffer

containing PBS with 3% BSA and 2% Triton X-100 and incubated for 2h at room temperature. Removed blocking buffer and added the first antibody diluted in PBS with 2% Triton X-100 and incubated for 2h at 4 ºC. Washed the cells twice in PBS and added the second antibody conjugated with fluorescence (FITC) and incubate for 1h at room temperature. Following to wash the cells three times in PBS and the slide was sealed using nail solution for observation by confocal microscopy.

2.11 Assay for protein dimerization

wt-EGFR or EGFR-K3R was transfected into HEK293 cells by liposome, respectively. After 48h expression, the transfectants were serum starved for 24h and then treated with 25 ng/ml EGF for 5 min. Washed the cells twice using pre-cold PBS and added cross-linking reagent BS^3 [Bis (sulfosuccinimidyl) suberate salt] 1.5 mM in PBS, then the ells were incubated for 2h on ice. After incubation, the cells were washed in PBS and collected for assay.

2.12 Cell growth assay

For *in vitro* cell growth assay, $1X10^4$ cells were seeded in a 6-well culture plate in DMEM containing 10% FBS and appropriate antibiotics. At specified time points, the cells were stained with trypan blue and counted. At least three independent experiments were performed and mean ± standard deviation (SD) was calculated and used for comparisons.

2.13 BrdU incorporation

For measuring cell DNA synthesis, $1X10^5$ MCF7 cells stably expressing either wt-EGFR or EGFR-K3R mutant were seeded in 6-well plates. After 30h serum starvation,

the cells were treated with 30 μM BrdU (BD transduction) in 10% FBS fresh medium with or without 25 ng/ml EGF for 16h, then an anti-BrdU monoclonal antibody (Upstate) was used to detect incorporated BrdU substrate in the cells. A FITC conjugated goat anti-mouse IgG antibody (Johnson Laboratory, USA) was used to amplify signals. Then DNA incorporated cells were counted by using a fluorescence microscope. At least three independent experiments were performed and mean ± SD was calculated and used for comparisons.

2.14 Soft agar assay

For anchorage independent cell growth assay, preparing 1.6% agarose solution in PBS and mixing equal volume of agarose and 2x DMEM containing 20% FBS and antibiotics. Added the mixture into a 6-well culture plate and let mixture become solid 0.8% bottom supporting gel. Then, $5x10^4$ cells were resuspended into 2X DMEM medium including 20% FBS and appropriate antibiotics and then mixed with an equal volume of 1.0% agarose gel. Finally, the mixtures were seeded in the 6-well plate on the bottom supporting gel. The cells were feed every 2-3 days with DMEM containing 10% FBS with appropriate antibiotics. The colonies were formed and counted after culturing for two consecutive weeks. At least three independent experiments were conducted and mean ± SD was calculated and used for comparisons.

2.15 Cell viability assay

To test the effect of drugs on tumor cell killing, $1X10^4$ cells were seeded in a 96-well plate and cultured for 24h. Then, treated with either SAHA or TKI (erlotinib, gefitinib, lapatinib) or different combinations as indicated for 72h. After that, added

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a final concentration of 0.5 mg/ml and continued culturing for another 2-4h. Then 100 μl of β-isopropanol was added into each well to dissolve precipitated substrate. Optical density (OD) was measured by a microplate reader (Bio-Rad, U.S.A.). At least three independent experiments were conducted and mean ± SD was calculated and used for comparisons (223).

2.16 Flow cytometry

To study the effect of HDACi on cell cycle, $1X10^5$ cells were seeded in a 6-well culture plate. After treatment, the cells were washed twice in pre-cold PBS and fixed by 50% ethanol for incubated for 1h at -20 ºC. Then the cells were centrifuged and the supernatant was removed. Following to add 500 μl propidium iodide to a final concentration of 10 μg/ml. The cells were analyzed by flow cytometry. At least three independent experiments were conducted and mean ± SD was calculated and used for comparisons.

2.17 *In vivo* mammary fat pad tumor cell injection

The nude mice were grouped randomly. A 17β-estradiol tablet was inoculated under the skin of each mouse 3 days prior to tumor cell injection. To compare the effect of wt-EGFR and EGFR-K3R mutant on cell tumorigenesis, $5X10^6$ MCF7 parental cells or stable clones were inoculated into mammary fat pad of nude mice. The tumor size was monitored and measured following standard procedures. Animal manipulation was in accordance with institutional guidance and policies.

2.18 *In vivo* animal treatment

To test the effect of drug on tumor cell growth *in vivo*, $5X10^6$ MDA-MB-468 cells were inoculated into mammary fat pad of each mouse. When tumor size reached 100 mm^3, then mice were grouped randomly as placebo or treatment groups. The treatment was initiated at the same day for each group. SAHA (20 mg/kg) and /or erlotinib (15 mg/kg) were administrated by oral daily. The tumor size was measured twice per week. Animal handling follows institutional policies and regulations accordingly (223).

2.19 Statistical analysis

Statistical analyses were performed by student's t-Test and ANOWA analysis. All data are described as mean ± SD. In general, $P<0.05$ is considered as statistical significance (223).

2.20 Clinical trial information

The list of ongoing clinical trials about HDAC inhibitors for the treatment of a variety of cancers can be found at: http://www.cancer.gov/clinicaltrials

CHAPTER 3 RESULTS

3 .1 Defining the role of EGFR acetylation

3.1.1 Identifying EGFR acetylation

It has been reported that HDACi induced EGFR phosphorylation. We considered that this effect might link with tumor cell resistance to HDACi (179). Recent studies demonstrated that EGFR interacted with HDAC6 in tumor cells that express EGFR (208, 209). Therefore, it is of interest in investigating likelihood of EGFR acetylation. Since A431 cells overexpress EGFR, this characteristics makes them as excellent candidates for studying EGFR modifications. By using A431 cells that are widely used cancer cells for studying EGFR function, we performed immunoprecipitation assay by immunoprecipitating (IP) with an anti-EGFR antibody and immunoblotting (IB) with an anti-acetyl Lysine antibody. EGFR acetylation was clearly demonstrated as showed in Figure 4A. And vice versa, in reciprocal immunoprecipitation by IP with an anti-acetyl-Lysine antibody and IB with an anti-EGFR antibody, we found similar results as showed in Figure 4A. In addition, an HDAC inhibitor, TSA, further enhanced acetylation of EGFR as showed in figure 4B. Together, these results suggest that EGFR acetylation occurred endogenously and was biologically meaningful PTM. This finding raises the question of whether or not EGFR acetylation is a universal modification in EGFR-expressing cancer cells. Is EGFR acetylation a common way that the cells adapt to environmental stimuli and/or cellular changes in order to survive? To address this question, we examined various EGFR-expressing cancer cell lines and fund that EGFR acetylation could be detected in various EGFR-expressing cancer cells (data not shown). This result suggests that EGFR acetylation may be a common phenomenon in cellular processes.

3.1.2 Determining acetylation sites of EGFR

Next, we worked on the enrichment of EGFR acetylation in order to identify acetylation sites, which is the most important step to understand the function of EGFR acetylation. There are two major approaches to address this issue. 1) MS/MS technique. 2) Mutation and deletion approach. MS/MS is current the most powerful and effective methodology to determinate protein modifications such as phosphorylation, ubiquitination, and acetylation. To identify all the possible modification sites, there is in need of covering all the peptide in the protein sequence. Proteolytic peptides behave differently during in-gel digestion and in machine. Some peptides respond much better than others. Therefore, it is almost impossible to cover 100% of protein sequence. This may be one of the possible reasons that we identified different EGFR acetylation sites from a report in the literature (178). In addition, cell type difference could be another reason that caused different acetylation of EGFR during the cellular processes. With the combination of MALDI-TOF and nano-HPLC MS/MS, we can reach up to 70% identification efficacy (225). Generally, a 3 pmole of protein or greater (appropriately 0.5 μg of EGFR) is helpful to increase sequence coverage and to identify maximum number of modification sites. To enrich such amount of protein, we used 150 mm culture plates to culture cells. To maximize the possibility of identifying EGFR acetylation sites, the cells were treated with multiple deacetylase inhibitors as stated in the section of Materials and Methods in order to prevent from rapid deacetylation by HDACs. Then, double immunoprecipitation using anti-EGFR and anti-acetyl-lysine antibodies was performed to enrich the portion of acetylated EGFR. Since epidermoid carcinoma cell line A431 is highly overexpressing EGFR, we were able to extracted sufficient EGFR from A431 cells by using a specific monoclonal antibody against

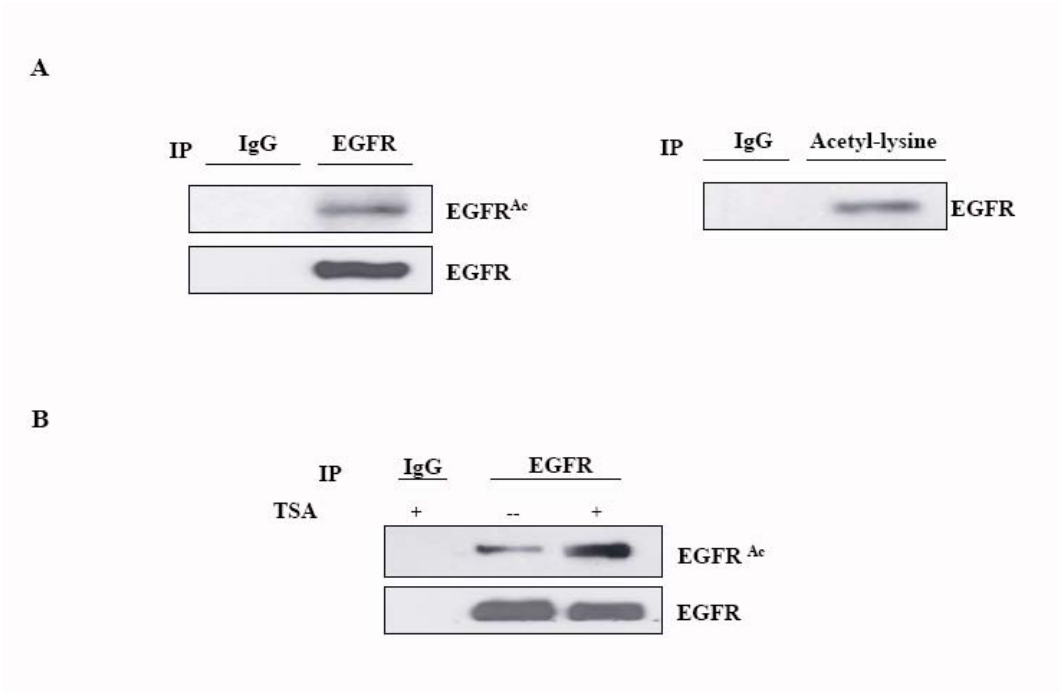
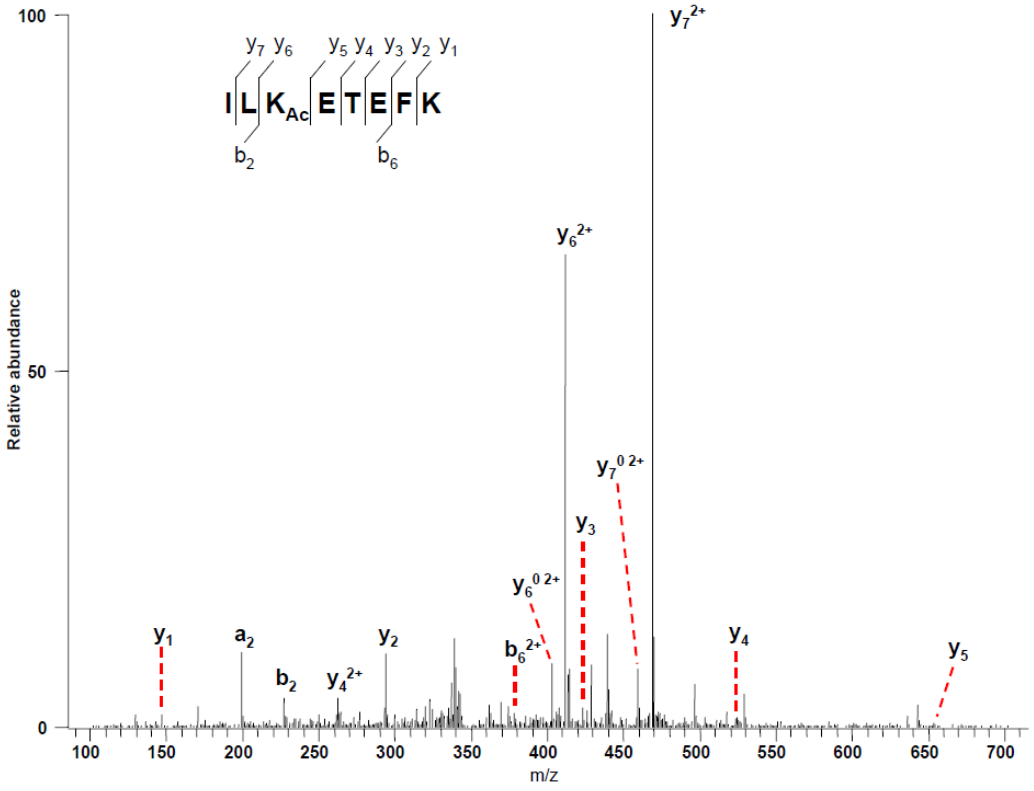


Figure 4 Acetylation of EGFR

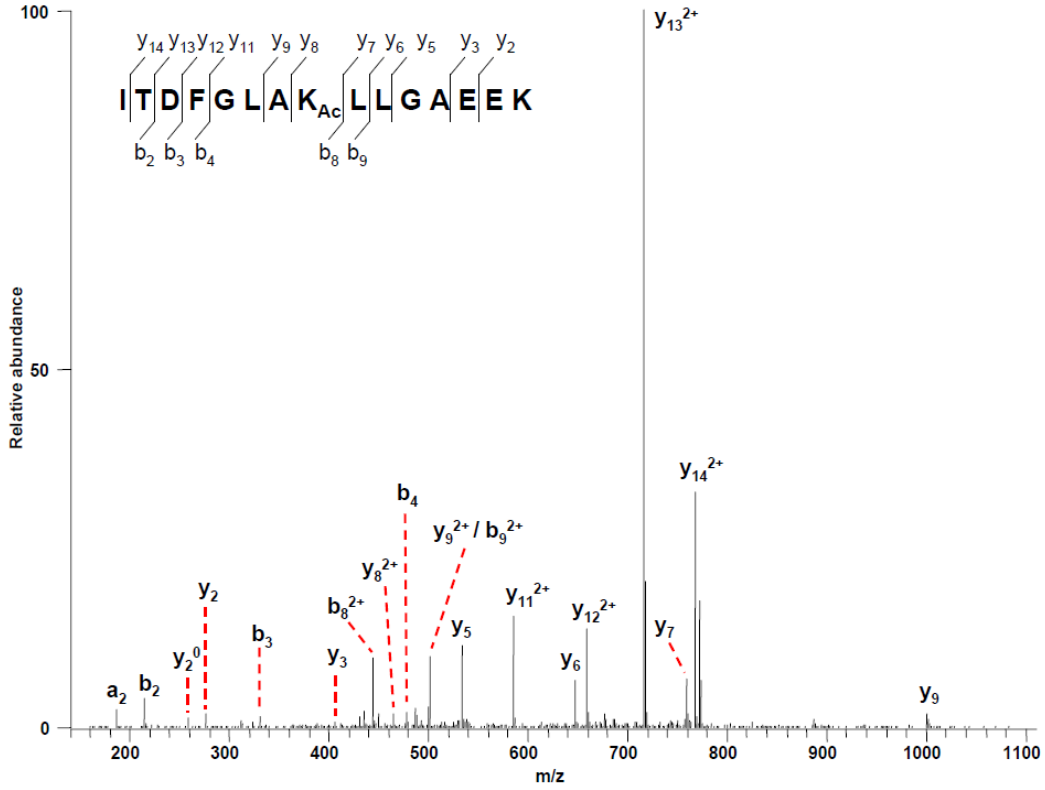
A total of 500 μg cell lysate was immunoprecipitated and immunoblotted with antibodies as indicated. EGFR acetylation was detected by using a polyclonal anti-acetyl-lysine antibody. A. A431 cells were cultured in DMEM containing 10% FBS and used to analyze endogenous EGFR acetylation. B. A431 cells were serum-starved and then treated with 20 μM TSA for 5h prior to collecting the cells.

(The figure 4 is adapted with permission from Elsevier Ltd)

A. K684 acetylation



B. K836 acetylation



C. K843 acetylation

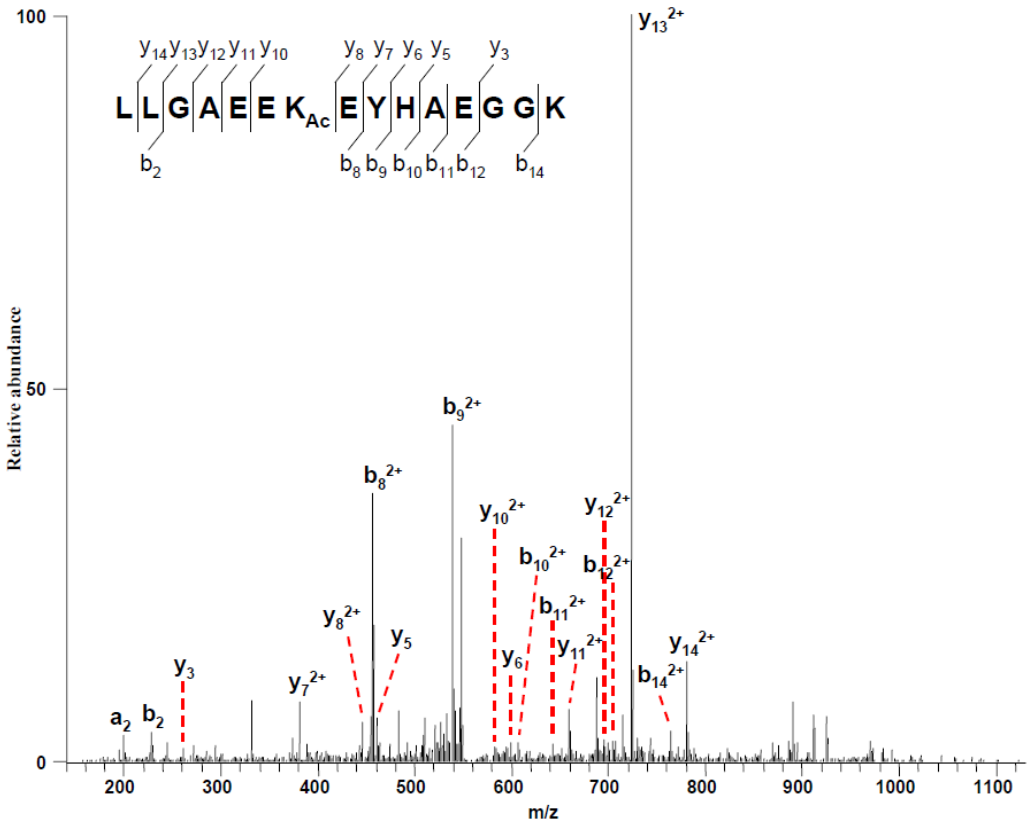


Figure 5 Acetylation of EGFR at K684, 836, and 843 sites

A431 cells pretreated with 2 μM TSA and 5 mM sodium butyrate for 24h were lysed and immunoprecipitated by an EGFR antibody and then were subjected to nano-HPLC-MS/MS analysis. A. Acetyl-K684. B. Acetyl-K836. C. Acetyl-K843. (Done by Dr. Yingming Zhao, The University of Texas, Southwestern Medical Center, Dallas, TX). (The figure 5 is adapted with permission from Elsevier Ltd)

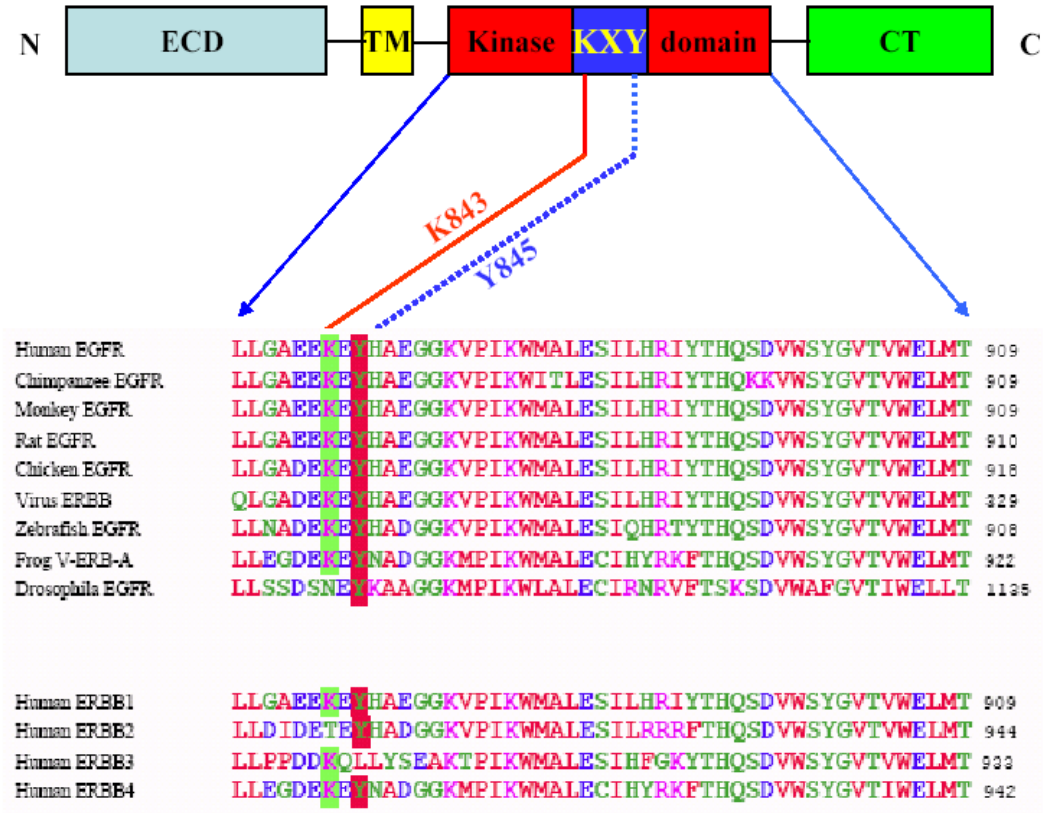


Figure 6 protein alignment of EGFR

K843 and Y845 are highlighted in figure. The alignment was performed by using ClustalW.

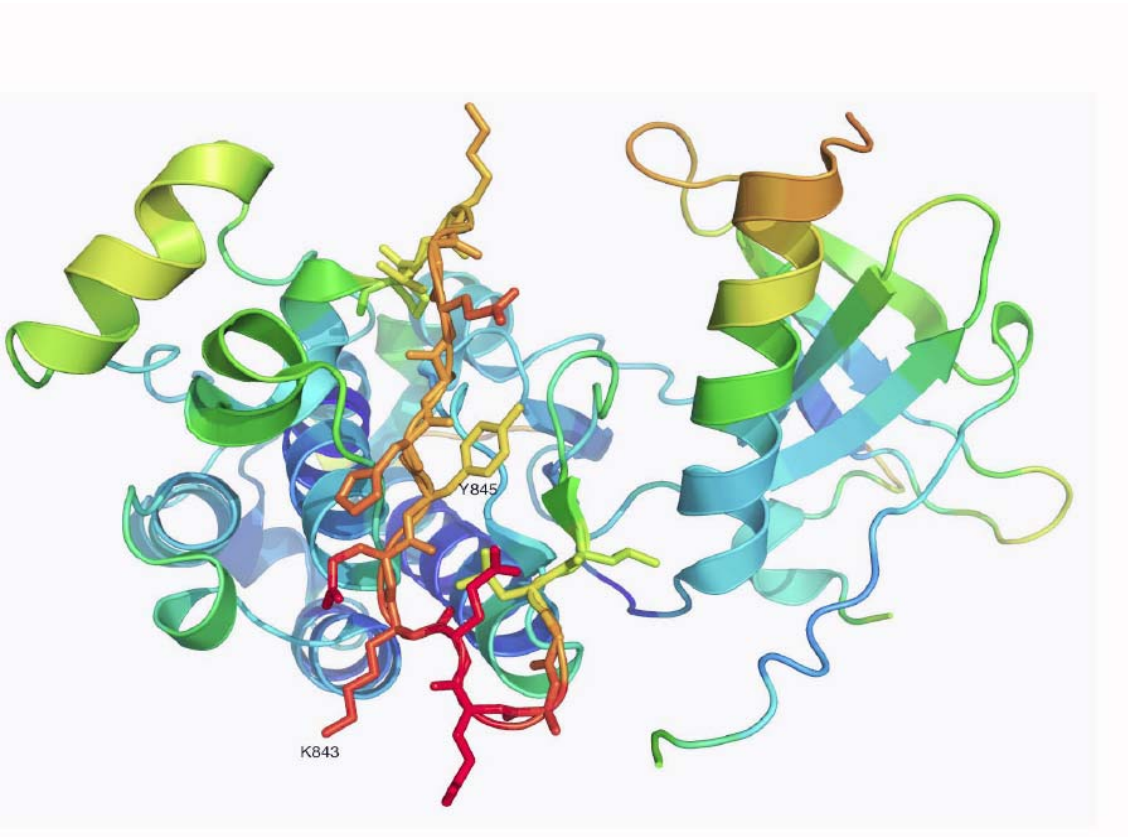


Figure 7 Structure of EGFR kinase domain

Tyrosine 845 and lysine 843 are showed in this figure. Both sites are highly conserved among various species. Lysine 684 and 836 are not shown in this figure (Courtesy by Dr. Xiaomin Chen, Department of Biochemistry, UT/MDACC).

EGFR. Meanwhile, we carefully enriched and preserved its acetylation by using multiple deacetylase inhibitors in all steps of cell and protein processes including treating the cells, lysing the cells, and performing immunoprecipitation. The extracted EGFR protein was subject to SDS-PAGE separation. The gel bands migrating at about 170 kDa position were excised and subject to trypsin in-gel digestion. We identified three EGFR acetylation sites that were all located in intracellular domain, namely K684, 836 and 843 (Figure 5). Based on current available EGFR kinase domain structure, we performed computer simulation analysis and identified that K843 and Y845 sites are highly conserved and physically close to each other (Figure 6). Y845 is a very important tyrosine phosphorylation site that regulates cell growth, proliferation, and cell cycle. K836 and 843 with Y845 have been reported to form enzymatic pocket that determines intrinsic EGFR kinase activity (226). Taken together, these results suggest that EGFR PTM on K836 and 843 along with Y845 may change the structure of this pocket and affect protein-protein interactions that subsequently result in change of related downstream signal pathways (Figure 7). The close proximity of three key amino acids may be one of reasons that alteration and interruption of protein modification may affect EGFR function. In addition, EGFR acetylation may potentially affect its phosphorylation due to the change of this triad including K836, K843, and Y845 sites.

3.1.3 CBP is responsible for acetylating EGFR

The following question we need to address is what enzyme(s) acetylates EGFR. Since CBP, p300, and PCAF are three well-characterized acetyltransferases and play an important role in regulation of gene expression in mammalian cells (227), this fact makes them as reasonable and potential candidates for determining the responsible enzyme that acetylates EGFR. Cotransfection of EGFR with either CBP or p300 or

PCAF into HEK293 cells was performed. Immunoprecipitation and immunoblot results demonstrated that only CBP dramatically induced EGFR acetylation while p300 and PCAF could not induce detectable acetylation of EGFR (Figure 8A). The knockdown of CBP but not p300 by siRNA resulted in significantly reduction of EGFR acetylation in A431 cells (Figure 8B). Acetylation–deficient EGFR-K3R mutant significantly reduced EGFR acetylation by CBP in HEK293 cells that ectopically expressed CBP and EGFR-K3R (Figure 8C), suggesting that CBP is an enzyme that can acetylate these three lysines on EGFR. Taken together, these data suggest that CBP is the enzyme that acetylates EGFR *in vivo*. In addition, we observed that CBP could increase EGFR tyrosine phosphorylation alongside EGFR acetylation as shown in Figure 8D. Moreover, we also demonstrated the interaction between CBP and EGFR (Figure 8E). All in all, these data support that CBP is a major acetylase that acetylates EGFR.

CBP is CREB binding protein and plays a key role in the regulation of gene expression. CBP is a transcription coactivator that is mainly located in the nucleus. The first obvious question is how CBP reaches to EGFR and then acetylates EGFR. It has been reported that there is small percent of CBP that remains in the cytoplasm in the non-stimulated cells, although the majority of CBP are mainly located in the nucleus, which may explain that EGFR acetylation occurred prior to ligand stimulation. However, in response to ligand stimulation, CBP rapidly moved out the nucleus and traveled towards cell membrane and then acetylated target proteins (228). We treated 24h serum starved A431 cells with 25ng/ml EGF for different time points and then performed cellular fractionation for immunoblot analysis and cell immunostaining for confocal microscopy analysis. The data demonstrated that CBP rapidly shuttled out the nucleus and moved into the cytoplasm as showed in Figure 9. Within a short period of time, the majority of CBP rapidly moved into the cytoplasm. This observation is very

similar to results reported in the literature (228). In addition, AG1478, a tyrosine inhibitor, was not able to block CBP shuttling from the nucleus into the cytoplasm. These suggest that it may be a general phenomenon of which ligand stimulation can cause CBP to travel out of the nucleus, then CBP moves towards cell membrane and performs its function. Since both interferon and EGF can trigger CBP shuttling, suggesting that this phenomenon is not an EGF-specific event.

3.1.4 Acetylation and protein stability

It has been shown that protein PTM, very often, affect protein stability (27, 229-231). To address whether EGFR acetylation affects its protein stability, we generated HEK293 cell stable clones that expressed either wt-EGFR or acetylation-deficient EGFR-K3R mutant. The stable clone cells were treated with 1 μM cycloheximide for the time points as indicated in Figure 10. Then the cells were collected and subjected to immunoblot analysis. Although EGFR-K3R significantly reduced lysine acetylation, the protein level of EGFR remained similar to wt-EGFR. Our observation suggests that EGFR protein stability is not significantly affected by EGFR acetylation.

3.1.5 Acetylation and protein dimerization

In response to ligand stimulation, EGFR generally forms dimers and then subsequently activates downstream signal pathways. The dimerization of EGFR is the key process to activate EGFR and EGFR-regulated downstream pathways. To investigate whether EGFR dimerization is affected by EGFR acetylation, HEK293 cells expressed either wt-EGFR or EGFR-K3R were treated with EGF, and then artificial dimers were formed by using BS^3 crossing linker reagent in these cells. The cells were

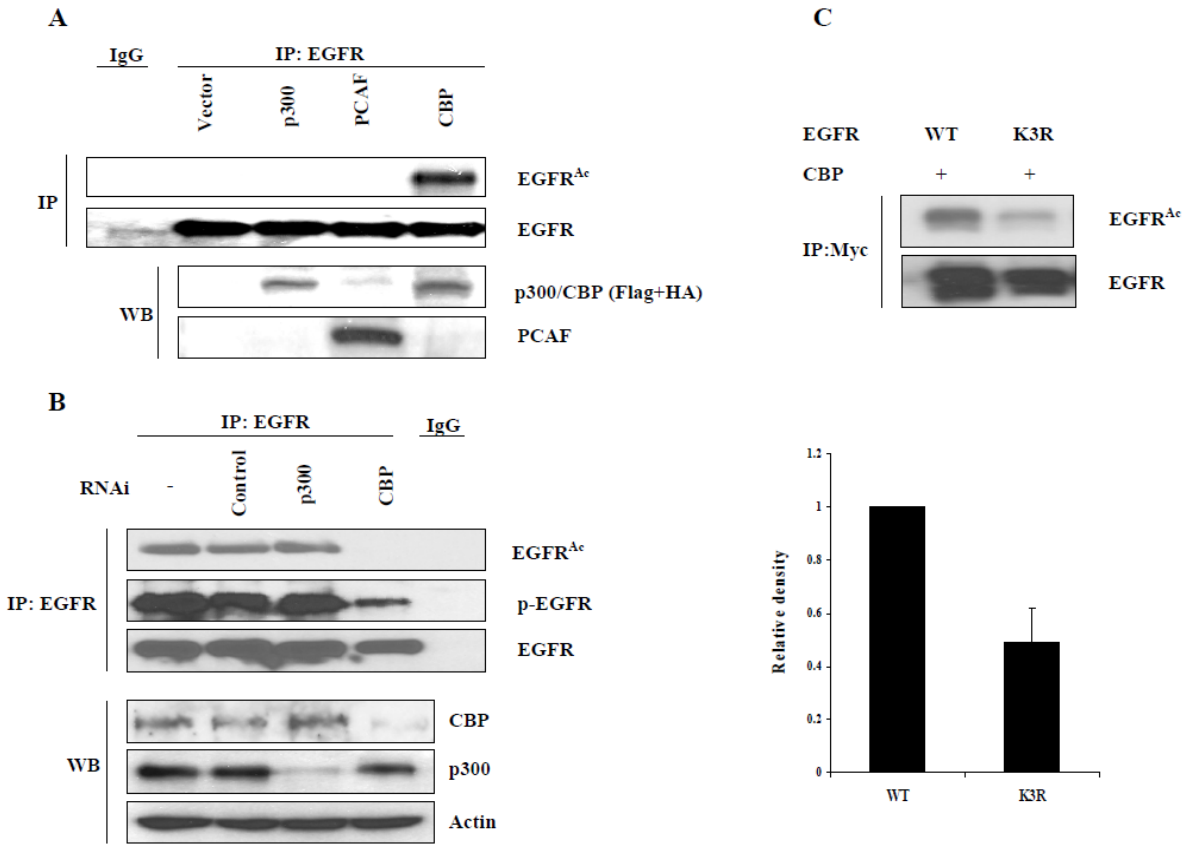
lysed and analyzed by Western blot. The gel shifting pattern of both wt-EGFR and EGFR-K3R was similar to each other as showed in Figure 11, suggesting that EGFR protein dimerization was not affected in acetylation-deficient EGFR-K3R mutant. This observation may further indicate that EGFR acetylation in the intracellular portion of EGFR least likely affects EGFR dimerization.

3.1.6 Acetylation and protein trafficking

EGFR is subject to frequent endocytosis and shuttling between membrane and inside of the cell. To compare whether EGFR acetylation is associated with EGFR protein endocytosis and localization, stable HEK293 cell clones for expression of either EGFR or EGFR-K3R were serum starved for 24h and then treated with 25 ng/ml EGF for 30 min. After fixation by ethanol, the cells were immunolabeled with an anti-EGFR monoclonal antibody and then detected by using a FITC-conjugated anti-IgG antibody. Finally, the cells were observed by a confocal microscopy. Our data demonstrated that EGFR surface protein expression and quantity were similar to each other as showed in Figure 12. In addition, in response to EGF, EGFR distribution pattern and localization were virtually no difference between wt-EGFR and EGFR-K3R. Together, tthese results suggest that EGFR acetylation may least likely affect its protein trafficking and shuttling.

3.1.7 Acetylation and protein interaction

Since K836 and K843 acetylation sites are physically located in the intrinsic enzymatic pocket of EGFR (226), the interruption of acetylation at K836 and 843 may intrinsically affect recruitment of EGFR adapter proteins and signaling proteins. Most



(The figure 8A-C is adapted with permission from Elsevier Ltd)

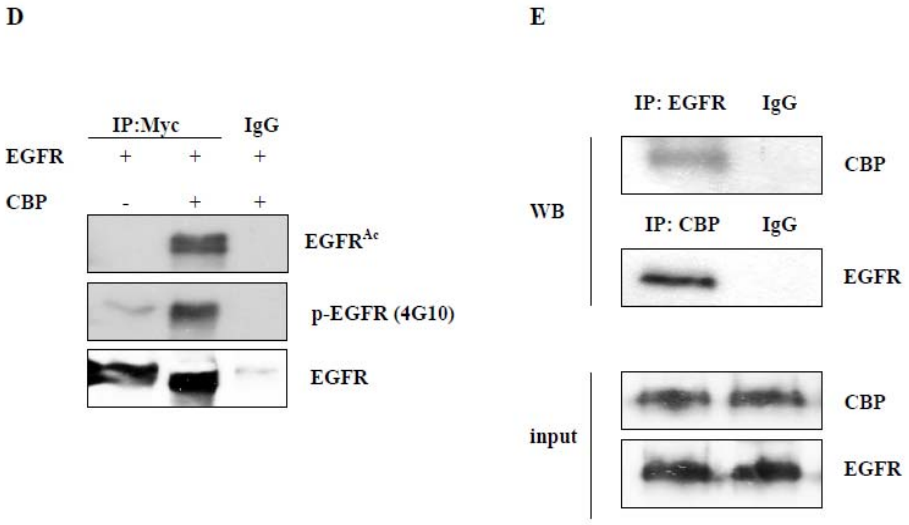
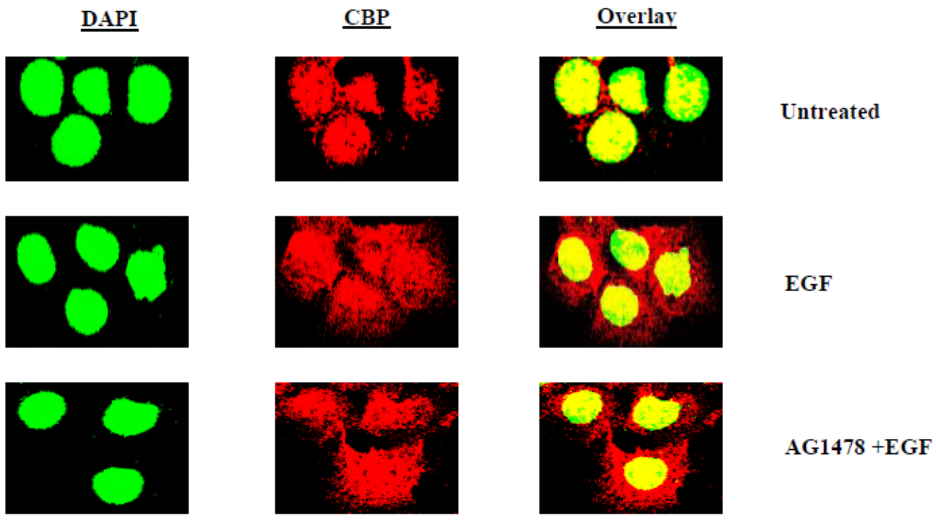
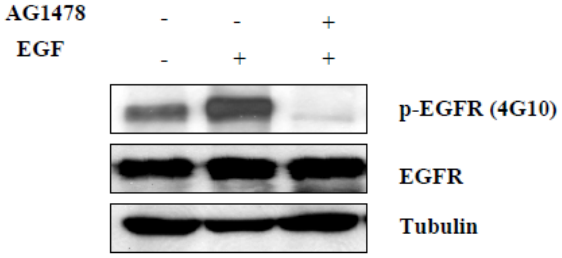


Figure 8 CBP acetylase is responsible for acetylating EGFR

A. wt-EGFR was co-transfected with CBP, p300 or PCAF into HEK293 cells, respectively. The cells were cultured for 48h after transfection, then immunoprecipitation and immunoblot were performed by using lysates from these transfectants. B. siRNA for silencing p300, or CBP was transfected into A431 cells by electroporation, respectively. The cells were cultured for 72h after transfection, then the cells were lysed for immunoprecipitation and immunoblot analysis. C and D. CBP was cotransfected with either wt-EGFR or EGFR-K3R mutant into HEK293 cells. The cell lysates were used for immunoprecipitation analysis. E. A431 cells were used for immunoprecipitation analysis.

A


DAPI
CBP
Overlay
Untreated
EGF
AG1478 +EGF


AG1478
EGF
- - +
- + +
p-EGFR (4G10)
EGFR
Tubulin

B

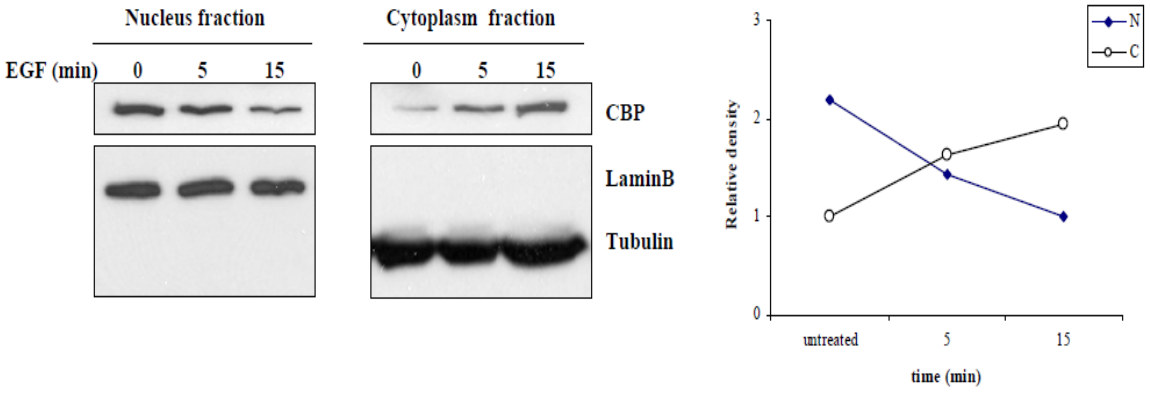


Figure 9 CBP travels from the nucleus to the cytoplasm in response to EGF stimulation

A. A431 cells were serum-starved for 24h prior to the assay. The cells were pre-treated with 5 μM AG1478 for 6h before adding 25 ng/ml EGF and probed with an anti-CBP antibody for immunostaining. Western blot was also performed to observe the effect of AG1478 on tyrosine phosphorylation of EGFR. B. The nucleus fraction and cytoplasm fraction of proteins from A431 cells were immunoblotted with indicated antibodies.

(The figure 9B is adapted with permission from Elsevier Ltd)

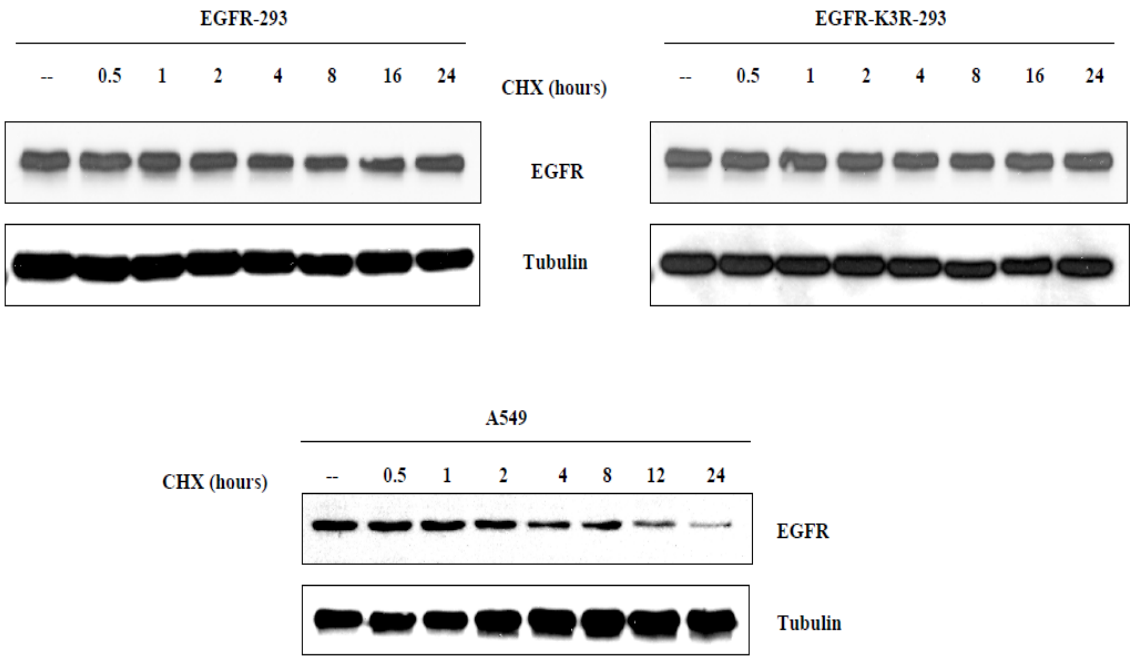


Figure 10 Comparison of EGFR protein stability between wt-EGFR and EGFR-K3R

wt-EGFR and EGFR-K3R mutant were transfected into HEK293 cells and stable clones were generated and used for assay. The cells were treated with 1 μg/ml cycloheximide for indicated time and then collected for immunoblot analysis. A549 cells were used as positive control.

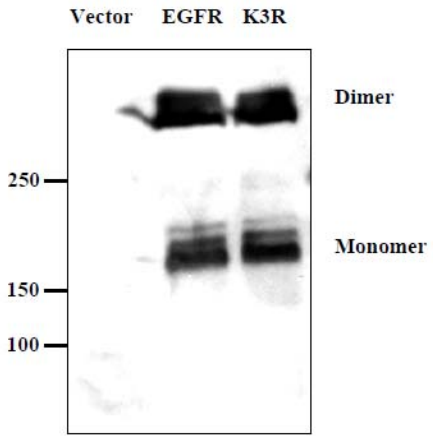


Figure 11 Comparison of EGFR dimerization between wt-EGFR and EGFR-K3R

wt-EGFR and EGFR-K3R were transfected into HEK293 cells. After 48h expression, the cells were treated with BS^3 cross-linker for 2h prior to collection. The cell lysates were used for immunoblot analysis.

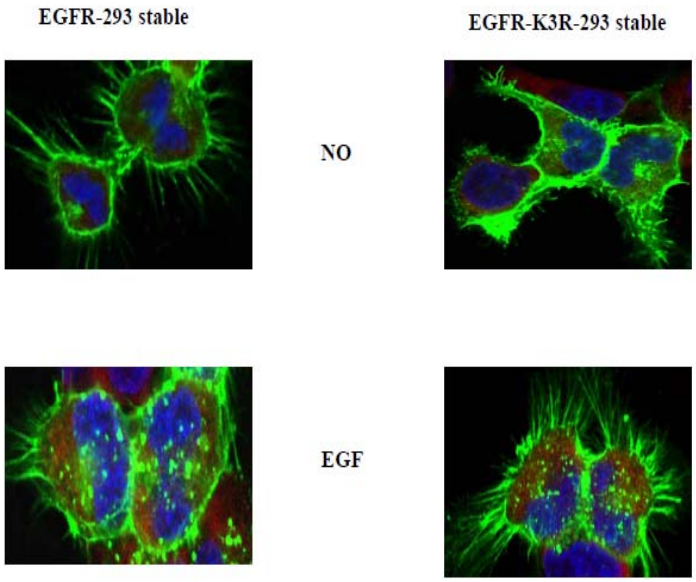


Figure 12 Comparison of protein localization between wt-EGFR and EGFR-K3R

HEK293 stable clones for expressing either wt-EGFR or EGFR-K3R were seeded into chamber slide and cultured for 24h. Then followed 24h serum starvation, the cells were treated with EGF for 30 min. The cells were fixed and subject to immunofluorescence staining by using anti-EGFR and FITC-conjugated anti-IgG antibodies. Confocal microscopy was employed to analyze the results (Done by Yi Du).

importantly, due to Y845 site at the center of the EGFR intrinsic activity pocket and phosphorylated by Src (232, 233), the mutation of K836 and K843 could possibly affect the interaction between EGFR and Src. In addition, it has also been reported that Src can promote destruction of c-Cbl that may implicate oncogenic synergy between Src and EGFR (177). To demonstrate the possible protein interaction affected by EGFR acetylation, we cotransfected Src with either wt-EGFR or EGFR-K3R mutant into HEK293 cells and performed immunoprecipitation analysis. Our data demonstrated that the binding capacity of EGFR-K3R with Src was reduced more than 50% compared with wt-EGFR (Figure13A). The wt-EGFR transfectants treated by HDACi TSA significantly increased the interaction between Src and EGFR (Figure 13B). Together, these findings suggest that EGFR acetylation affects its interaction with Src. Consistent with the literature, these data demonstrate that the change of EGFR modification could significantly affect the interaction between EGFR and its interacting proteins.

3.1.8 EGFR acetylation enhances its tyrosine phosphorylation

To understand how EGFR acetylation affects its function, we compared phosphorylation of wt-EGFR with EGFR-K3R acetylation-deficient mutants. Either wt-EGFR or EGFR-K3R mutant was transiently transfected into HEK293 cells. The transfectants were grown in regular cell culture condition for 48h. Then the cells were collected and subject to immunoblot analysis. Meanwhile, wt-EGFR or EGFR-K3R was transfected into MCF7 breast carcinoma cells. The stable cell clones were selected and pooled for further analysis.

To compare EGFR activation between wt-EGFR and EGFR-K3R in normal growth environment, we grew both HEK293 and MCF7 transfectants for expression of either wt-EGFR or EGFR-K3R in normal DMEM with 10% FBS. The cells were

immunoblotted for analyzing EGFR tyrosine phosphorylation. Our result demonstrated that tyrosine phosphorylation of EGFR-K3R was significantly reduced compared with wt-EGFR (Figure 14). As the data will be shown later, TSA or SAHA increased EGFR phosphorylation. Considering that CBP increased EGFR phosphorylation and the knockdown of CBP significantly reduced EGFR phosphorylation (Figure 8), we concluded that EGFR acetylation affected its tyrosine phosphorylation. In addition, compared with wt-EGFR, EGFR-K3R significantly reduced its acetylation by CBP as showed in Figure 8C, further supporting that EGFR acetylation is associated with its tyrosine phosphorylation. These data indicate an intrinsic correlation between phosphorylation and acetylation on EGFR.

3.1.9 EGFR acetylation augments cell growth and DNA synthesis

To observe whether EGFR acetylation is indeed affected cellular function, we generated MCF7 stable clones and performed a series of cell-based function assays. *In vitro* cell growth result showed that acetylation-deficient EGFR-K3R significantly lost its ability to stimulated cell growth compared with wt-EGFR (Figure 15), although the expression of EGFR was actually similar to each other. Together, suggesting that acetylation status of EGFR but protein expression level affected cell growth.

Meanwhile, MCF7 stable cell clones were used for investigating whether EGFR acetylation affected DNA synthesis. The results show that acetylation-deficient EGFR-K3R mutant significantly reduced its ability to stimulate cell DNA synthesis in compared with wt-EGFR (Figure 16). Together, these results suggest that EGFR acetylation could significantly affect its ability to regulate cell growth and DNA synthesis.

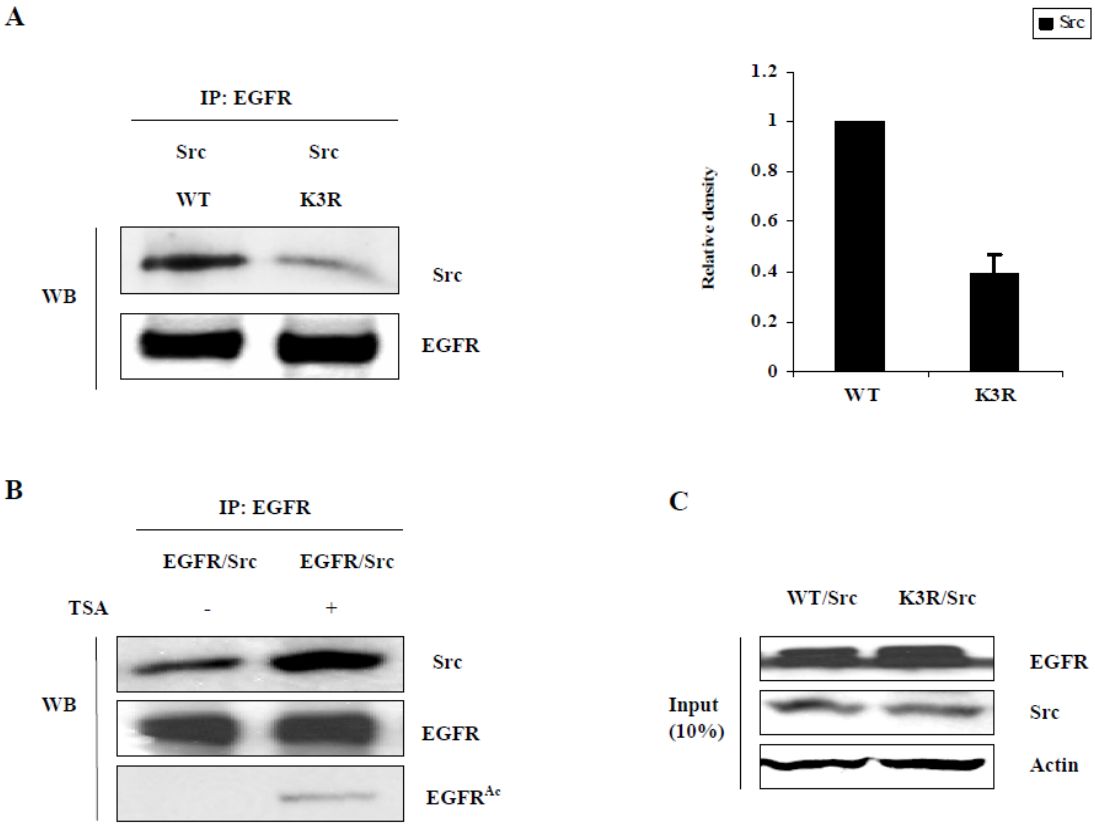


Figure 13 Protein interaction between EGFR and Src

Either wt-EGFR or EGFR-K3R was cotransfected with Src into HEK293 cells. The transfectants were pre-treated by 20 μM TSA for 5h. A total of 500 μg cell lysate was immunoprecipitated and immunoblotted with antibodies as indicated. A. IP: EGFR. B. IP: EGFR C. input control.

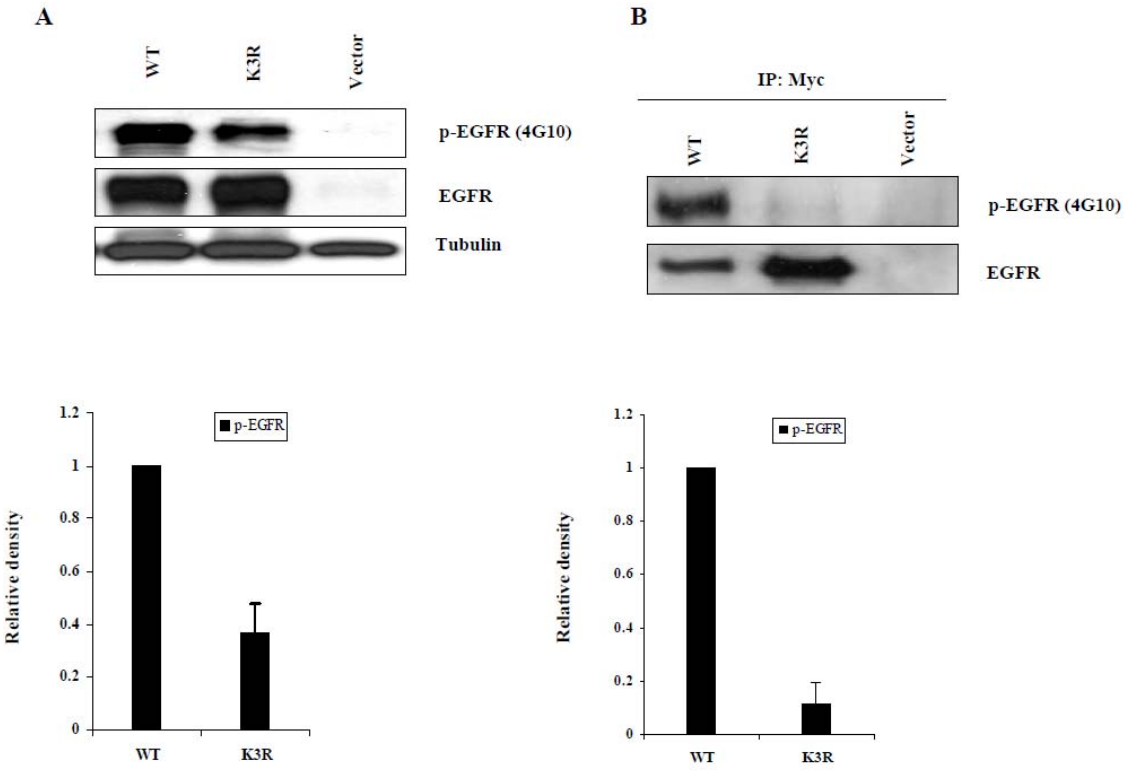
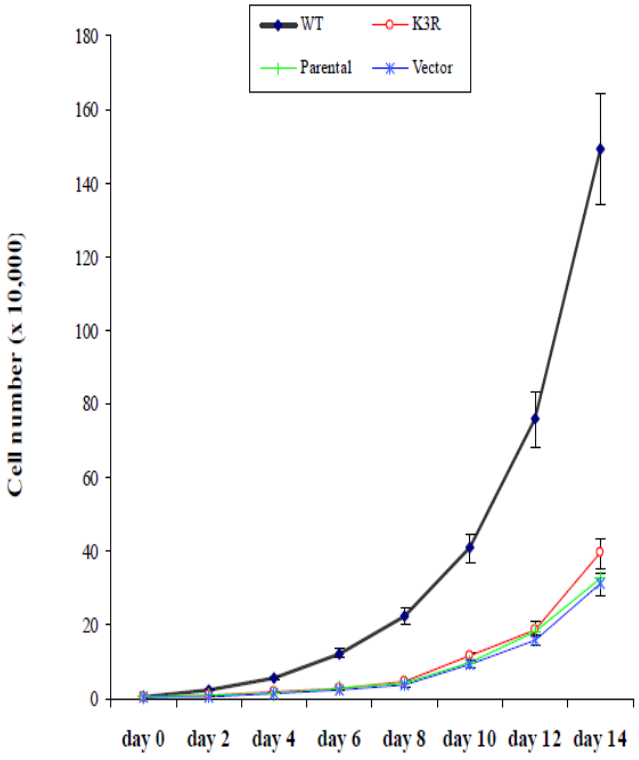
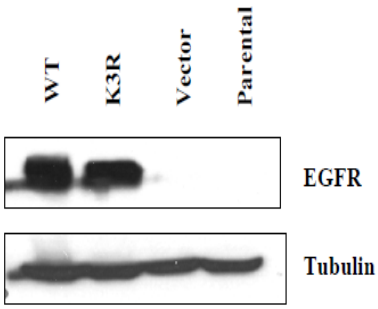


Figure 14 Acetylation-deficient EGFR-K3R impairs phosphorylation of EGFR

wt-EGFR or K3R mutant was transfected into different cells. The transfectants were culture for 48h in DMEM containing 10% FBS prior to collection. A. HEK293 cells. A total of 20μg of protein lysate was used for immunoblot analysis and probed with antibodies as indicated. B. MCF7 stable cell clone. Immunoprecipitation and immunoblot were performed by using antibodies as indicated. The quantitation of protein levels were normalized by densitometry and calculated as mean ± SD based on at least three independent data sets.

A


WT
K3R
Parental
Vector
Cell number (x 10,000)
180
160
140
120
100
80
60
40
20
0
day 0
day 2
day 4
day 6
day 8
day 10
day 12
day 14


WT
K3R
Vector
Parental
EGFR
Tubulin

B

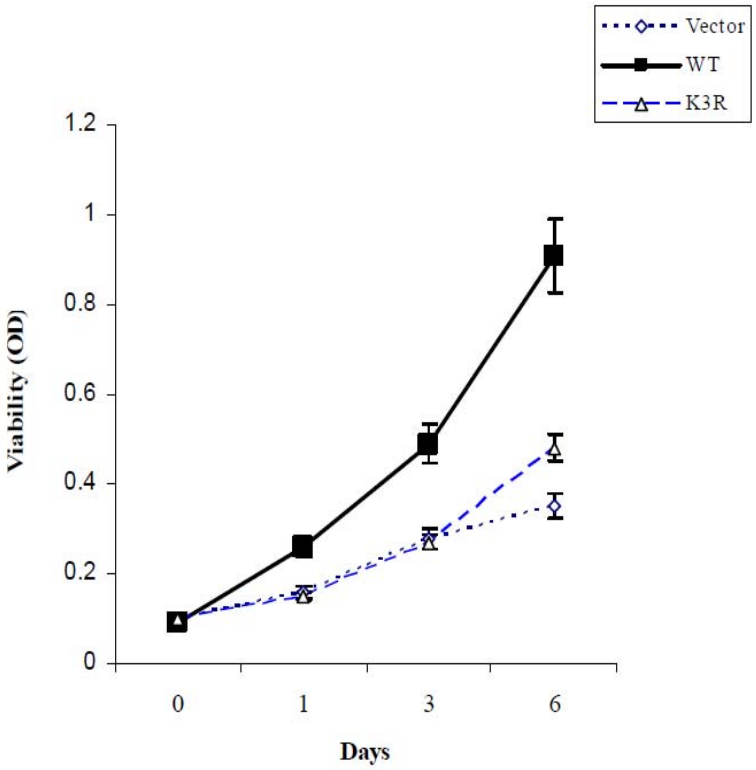


Figure 15 EGFR acetylation is associated with enhanced cell growth

For function assays, MCF7 cell stable clones that express wt-EGFR or EGFR-K3R were established and used to perform the following function assays. A. $1x10^4$ MCF7 parental cells or transfectants were plated into 6-well plates and subjected to cell number counting at different time points as indicated. The expression levels of wt-EGFR and EGFR-K3R were showed as well. B. a total of 1,000 cells were seeded into 96-well culture plates. MTT assay was performed.

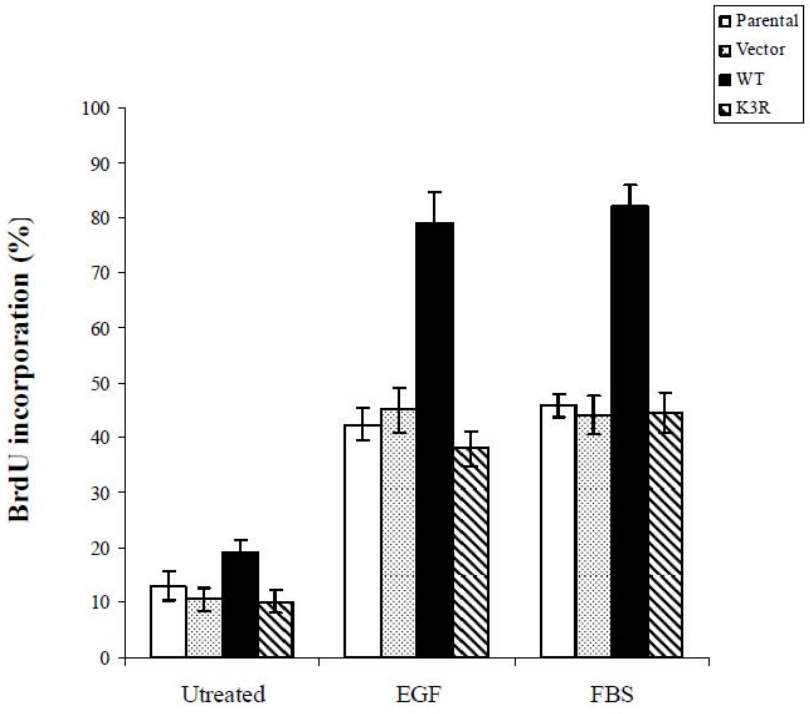


Figure 16 EGFR acetylation is associated with increased cell DNA synthesis

A total of $5x10^5$ MCF7 parental cells or stable transfectants were seeded into 6-well culture plates and cultured to reach 90% confluence. The cells then were serum-starved for 36h and treated with BrdU for 18h prior to assay. All data were calculated as mean ± SD and statistically analyzed by ANOWA and student's t-Test. $P<0.05$ is considered as statistical significance.

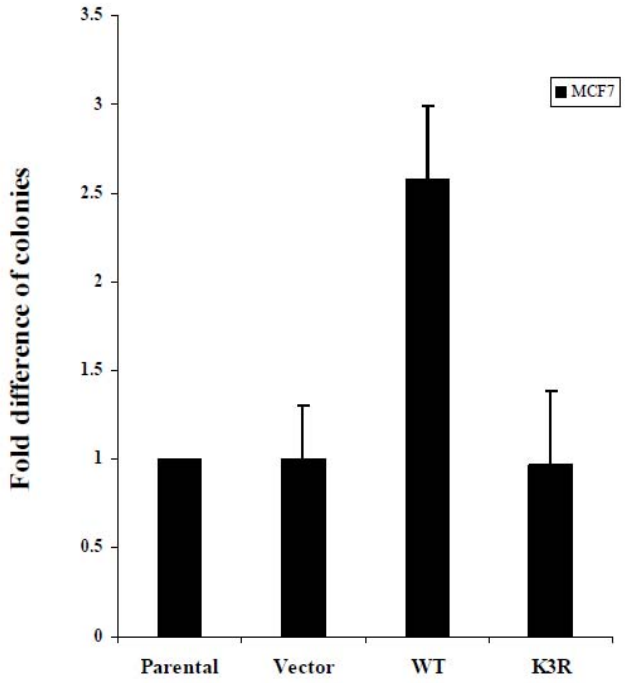


Figure 17 EGFR acetylation is associated with increased cell anchorage independent growth

A total of $5x10^4$ MCF7 parental cells or transfectants were placed on the top soft agar gel in 6-well plates and cultured for 2-3 weeks. The cell colonies were calculated as described in materials and methods. All data were calculated as mean ± SD and statistically analyzed by ANOWA and student's t-Test. $P<0.05$ is considered as statistical significance.

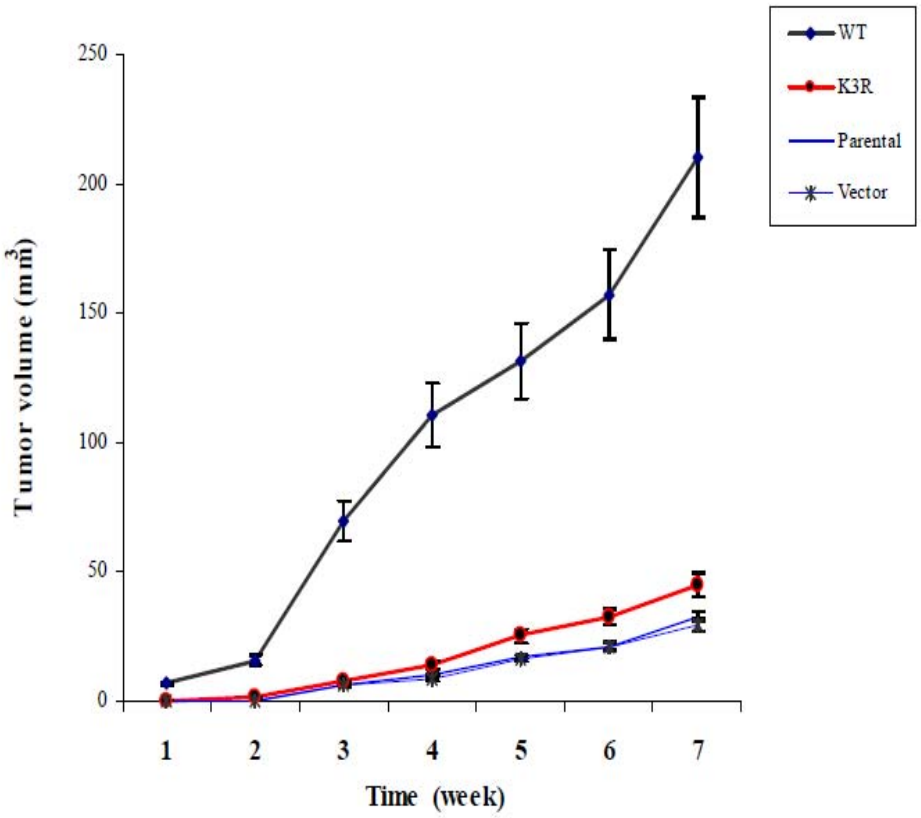


Figure 18 EGFR acetylation is associated with increased cell tumorigenesis *in vivo*

MCF7 stable clone cells for expressing EGFR or EGFR-K3R were inoculated into mammary fat pads of nude mice. The tumor size was measured by a standard method as stated in materials and methods section. All data were calculated as mean ± SD and statistically analyzed by ANOWA and student's t-Test. $P<0.05$ is considered as statistical significance.

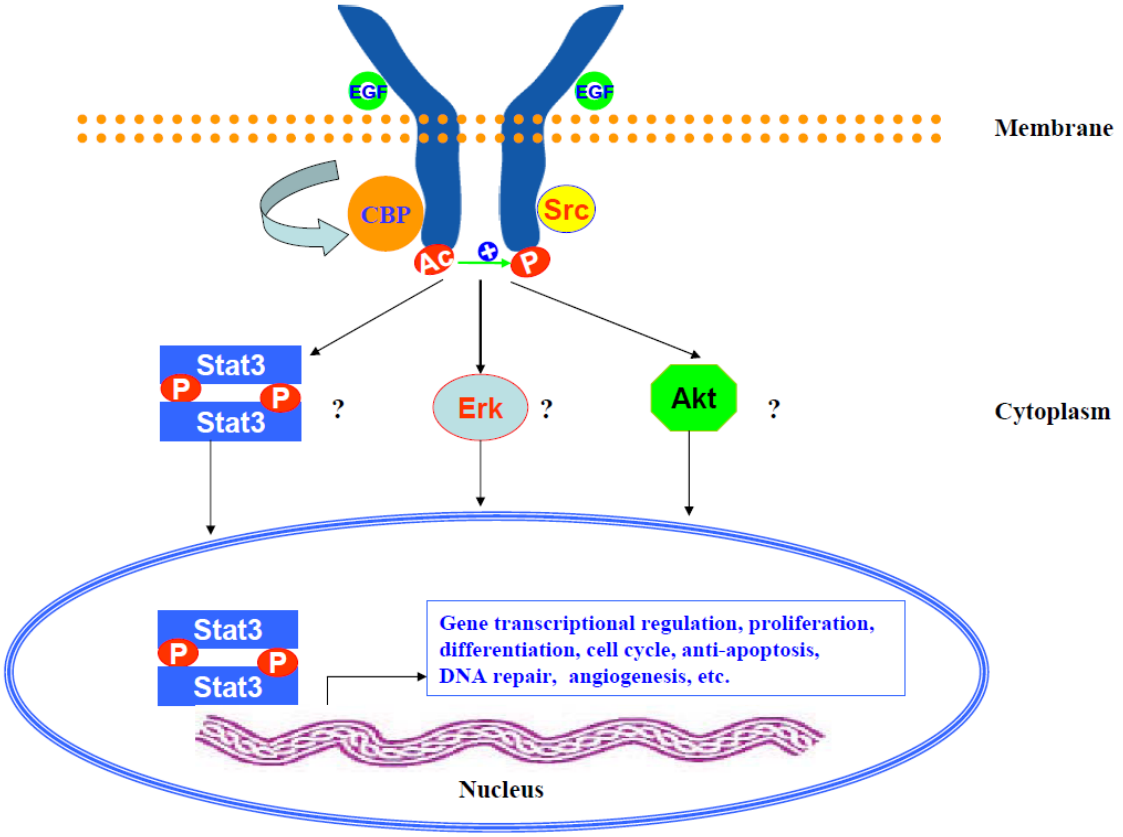


Figure 19 Proposed model for EGFR acetylation-induced cell growth and tumorigenesis

Upon EGF stimulation, EGFR is acetylated by CBP, which resulted in the increase of Src interaction and EGFR phosphorylation. These reactions may cause initiation of EGFR activation and subsequently activate EGFR downstream signal pathways.

3.1.10 EGFR acetylation is associated EGFR-induced tumorigenesis

To further study whether EGFR acetylation could impact cell tumorigenesis, we performed soft agar assay to observe cell anchorage independent growth by using MCF7 stable cell clones. The result demonstrated that EGFR acetylation-deficient mutant lost its ability to augment EGFR-induced cell anchorage independent growth in the presence of either EGF or FBS as shown in Figure 17.

3.1.11 EGFR acetylation is linked with EGFR-induced tumor burden

To investigate whether the impairment of EGFR acetylation could possible affect its oncogenic function *in vivo*, we generated MCF7 stable clones which could be used for *in vivo* animal tumor growth. On the basis of observations above, the loss of function of acetylation-deficient EGFR-K3R in stimulating cell growth, DNA synthesis, and anchorage independent growth could consequently result in the reduction of its tumorigenic ability. In this regard, we further investigated whether EGFR-K3R could support breast cancer MCF7 cell to grow tumor *in vivo* by using orthotopic animal model. MCF7 stable clones for expression of either wt-EGFR or EGFR-K3R mutant were injected into mammary fat pads of nude mice and tumor sizes were measured. As data showed in Figure 18, EGFR-K3R almost completely lost its ability to stimulate breast cancer MCF7 cells to grow tumor in nude mice. Together, our *in vitro* and *in vivo* results supported that EGFR acetylation might play an essential role in regulating cell growth, DNA synthesis, and tumorigenesis.

3.1.12 Proposed model

Based on our observations and knowledge from the current literature (4, 21, 22), we proposed a model for illustrating a possible mechanism by which EGFR acetylation

regulates cell growth and tumorigenesis. As schematic diagram shown in Figure 19, in response to ligand stimulation, CBP is rapidly recruited to close proximity of EGFR in the cytoplasm and then acetylates EGFR. The acetylated EGFR then enhances Src interaction with EGFR and together substantially augments EGFR phosphorylation. The augmented EGFR phosphorylation subsequently turns on EGFR-regulated downstream signal pathways and results in facilitated cell growth and tumorigenesis.

3 .2 Clinical implications

3.2.1 EGFR interacts with HDAC6

A431 cell lysate was immunoprecipitated by an anti-EGFR monoclonal antibody and immunoblotted by an anti-HDAC6 antibody. The result showed that EGFR interacted with HDAC6. In reciprocal immunoprecipitation, the similar result was observed (Figure 20). Together, suggesting that EGFR and HDAC6 are physically associated with each other inside a cell that overexpresses EGFR.

3.2.2 HDAC6 is overexpressed in various cancer cells

To examine whether HDAC6 expression is linked with cancers, we collected various types of cancer cell lines. The cells were cultured with DMEM containing 10% FBS except HBE4 cells and then lyzed for immunoblot analysis. The result showed that all cell lines expressed different levels of HDAC6 protein (Figure 21). However, cancer cells expressed much higher levels of HDAC6 compared with non-cancerous cells. For example, breast cancer cells MDA-MB-468 and lung cancer cells A549 exhibited high expression levels of HDAC6. In addition, it has been reported that HDAC6 is required for cell transformation and tumorigenesis (234). Together, the data

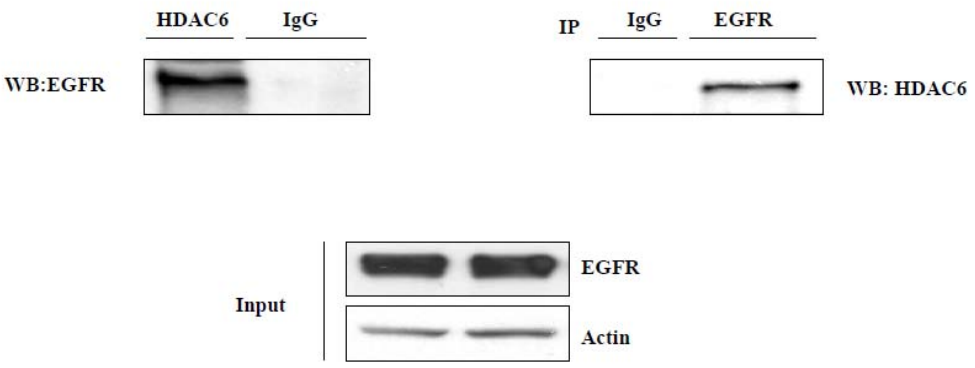


Figure 20 The interaction between EGFR and HDAC6 is associated with EGFR-expressing cancer cells

A total of 500 μg A431 cell lysate was immunoprecipitated and immunoblotted with antibodies as indicated.

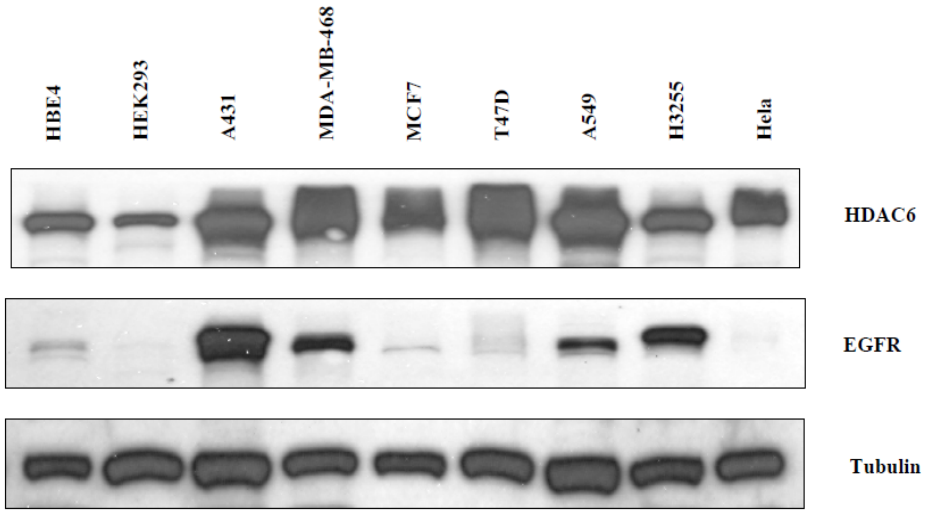


Figure 21 HDAC6 is highly expressed in various cancer cells

The different cancer cell lines were lysed and the cell lysates were subject to immunoblot analysis using antibodies as indicated.

suggest that HDAC6 may be associated with cancers. Although not all cancer cells with high level of HDAC6 express significant higher level of EGFR, the cancer cells with high EGFR expression generally exhibit high level of HDAC6, implying that there is a correlation between HDAC6 and EGFR in the cell tumorigenic processes.

3.2.3 EGFR-bearing cancer cells resist to HDAC inhibitors

A431 cells were treated with SAHA for 72h and MTT assay was performed to determine the survival rate of the treated cells. Unexpectedly, we found the SAHA had minimal effect on growth and survival of A431 cells that express high level of EGFR (Figure 22A). Immunoprecipitation and immunoblot analysis demonstrated that SAHA-treated A431 cells showed an increase in EGFR acetylation. By contrast, nicotinamide, a class III HDACi, had minimal effect on EGFR acetylation (Figure 22B).

To examine whether SAHA induced abnormal cell growth in A431 cells is general phenomenon in subtype of EGFR-expressing cancers, we use another EGFR-expressing MDA-MD-468 breast cancer cells. The cells were treated with SAHA for 72h and MTT assay was performed to determine the survival rate of the treated cells. The similar result was observed (Figure 23A). In contrast to this result, MDA-MB-453 breast cancer cells with low level of EGFR were sensitive to SAHA treatment (Figures 22A and 23A). To investigate individual EGFR acetylation site upon SAHA treatment, we generated an antibody that was against lysine 843 acetylation on EGFR. Immunoblot analysis demonstrated that SAHA-treated MDA-MB-468 cells showed higher level of EGFR-K843 acetylation (Figure 23B). Taken together, these result show that SAHA-enhanced EGFR acetylation may be associated with HDACi resistance in the treatment of EGFR-expressing cancers.

3.2.4 Knockdown of EGFR renders cancer cells sensitive to SAHA

To further understand the role of EGFR acetylation in SAHA-induced phosphorylation and resistance to SAHA treatment, we examined the effects of SAHA on the regulation of EGFR modification in high EGFR-expressing MDA-MB-468 and A431 cancer cells. As data showed in Figure 22 and 23, SAHA induced EGFR acetylation and phosphorylation that resulted in cell resistance to SAHA treatment in A431 and MDA-MB-468 cells. We also asked if the deletion of EGFR from these cells could re-sensitize the cells to respond to SAHA treatment. The knockdown of EGFR by siRNA could make the cells to lose EGFR-dependent survival pathway, therefore, the cancer cells may become sensitive to SAHA treatment. As expected, our result demonstrate that the knockdown of EGFR by siRNA rendered the cells sensitive to SAHA by using SAHA-resistant MDA-MB-468 cells (Figure 24). Together, these data suggest that EGFR is indeed involved in SAHA resistance in MDA-MB-468 cells.

3.2.5 Overexpression of EGFR renders cancer cells resistant to SAHA

To further prove that EGFR will allow cells to regain resistance to SAHA, we overexpressed EGFR in MDA-MB-453 breast carcinoma cells to study whether the cells could become resistant to SAHA treatment. MDA-MB-453 cells are originally very sensitive to SAHA treatment and express very low level of EGFR that is virtually undetectable. After introducing EGFR into the cells, MDA-MB-453 cells became much more tolerant to SAHA treatment as showed in Figure 25, suggesting that EGFR was indeed contributed to SAHA resistance.

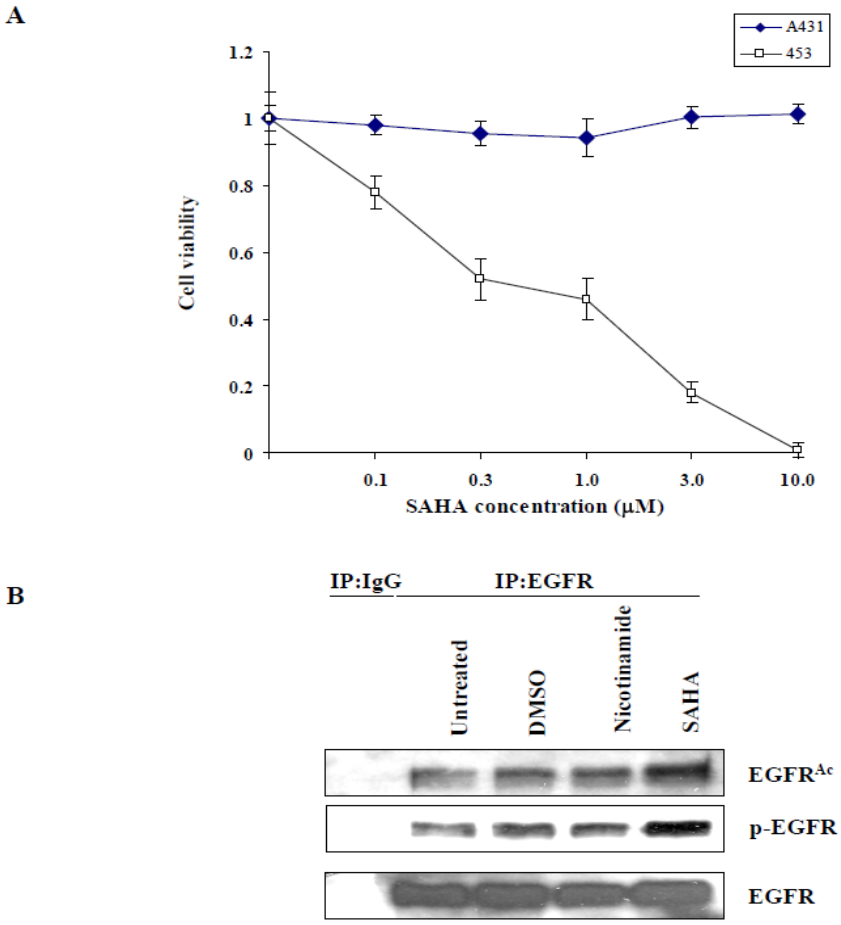


Figure 22 Acetylation of EGFR is associated with cancer cell resistance to HDACi

A. A431 or MDA-MB-453 cells were treated with SAHA for 72h as doses indicated. B. A431 cells were serum-starved then treated with 5 μM SAHA or 4 mM Nicotinamide for 5h prior to collecting cells. Then, immunoprecipitation and immunoblot were performed using antibodies as indicated.

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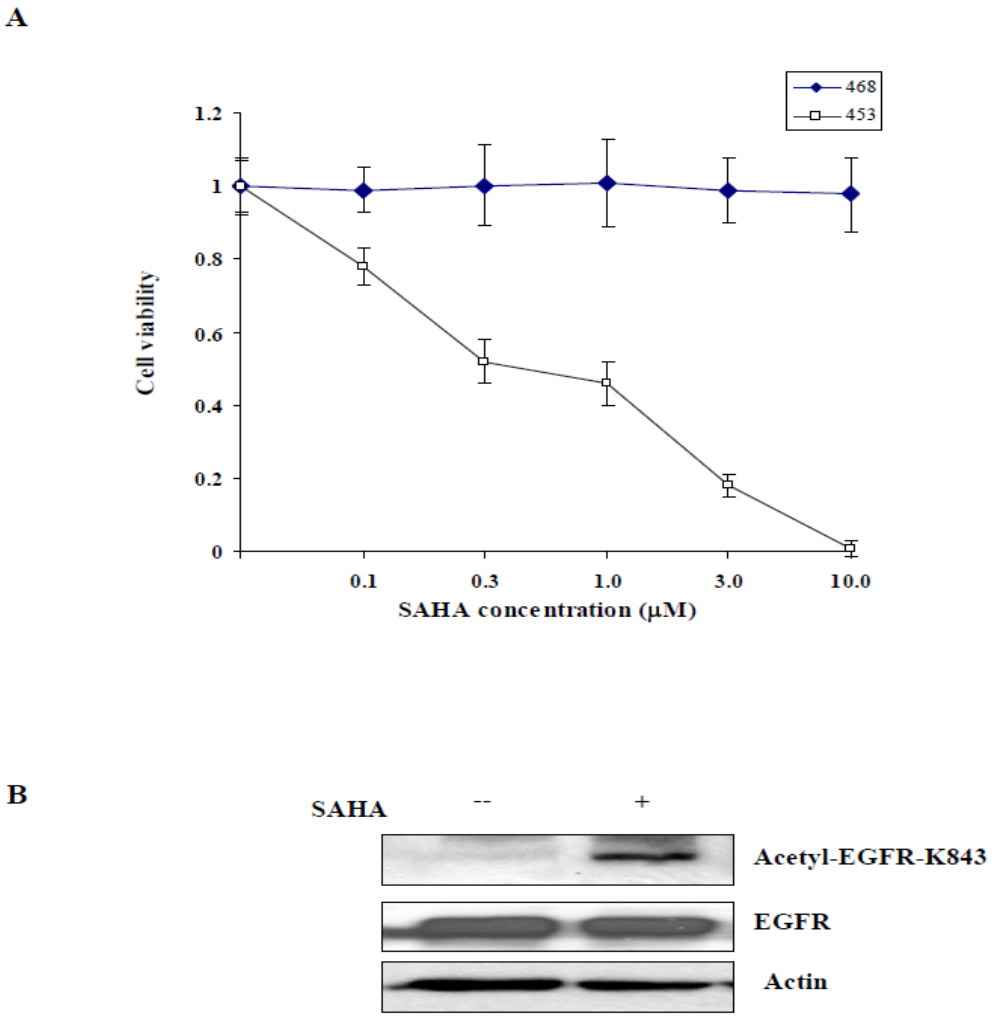

Figure 23 Acetylation of EGFR is associated with cancer cell resistance to HDACi

A. MDA-MB-468 or MDA-MB-453 cells were treated with SAHA for 72h with different concentrations as indicated. B. MDA-MB-468 cells were treated with 5 μM SAHA for 24h. Then, immunoblot was performed using antibodies as indicated.

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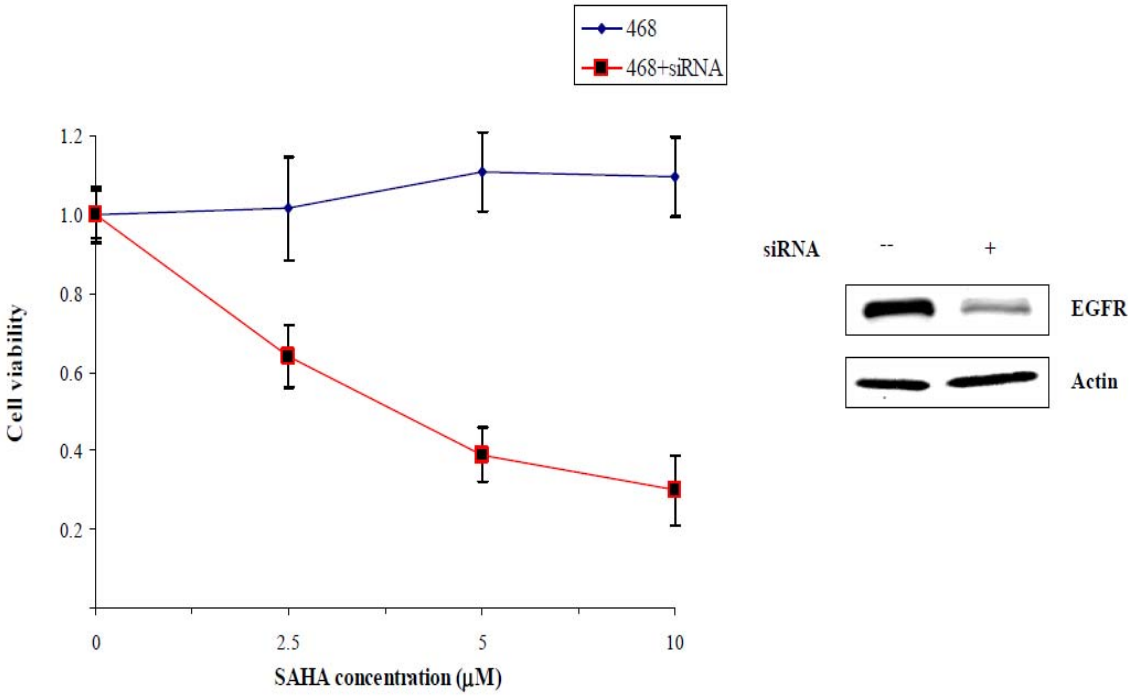

Figure 24 Knockdown of EGFR renders tumor cells sensitive to SAHA

siRNA for knockdown of EGFR was transfected into MDA-MB-468 cells by electroporation. After 72h, the cells were treated with SAHA as indicated concentrations. MTT assay was performed for measuring cell viability. All data were calculated as mean ± SD and statistically analyzed by ANOWA and student's t-Test. $P<0.05$ is considered as statistical significance

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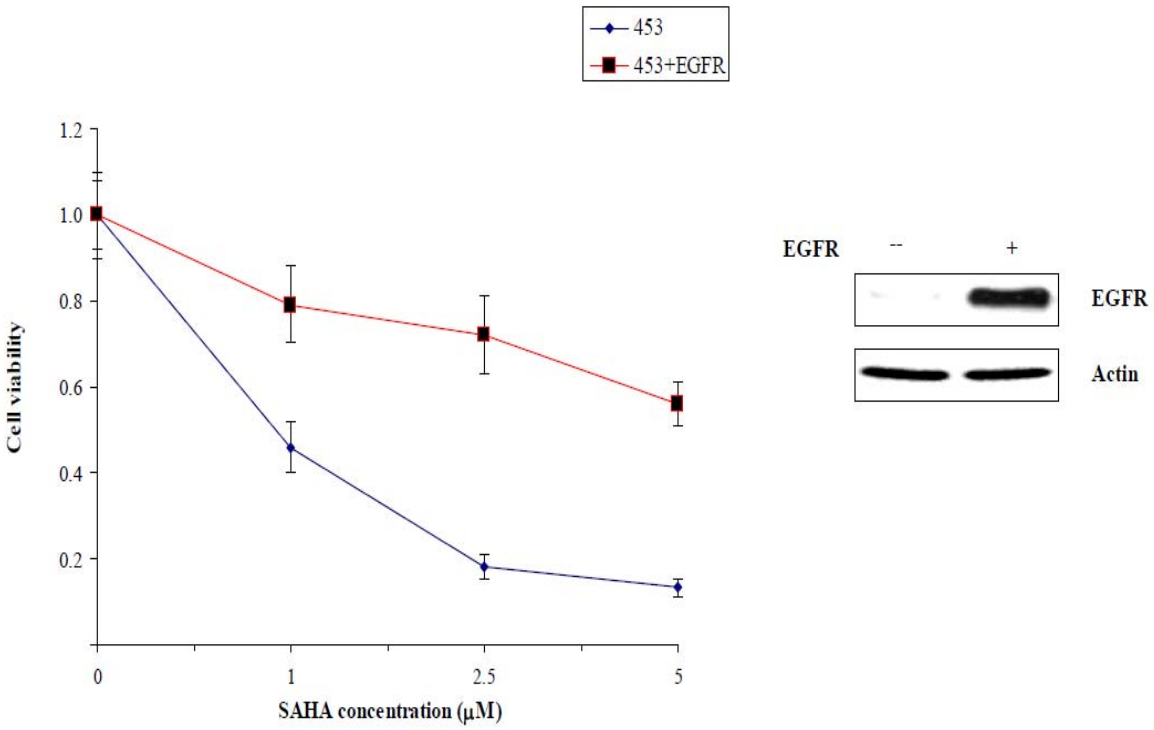

Figure 25 Overexpression of EGFR renders tumor cells resistant to SAHA

wt-EGFR for expression of EGFR was transfected into MDA-MB-453 cells. The stable cells were pooled and the cells were treated with SAHA as indicated concentrations. MTT assay was performed for measuring cell viability. All data were calculated as mean ± SD and statistically analyzed by ANOWA and student's t-Test. $P<0.05$ is considered as statistical significance

(The figure 25 is adapted with permission from Elsevier Ltd)

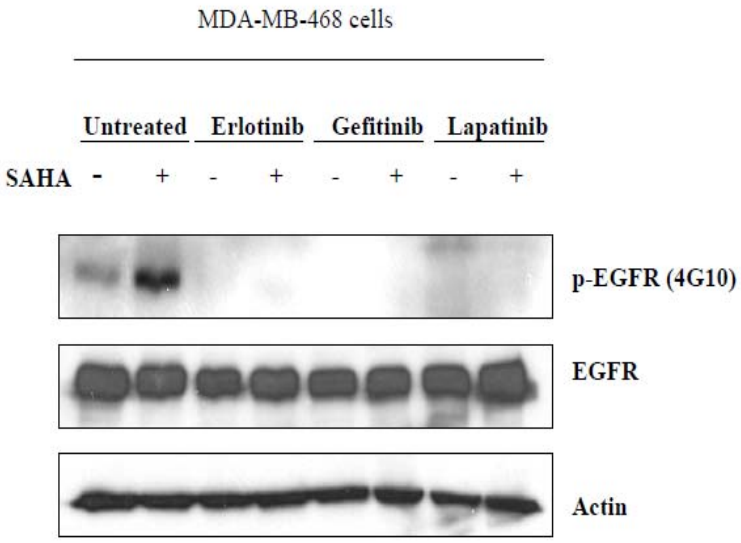

Figure 26 SAHA augments EGFR tyrosine phosphorylation in breast cancer cells

MDA-MB-468 cells were treated with 2.5 μM SAHA and/or 10 μM erlotinib or gefitinib or lapatinib for 24h prior to collection. The cell lysates were resolved on 8% SDS-PAGE and probed with antibodies as indicated.

3.2.6 SAHA-induced EGFR phosphorylation is linked with resistance

To understand the mechanism by which SAHA enhances cancer cell growth, we performed Western blot analysis using MDA-MB-468 breast cancer cells and found that SAHA indeed augmented EGFR phosphorylation, suggesting that SAHA-enhanced breast cell growth and survival were, at least in part, due to SAHA-enhanced EGFR phosphorylation that activated downstream survival pathway (Figure 26). Our observation is also consistent with literature report (179). These observations suggest that SAHA has an opposite effect due to the enhancement of EGFR phosphorylation that contributes to tumor cell survival and growth. Given the fact that SAHA enhances phosphorylation of EGFR and could possibly augment tumor cell survival, we rationally considered that SAHA-enhanced EGFR phosphorylation is associated with protein acetylation, because SAHA as a HDAC inhibitor generally preserves protein acetylation. As shown in Figures 22B and 23B, SAHA-treated A431 cells and MDA-MB-468 cells all demonstrate elevated levels of EGFR acetylation. Combined the data from Figures 22, 23, 24, 25, and 26, all of these evidence supported that EGFR acetylation-enhanced phosphorylation contributed to SAHA resistance in the cancer cells that overexpress EGFR. These results suggested that SAHA-enhanced EGFR acetylation might be correlated with EGFR phosphorylation that contributed cancer cells to resist to SAHA treatment as a result.

3.2.7 SAHA treatment and cell cycle change

To study cell cycle changes after the treatment of SAHA, MDA-MB-468 cells were treated by SAHA for 72h as doses indicated. The results showed that SAHA induced cell cycle changes that mainly arrested in G2/M phase (Figure 27). With increasing

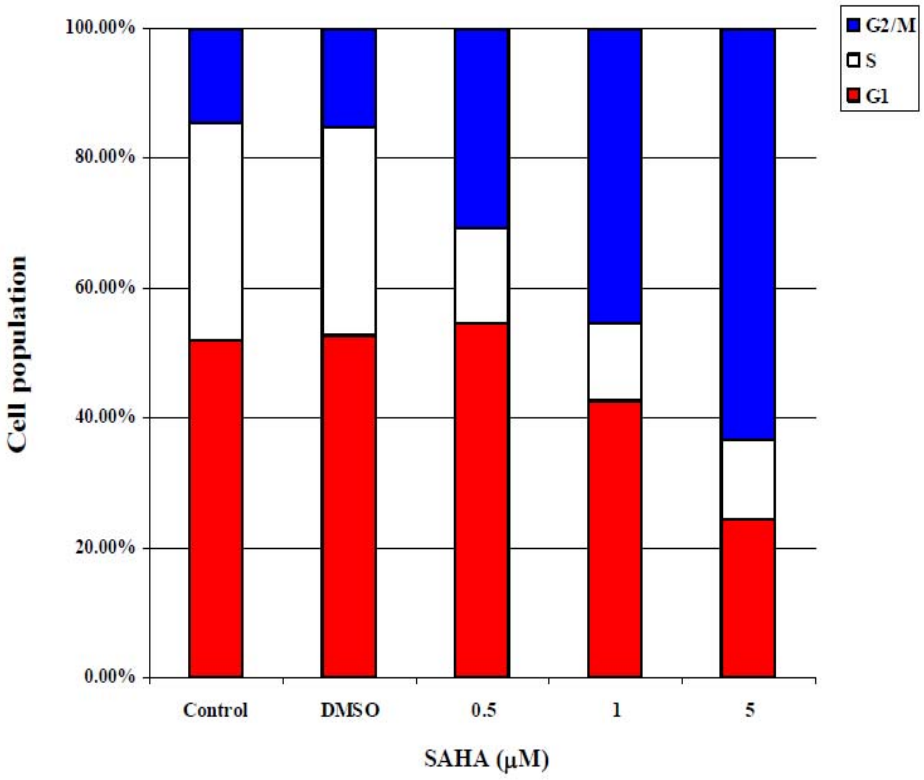

Figure 27 Cell cycle change after SAHA treatment

MDA-MB-468 cells were treated with SAHA for 72h and stained with PI. The cells were analyzed by flow cytometry.

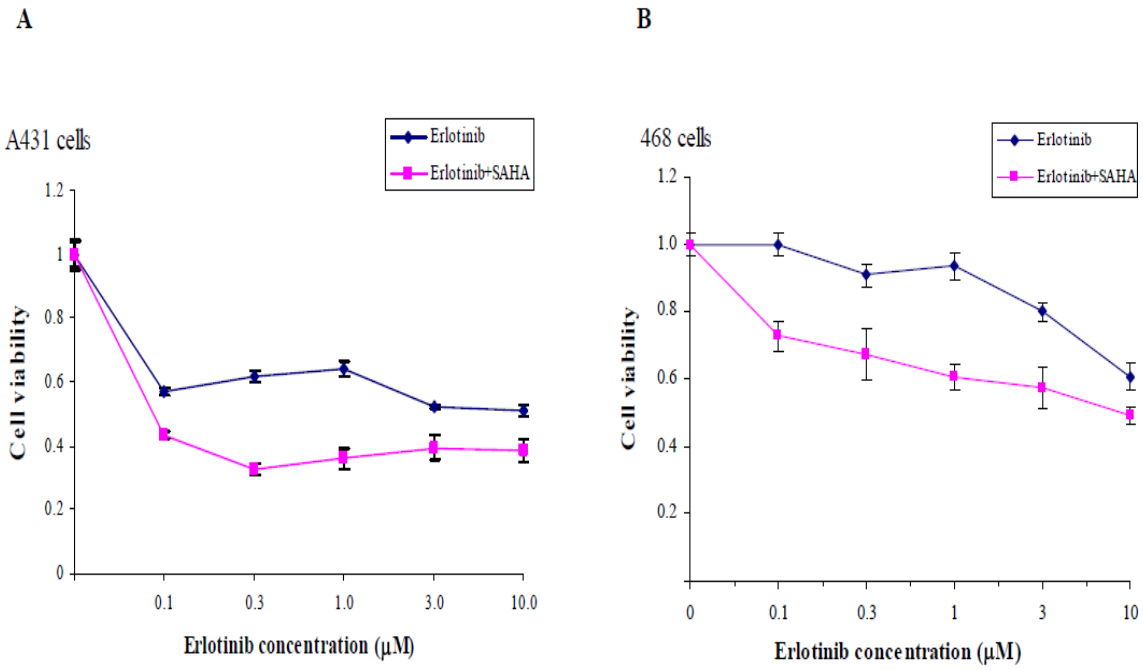

Figure 28 Combination of SAHA and EGFR inhibitors offsets cancer resistance to SAHA

An MTT Assay was performed by plating $1x10^4$ cells into 96-well plates. The cells were cultured for 72h in the presence of indicated TKI and/or SAHA. All treatments were set up at least as triplets. The data represent three independent experiments. The cells were treated with 2.5 μM SAHA in combination of erlotinib with indicated concentrations for 72h. A. A431 cells were treated with erlotinib with or without SAHA. B. MDA-MB-468 cells were treated with erlotinib with or without SAHA. All data were calculated as mean ± SD and statistically analyzed by ANOWA and student's t-Test. $P<0.05$ is considered as statistical significance.

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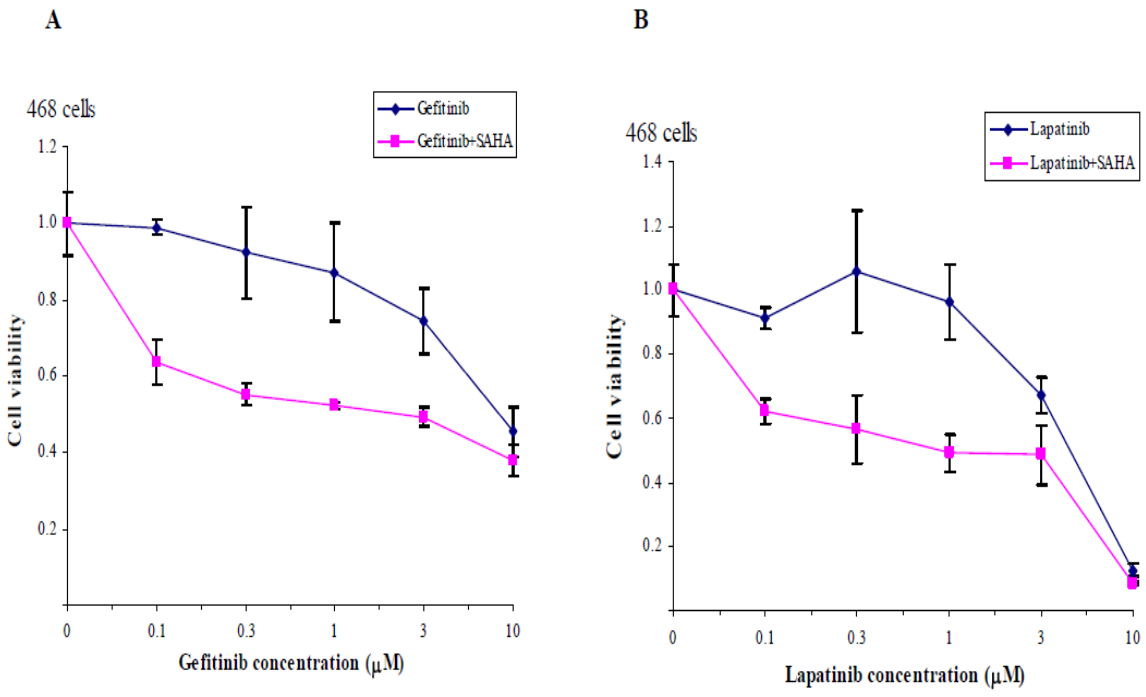

Figure 29 Combination of SAHA and EGFR inhibitors offsets cancer resistance to SAHA

An MTT Assay was performed by plating $1x10^4$ MDA-MB-468 cells into 96-well plates. The cells were cultured for 72h in the presence of indicated TKI and/or SAHA. All treatments were set up at least as triplets. The data represent three independent experiments. The cells were treated with 2.5 μM SAHA in combination of either gefitinib or lapatinib with indicated concentrations for 72h. A. the cells were treated with gefitinib with or without SAHA. B. the cells were treated with lapatinib with or without SAHA. All data were calculated as mean ± SD and statistically analyzed by ANOWA and student's t-Test. $P<0.05$ is considered as statistical significance.

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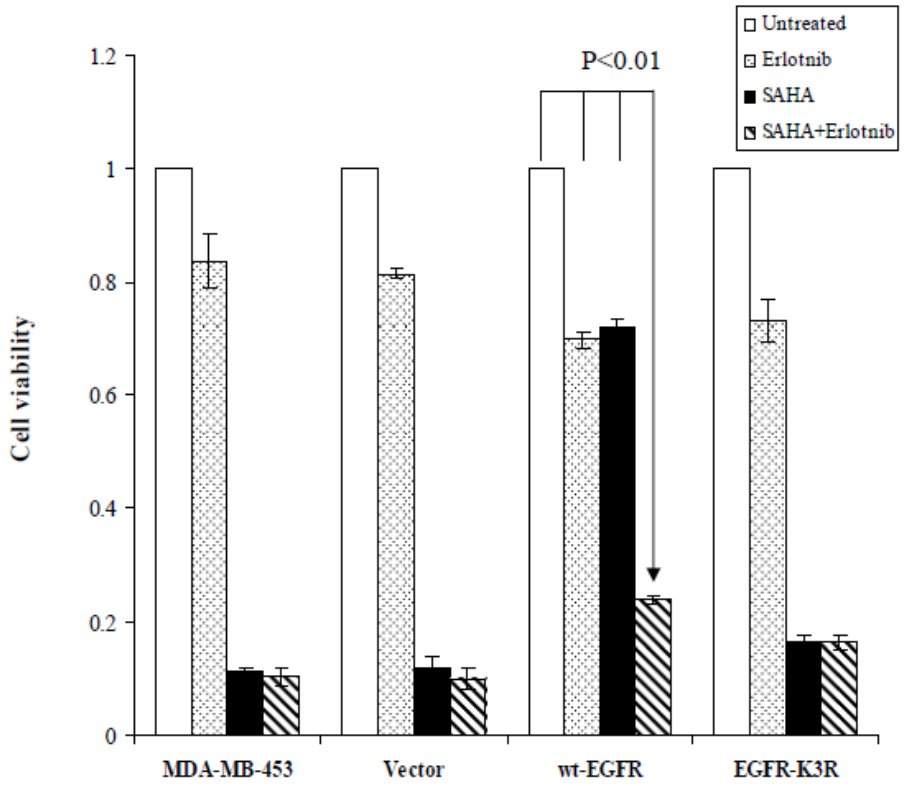

Figure 30 Acetylation-deficient EGFR-K3R mutant abrogates SAHA-induced resistance

Either wt-EGFR or EGFR-K3R was transfected into MDA-MB-453 cells. After expression and selection with corresponding antibiotics, MTT Assay was performed by plating $1x10^4$ cells into 96-well plates and MDA-MB-453 cells were cultured for 72h in the presence or absence of indicated TKI and/or SAHA. All treatments were set up at least as triplets. The data represent three independent experiments. For combination therapy, the cells were treated with 2.5 μM SAHA in combination of 10 μM erlotinib for 72h. All data were calculated as mean ± SD and statistically analyzed by ANOWA and student's t-Test. $P<0.05$ is considered as statistical significance.

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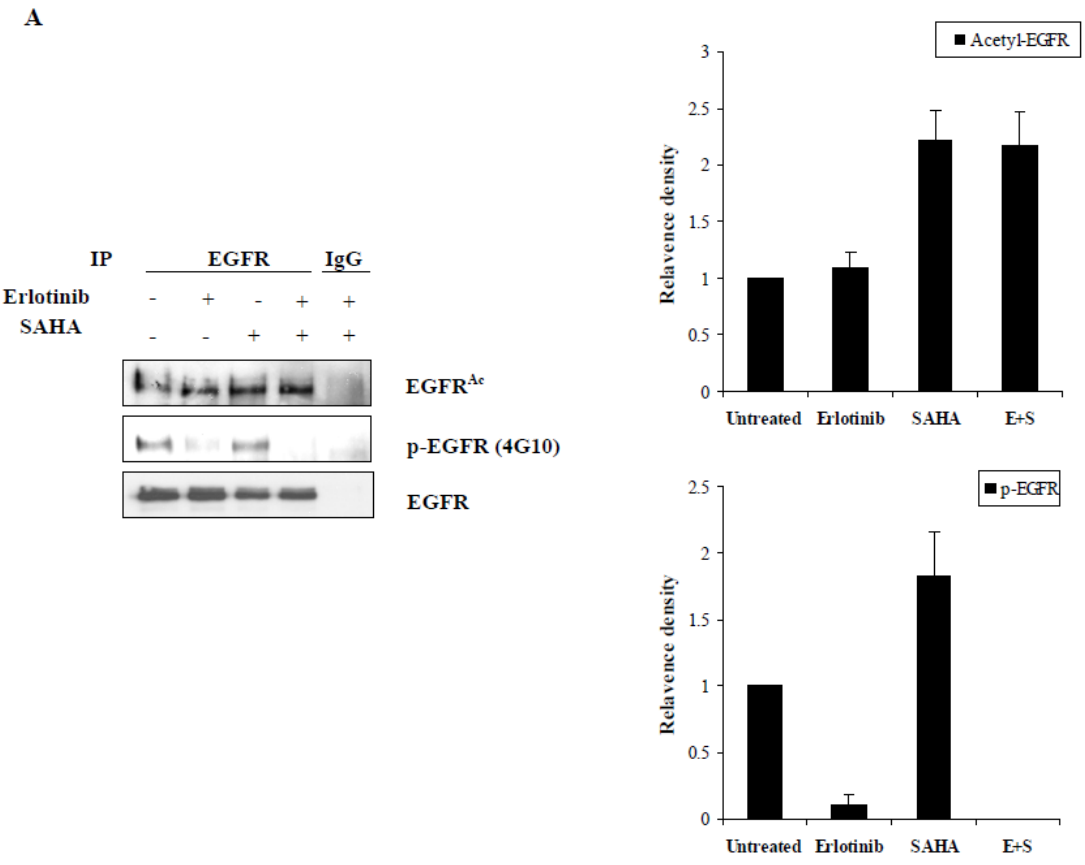
A
IP
EGFR
IgG
Erlotinib
SAHA
EGFR^Ac
p-EGFR (4G10)
EGFR
Acetyl-EGFR
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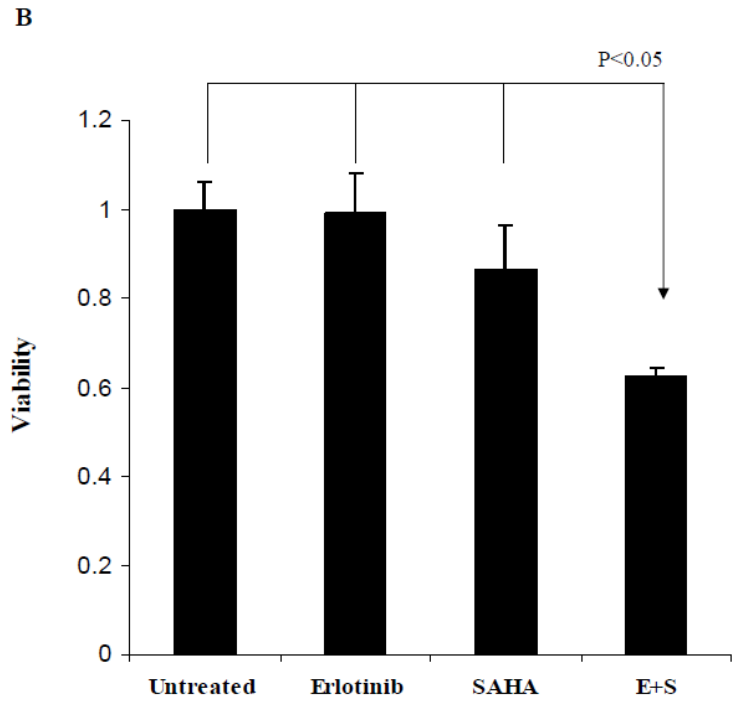
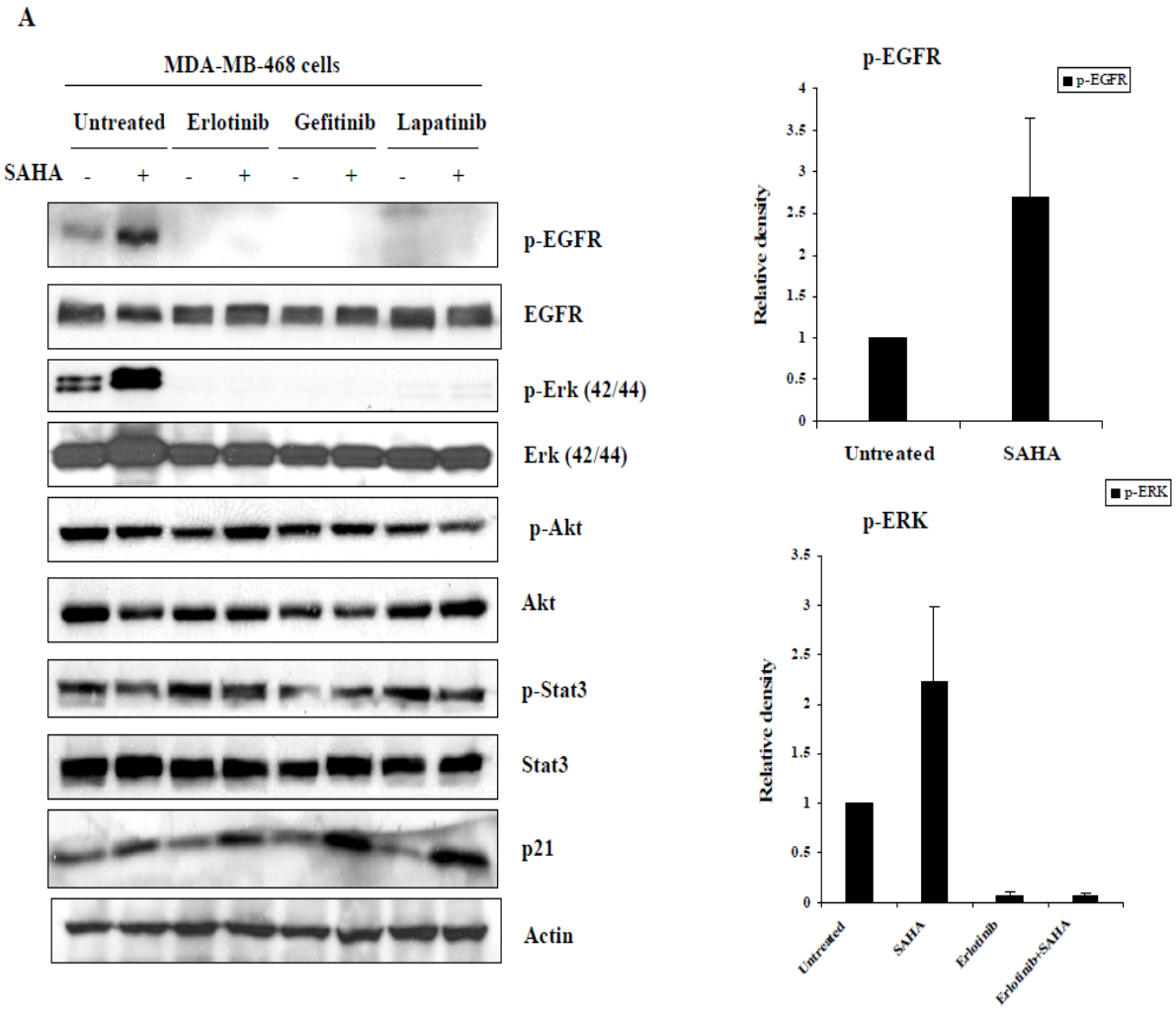


Figure 31 Acetylation of EGFR may be related to HDACi-mediated sensitization to TKI

A. T47D cells were treated with 5 μM SAHA and/or 10 μM erlotinib for 24h prior to collection. Immunoprecipitation was performed and the immunocomplexes were resolved on 8% SDS-PAGE and probed with antibodies as indicated. B. T47D cells were treated with 5 μM SAHA and/or 10μM erlotinib for 72h prior to MTT assay. E+S: erlotinib and SAHA.


A
MDA-MB-468 cells
Untreated
Erlotinib
Gefitinib
Lapatinib
SAHA - + - + - + - +
p-EGFR
EGFR
p-Erk (42/44)
Erk (42/44)
p-Akt
Akt
p-Stat3
Stat3
p21
Actin
p-EGFR
p-EGFR
Relative density
Untreated
SAHA
p-ERK
p-ERK
Relative density
Untreated
SAHA
Erlotinib
Erlotinib+SAHA

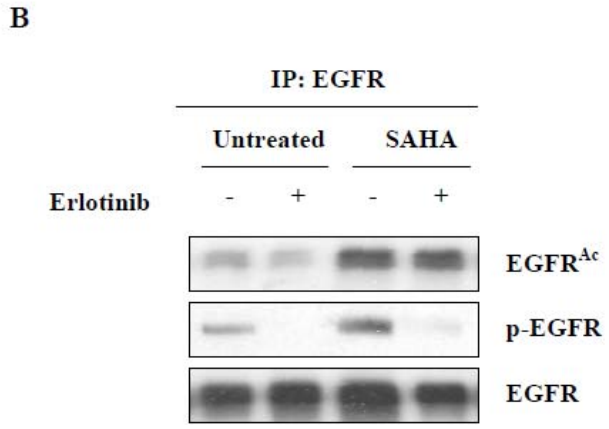


Figure 32 The signal pathways affected by combining SAHA and TKI in breast cancer cells

MDA-MB-468 cells were treated with SAHA 2.5 μM SAHA and/or 10 μM erlotinib or gefitinib or lapatinib for 24h. A. The cell lysates were resolved on 8% SDS-PAGE and probed with antibodies as indicated. The quantitation of protein levels were normalized by densitometry and calculated as mean ± SD based on at least three independent data sets. B. The cell lysates from MDA-MB-468 cells were used for immunoprecipitation analysis. Western blot was performed by using antibodies as indicated.

SAHA concentration, more cells were accumulated at G2/M phase. All these data were consistent with literature reports.

3.2.8 SAHA sensitizes tumor cells to respond to TKI *in vitro*

Since SAHA-enhanced EGFR acetylation induces its phosphorylation, we rationalize that EGFR signaling pathways may become dominant survival factors. The blockade of EGFR may potentially benefit for targeting cancer cells that express EGFR based on oncogene addiction concept (235-237). Therefore, we tested the combination of different TKI with SAHA. A431 cells or MDA-MB-468 cells were treated with SAHA and/or erlotinib as doses indicated for 72h, and then cell viability was measured by MTT assay. The results demonstrated that the combination of erlotinib and SAHA showed more effective killing in both EGFR-expressing cancer cells compared with erlotinib alone (Figure 28). In addition, we tested different TKI to treat EGFR-expressing cancer cells. MDA-MB-468 cells were treated using SAHA with either gefitinib or lapatinib as doses indicated for 72h. The similar results were observed as showed in Figure 29. In addition, SAHA significantly lowered TKI dosage in the treatment of these EGFR-expressing cancer cells, especially in low dose ranges, SAHA demonstrated higher sensitization effects on cancer cells treated with TKI. Taken together, our observations demonstrate that the combination of both HDAC and TKI could significantly benefit for cancer therapy in cancer cells that overexpress EGFR.

To further investigate whether EGFR acetylation is associated with SAHA-induced resistance, we transfected wt-EGFR or EGFR-K3R into MDA-MB-453 cells. After 48h expression, the cells were selected with blasticidin for two weeks. The survival cells were then pooled for MTT assay. The results showed that only wt-EGFR could render SAHA-sensitive MDA-MB-453 cells resistant to SAHA but not acetylation deficient

EGFR-K3R mutant (Figure 30). Acetylation-deficient EGFR-K3R mutant did not have ability to help the cells to gain resistance to SAHA. These data further suggested that acetylation of EGFR may link with SAHA resistance. Combined the data that we observed previously, the augmented EGFR acetylation by SAHA consequently enhanced phosphorylation of EGFR, which may be responsible for SAHA resistance in EGFR-expressing cancer cells.

In addition to using EGFR overexpressing cancer cell lines, we also employed low EGFR-expressing breast cancer cell line T47D to investigate how the cells responded to SAHA. T47D cells are insensitive to both TKI and SAHA treatment overall. As shown in Figure 31, SAHA treatment increased EGFR acetylation as well as EGFR tyrosine phosphorylation (Figure 31A). There was no effect of erlotinib on EGFR acetylation as shown in Figure 31A. As a result, T47D cells became to respond to erlotinib treatment and showed about 40% killing effect in combination with SAHA and erlotinib (Figure 31B). This phenomenon may be also explained by oncogene addiction concept.

To understand the underlying molecular mechanism by which cancer cells were sensitized to respond to TKI in SAHA-treated cells that overexpress EGFR, MDA-MB-468 cells were treated using SAHA with either erlotinib or gefitinib or lapatinib for time and doses indicated. As expected, SAHA increased EGFR phosphorylation in MDA-MB-468 cells (Figure 32A). Consistent with previous observation, SAHA but not erlotinib increased EGFR acetylation in MDA-MB-468 cells (Figure 32B). In addition, our observations suggest that Erk as a major downstream signal molecule was affected. In other words, MAPK signaling pathway was involved in SAHA-induced sensitization to TKI treatment in MDA-MB-468 breast cancer cells (Figure 32A). In addition, p21 protein expression was significantly increased upon the combination therapy. These observations are consistent with reports in the literature (208, 209).

3.2.9 SAHA sensitizes cancer cells to respond to TKI *in vivo*

To further prove that combination therapy is indeed the best option for the treatment of cancers that overexpress EGFR, we performed *in vivo* animal study. MDA-MB-468 cells were injected into mammary fat pads of nude mice. After tumor grew to designated sizes, the treatment was initiated. A single drug or combination was administrated by oral daily. The tumor sizes were measured twice a week. The result showed that the combination of SAHA and erlotinib significantly reduced tumor burden in mice compared with either SAHA or erlotinib alone (Figure 33). Either SAHA or TKI alone was less efficient to reduce tumor growth. These data indicated that the combination of SAHA and TKI actually achieved significant inhibition of tumor growth *in vivo*, which might be attributed to SAHA-induced EGFR phosphorylation that triggered sensitization of cancer cells to TKI. In addition, Western blot results demonstrated that SAHA alone could not inhibit Erk activity. However, combined SAHA and TKI inhibited breast cancer MDA-MB-468 cell growth through downregulating Erk phosphorylation (Figure 32). Together, suggesting that the sensitization effect might be attributed to SAHA-activated Erk that resulted in high response to TKI that mainly targeted EGFR/Erk signaling pathway in EGFR-expressing cancers. All in all, our *in vitro* cell based data and animal study suggest that SAHA had potentials that could sensitize EGFR-expressing cancer cells to respond to TKI. This sensitization action triggered by SAHA rendered TKI more effective to kill cancer cells that express EGFR.

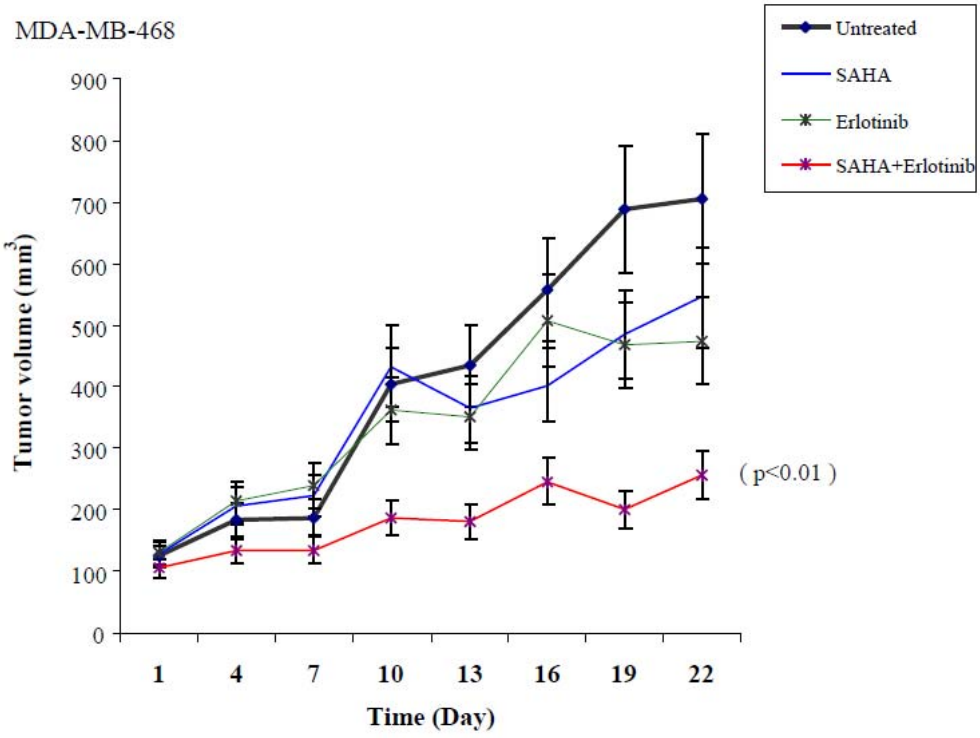


Figure 33 The combination of SAHA and TKI increases therapeutic efficacy in the treatment of breast cancer in an orthotopic animal model

MDA-MB-468 cells were injected into nude mice through mammary fat pad injection. After the tumors grew to designated size, the drugs were administrated by oral daily. The tumor volume was measured twice per week as indicated. All data were calculated as mean ± SD and statistically analyzed by ANOWA and student's t-Test. $P<0.05$ is considered as statistical significance.

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CHAPTER 4 DISCUSSION, SUMMARY, AND FUTURE DIRECTIONS

Discussion

Protein phosphorylation is the well understood field of protein posttranslational modifications (PTM), in which there are well-established models to study protein function in cellular regulation (65, 123, 145, 147, 148, 151, 189). As we knew, protein phosphorylation is the most extensively studied filed . Almost all aspects of cellular function and regulation are involved in protein phosphorylation. For example, cell membrane protein regulation, signaling transduction, cell cycle regulation, DNA repairs, transcription and translation, protein stability and degradation, and etc (147, 148, 189). Accordingly, a variety of proteins need to be phosphorylated before functioning. The switch and balance between protein phosphorylation and dephosphorylation control and dictate protein function and stability (123, 151). The signaling transduction dictated by protein phosphorylation is well-established area that demonstrated that protein phosphorylation is a key and central even which is responsible for signaling cascades and protein function. The loss of even a single protein phosphorylation could deviate entire signaling pathway and result in severe consequence including tumorigenesis and genetic deficiency (65, 148, 151). From the cell surface to the nucleus, there is no exception that protein phosphorylation is essential for protein regulation and function. The cell membrane proteins such as tyrosine kinase receptors need to be phosphorylated upon ligand stimulation. Only after phosphorylation event occurs in membrane protein, membrane proteins could possibly form different structure and generate signaling cascades for cell cycle change and cell growth. Even membrane protein turnover, phosphorylation is also an essential step for protein degradation and trafficking. The cytoplasmic proteins mainly play a role in transferring cellular signals into the nucleus. Although some cytoplasmic proteins could be directly shuttled into the nucleus and interact with transcription factors to

regulate DNA replication, RNA transcription, and protein translation, however, the majority of cytoplasmic proteins still acts through phosphorylation cascades that pass signals to downstream molecules. The MAPK, Akt, and Stat signal pathways are good examples to illustrate this scenario. As far as transcription and nuclear proteins, they play a role in finally executing surface signals through the regulation of nuclear function that is in the center of the cells.

It has been shown that the majority of protein methylation and acetylation occurs in histone proteins that regulate transcription and translation. Recently, more and more studies have found that many non-histone proteins are also methylated and acetylated including transcription factors and membrane proteins (167, 238). For example, p53 was reported to subject to methylation and acetylation modification that played an important role in regulating its protein stability and function (239-242). Another report demonstrated that membrane interferon-α receptor 2 was acetylated by CBP in response to interferon-α ligand stimulation (228). These findings show that not only histone proteins but also most of cellular proteins are subjected to methylation and acetylation, which likely play a role in regulating cell behavior. The recent discovery of various non-histone proteins as acetylation substrates (124, 144, 228, 243, 244) provides plausible evidence that protein acetylation may be critical for cellular processes and signal transduction (124, 228, 245). The involvement of protein acetylation in cellular processes has widely spread in almost every aspect of the cells including membrane, organelles, cytoplasm, and the nucleus (124). Therefore we raise a question: whether does protein acetylation have its own signaling pathway or just simply participate in protein phosphorylation-regulated signaling pathway? This question remains elusive. The gain of insights about this question may help us to further understand how proteins

interact with one another and how protein codes will be marked and regulated. To this end, understanding protein acetylation may be able to help us to develop more effective therapeutic drugs to treat related diseases.

In our study, we demonstrated that EGFR could be acetylated by CBP with knockdown and overexpression approaches. Three acetylation sites in EGFR were identified. We also observed that EGF rapidly triggered CBP to shuttle out the nucleus and approach to EGFR in close proximity of cell membrane. This observation was similar to previously reported that INF-α triggered CBP moved from the nucleus into the cytoplasm and then acetylated INF-αR2 (228). Although CBP is mainly located in the nucleus, however, there is small percentage of CBP located into the cytoplasm, which may explain basal levels of EGFR acetylation prior to EGF stimulation. In fact, the EGFR overexpressing cells exhibit relative high level of EGFR acetylation without ligand stimulation that may be attributed to the remains of CBP in the cytoplasm. However, it is unclear what role EGFR acetylation plays in this stage. One possibility is that acetylation of EGFR prior to ligand stimulation may be responsible for initiation of EGFR phosphorylation and dimerization. Since lysine 836 and 843 are physically close to Y845, which is one of reported Src phosphorylation sites (233). We speculate that the triad formed by K836, 843 and Y845 may be an important structure for recruiting Src to initiate EGFR activity. Several reports about EGFR structure and function demonstrated that EGFR membrane structure but not ligand-induced tyrosine phosphorylation was critical and essential for initiating EGFR dimerization and activation (16, 226, 246). The mutation of these critical structure sites could abrogate EGFR activation. These reports seem consistent with our observations. As far as the role of Src in EGFR activation and function, there are a few reports in which

demonstrated different observations and results. One report showed that Src was required for Y845 phosphorylation and autophosphorylation at Y1068 on EGFR. Src inhibitor reduced Y845 phosphorylation and Y1068 autophosphorylation (188). These observations suggested that Src might be required for EGFR transphosphorylation, autophosphorylation, activity, and function. However, there is another seemingly controversial report in which showed that Src was not required for EGFR kinase activity (187). In that report, the authors demonstrated that although Src-phosphorylated Y845 on EGFR was required for regulating cell growth, DAN synthesis, and tumorigenesis, Y845 phosphorylation status had nothing to do with EGFR kinase activity. In comparison of wt-EGFR and EGFR-Y845F mutant, the kinase activity was similar to each other in an *in vitro* kinase assay. Interestingly, there is a report in which even showed that the position and structural proximity of the key amino acids in enzymatic pocket but not tyrosine phosphorylation on EGFR was responsible for the initiation of EGFR dimerization and activation (16). It seems that there was no a common ground with respect to how to explain the initiation of EGFR activity. Therefore, the questions are raised as follows: 1. what event is the first step and critical for EGFR activation, dimerization or phosphorylation? 2. As Src is the only currently known kinase that tyrosine phosphorylates EGFR, is there a role of Src in activating EGFR? 3. What kind of relationship is among Src-associated EGFR transphosphorylation, autophosphorylation, and EGFR kinase activity? There are no convincing answers yet based on the literature that we can find about EGFR study. However, the further understanding of these issues may be important for decoding EGFR acting mechanisms and targeting EGFR-associated diseases including cancers.

Broadly speaking, PTM of protein usually affect protein stability, protein trafficking, and protein-protein interaction. In our study, in comparison of wt-EGFR

and acetylation-deficient EGFR-K3R mutant, the protein stability and trafficking were not affected. The difference that we observed was protein-protein interaction. Several critical proteins such as Src showed weak interaction with EGFR-K3R mutant. An average of 50% reduction of Src protein binding was observed in EGFR-K3R mutant compared with wt-EGFR. Considering the loss of tyrosine phosphorylation in EGFR-K3R, there may have some intrinsic links between acetylation and phosphorylation of EGFR. The cellular study results suggest that EGFR acetylation affected its phosphorylation and protein interaction, which proved by EGFR-K3R mutant and HDACi treatment. These observations may be further evidence that EGFR acetylation was important for the initiation of EGFR activation through regulating its phosphorylation. In addition, the treatment of EGFR-expressing cells with TKI did not change EGFR acetylation level, which may suggest that EGFR acetylation occurs before its phosphorylation. However, further study is needed to demonstrate whether EGFR acetylation is essential for its phosphorylation. The understanding of dependency or causal relationship between EGFR acetylation and phosphorylation could be very useful for deciphering EGFR function and designing therapeutic strategies to target EGFR-associated cancers.

EGFR acetylation also played an important role in regulating EGFR/Src protein interaction. Based on our observations, we speculate that the acetylated EGFR may be required for the recruitment of Src. As a result of Src binding to EGFR, the activation of EGFR will be initiated. In other words, what EGFR acetylation enhances Src recruitment may be essential for the initial activation of EGFR. Our observation of which acetylation deficient EGFR-K3R lost EGFR kinase activity further supports this postulation (data not shown). In addition, considering that CBP-catalyzed EGFR acetylation consequently augmented EGFR phosphorylation, we consider that these

observations support the notion of which EGFR acetylation may be required for the initiation of EGFR activity.

HDACi have become a hot spot in therapeutic application recently, especially in cancer treatment. With HDAC class I and II inhibitor SAHA being approved for treating lymphoma by FDA, more HDACi are under developing and/or undergoing clinical trial (167, 169, 174). However, our observations also raise a safety issue about application of HDACi in EGFR-expressing diseases. On the basis of enhanced EGFR phosphorylation by SAHA-induced EGFR acetylation, it may be general phenomenon that HDACi could augment EGFR phosphorylation and then subsequently trigger EGFR activation and oncogenic function in the cancer cells expressing EGFR. The consequence may be severe. To avoid this unwanted problem, our results imply that TKI should be included in the intervention. Interestingly, HDACi can actually sensitize the cells to respond to TKI, which can be explained by oncogene addiction concept. Our *in vitro* and *in vivo* data all support that SAHA has sensitization effect on EGFR-expressing cancer cells. We considered what SAHA sensitized EGFR-expressing cancer cells to respond to TKI was a case that could be explained by oncogene addiction concept. According to this theory, in response to environment or therapeutic intervention, the affected cancer cells would adapt to survive by depending on a single intensified signal pathway, namely acquired dependency. Due to the increase of dependency to this surrogate survival pathway, the affected cells became more susceptible to the inhibitors that target this more demanding pathway. Therefore the sensitization effect can be achieved by the addition of inhibitors to block such a pathway. The expression of wt-EGFR but not acetylation-deficient EGFR resulted in the gain of resistance to SAHA in SAHA-sensitive cells, suggested that EGFR acetylation could be an important mechanism that caused cell resistance to SAHA.

The study on EGFR acetylation unveiled insights about mechanism(s) of possible acetylation-regulated phosphorylation on the cell surface tyrosine kinase receptors. Meanwhile, acetylation-augmented EGFR phosphorylation "has an important clinical implication, predicting that EGFR-expressing cancer cells may be resistant to a single HDAC inhibitor treatment" (223). One of the possible mechanisms is due to enhanced EGFR/Src interaction and/or acetylation-enhanced EGFR tyrosine phosphorylation. A therapeutic regimen of combined HDACi with TKI could take advantage of HDACi-induced EGFR phosphorylation, which makes EGFR more accessible and vulnerable to TKI according to the oncogene addiction concept. The underlying molecular mechanism may mainly act through Erk signal pathway in various cancer cells as reported in the literature (247-252). Although EGFR-regulated Akt and Stat signaling pathways were also involved in various cancer cells in the treatment of TKI, however, Erk pathway has always been involved in most cases. In addition, HDACi-enhanced phosphorylation of Erk was also reported in the literature recently (249). Consistent with this report, we also demonstrated that SAHA induced Erk phosphorylation in EGFR-expressing breast cancer cells. The HDACi-activated Erk pathway may provide a surrogate survival pathway for the cancer cells treated by HDACi alone to survive. Our observation suggest what SAHA sensitized cancer cells to respond to TKI was also mainly through down-regulation of Erk signaling. In the cells treated with SAHA, Erk phosphorylation was elevated. However, the elevated Erk was effectively inhibited by the addition of TKI, since TKI mainly target Erk signaling pathway (21, 253-259).

Currently, using deacetylase inhibitors as therapeutic drugs is an extensively explored field (126, 171, 174, 260, 261). There are more than 160 worldwide multi-center clinical trials (this number is increasing steadily) listed on the NCI website. Among those trials, more than 50% are involved in assessing SAHA for the treatment

of various cancers. The combination of SAHA and TKI is also included in those multi-center clinical trials. Since SAHA seems to be a very promising anti-cancer agent with the property of excellent bioavailability, low toxicity and few side effects. In addition, SAHA can be orally administrated that makes SAHA practically more attractive. However, there is safety issues emerged from EGFR-expressing cancers. Our observations by using SAHA *in vitro* and *in vivo* not only raised a safety issue about SAHA, but also we provided a possible explanation about the underlying mechanism. Our study provided an insight into direct involvement of EGFR acetylation in response to HDACi treatment to human cancers, which suggested that using these inhibitors alone could have adverse effects on EGFR-expressing cancers. In this scenario, the combination of HDACi and TKI may be a better option to prevent potential risks from patient treatment provided that HDACi regimen becomes necessary. Our *in vivo* animal study also supported that the combination of SAHA and TKI actually achieved better therapeutic outcome compared with an individual agent alone. These observations are consistent with reports in the literature (222). Given the fact of the potent pharmacological effect and increasing popularity of HDACi such as SAHA for cancer treatment, our observations suggested when using HDACi alone to treat EGFR-addicted cancers, caution should be taken due to the potential HDACi-enhanced EGFR oncogenic function and combination therapy may need to be considered accordingly.

Summary

EGFR, as an essential growth and survival factor, plays an important role in cancers of the lung, breast, brain, ovary, skin, and head and neck. The modification patterns of EGFR are critical for its function and the understanding of these EGFR modifications could help us to design the optimal therapeutic strategies for targeting various EGFR-

associated cancers and/or non-cancerous diseases. We observed that EGFR acetylation as a novel protein posttranslational modification might play a very important role in the early regulation of EGFR signaling pathways. We identified that CBP was an acetylase responsible for EGFR acetylation. Our data suggest that EGFR acetylation mainly affects its protein interaction. EGFR dimerization, protein stability, trafficking, and localization were not significantly different between wt-EGFR and acetylation-deficient EGFR-K3R mutant. Our study demonstrated that EGFR acetylation upregulated its phosphorylation and subsequently augmented cell growth and tumorigenesis.

We also expanded our study into translational field in order to understand the indication of EGFR acetylation in the clinical settings. As we demonstrated as before, what HDACi including SAHA treated EGFR-expressing cancer cells could subsequently gain resistance to HDACi due to the augmented EGFR phosphorylation. The combination of HDACi and TKI can effectively inhibited cell growth and tumorigenesis, due to the nature of that TKI could block the acetylation-augmented phosphorylation and activation of EGFR, while synergistically enhanced SHAH-induced p21 protein expression. In addition, we also demonstrated that MAPK signaling pathway was downregulated in this combination approach. Together these actions resulted in cancer cell death. Our study provides an experimental rationale that supports combination therapy of HDACi and TKI in the treatment of a subtype of cancers that overexpress EGFR.

Future Directions

There are still several unaddressed questions that need to be addressed regarding EGFR acetylation. First of all, our study provided evidence that elucidated EGFR acetylation was an early event prior to its phosphorylation. It has been reported that

more than 80 proteins interact with EGFR prior to activation, suggest that EGFR is actually involved in dynamic protein-protein interactions (208). It is of interest to understand how the initiation of EGFR activation occurs and how the protein interactions are regulated.

Second, acetylation-enhanced EGFR phosphorylation plays an important role in regulating cell growth and tumorigenesis. Besides these function, is there addition function with respect to EGFR acetylation? It is also of interest to understand how these modifications reconcile one another and together regulate EGFR function.

Third, it is still unclear how EGFR acetylation, autophosphorylation, and transphosphorylation by Src interact in EGFR activation and function. Little is known about the relationship of the kinase activity of EGFR with these modifications. However, the understanding of these questions can be very important for studying EGFR function and developing therapeutic strategies for targeting EGFR-expressing cancers.

Lastly, it is also very important to understand how EGFR acetylation is regulated. Is EGFR acetylation inducible? The insights about the regulation of EGFR acetylation will be helpful for clinical applications. For example, to guide physicians to design HDACi regimens, while to avoid adverse effects in the treatment of EGFR-expressing cancers.

In summary, the aberrant expression of EGFR is involved in various cancers. According to the literature, what the mutations and modifications of EGFR affect its function mainly acts through the regulation of EGFR phosphorylation and downstream signal pathways. Gaining knowledge of EGFR modifications could help us to better understand how cancer resistance occurs and develop alternative strategies to treat

theses cancers. Therefore, the elucidation of EGFR modifications could potentially benefit for the treatment of EGFR-related cancers.

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