

Extracellular Signal-regulated Kinase (ERK) Phosphorylates Histone Deacetylase 6 (HDAC6) at Serine 1035 to Stimulate Cell Migration*

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Background: HDAC6 plays an important role in cell migration.

Results: ERK interacts with and phosphorylates HDAC6 to promote cell migration.

Conclusion: ERK signaling pathway promotes cell migration, in part, through phosphorylating HDAC6.

Significance: Inhibition of HDAC6 activity as well as the EGFR-Ras-Raf-MEK-ERK signaling pathway may cooperatively reduce cell migration.

Histone deacetylase 6 (HDAC6) is well known for its ability to promote cell migration through deacetylation of its cytoplasmic substrates such as α -tubulin. However, how HDAC6 itself is regulated to control cell motility remains elusive. Previous studies have shown that one third of extracellular signal-regulated kinase (ERK) is associated with the microtubule cytoskeleton in cells. Yet, no connection between HDAC6 and ERK has been discovered. Here, for the first time, we reveal that ERK binds to and phosphorylates HDAC6 to promote cell migration via deacetylation of α -tubulin. We have identified two novel ERK-mediated phosphorylation sites: threonine 1031 and serine 1035 in HDAC6. Both sites were phosphorylated by ERK1 *in vitro*, whereas Ser-1035 was phosphorylated in response to the activation of EGFR-Ras-Raf-MEK-ERK signaling pathway *in vivo*. HDAC6-null mouse embryonic fibroblasts rescued by the non-phosphorylation mimicking mutant displayed significantly reduced cell migration compared with those rescued by the wild type. Consistently, the nonphosphorylation mimicking mutant exerted lower tubulin deacetylase activity *in vivo* compared with the wild type. These data indicate that ERK/HDAC6-mediated cell motility is through deacetylation of α -tubulin. Overall, our results suggest that HDAC6-mediated cell migration could be governed by EGFR-Ras-Raf-MEK-ERK signaling.

Histone acetyltransferases and histone deacetylases (HDACs)³ are enzymes involved in modifying core histones through the addition or removal of an acetyl group from the lysine residue on histone tails. Acetylation of core histones usually results in transcriptional activation, whereas deacetylation of core histones causes transcriptional repression (1, 2). Apart from histones, it is well known that HDACs also modify nonhistone proteins which in turn regulate the functionality of those proteins (3). HDACs are grouped into four classes based on their similarity to yeast homologs and the usage of cofactors. They are class I (HDACs 1, 2, 3, and 8), class II (HDACs 4, 5, 6, 7, 9, and 10), class IV (only HDAC11), and the silent information regulator 2 (sir2)-related family which represents the class III HDACs consisting of sirtuins 1–7 (1, 2, 4). The classes I, II, and IV of HDACs require Zn²⁺ to be active, whereas the class III HDACs bearing no sequence similarity to class I, II, and IV need NAD⁺ as a cofactor (2). The class I HDACs are ubiquitously expressed in many cell types and are found almost exclusively in the nucleus. However, the expression pattern of class II HDACs is more restricted, and they are able to shuttle between the nucleus and the cytoplasm in response to cellular signals (1, 5–7). HDAC6 which belongs to the class II HDACs is unique among HDACs in that it is the only HDAC that contains a complete duplication of the large class I/II HDAC homology domain. HDAC6 consists of both a nuclear export signal located in the N terminus and a cytoplasmic anchorage motif known as SE-14 motif in the C terminus (8, 9) and a zinc finger motif, which binds to mono- and polyubiquitin chains (9, 10). HDAC6 is recognized for deacetylating cytoplasmic proteins,

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³ The abbreviations used are: HDAC, histone deacetylase; DN, dominant negative; EGFR, epidermal growth factor receptor; MEF, mouse embryonic fibroblast; TDAC, tubulin deacetylase.

including α -tubulin (11, 12), cortactin (13), and HSP90 (14). HDAC6 associates with microtubules through its HDAC domain and is capable of deacetylating α -tubulin at lysine 40 both *in vivo* and *in vitro* (12). It is generally believed that deacetylation of microtubules and cortactin by HDAC6 influences microtubule-dependent and actin-dependent cell motility, respectively (11, 13).

Recently, phosphorylation sites within HDAC6 as well as kinases that are responsible for phosphorylating these sites have started to emerge. For instance, glycogen synthase kinase 3 β has been reported to phosphorylate the serine 22 site located in the N terminus of HDAC6 (15). It has been suggested that glycogen synthase kinase 3 β enhances HDAC6 deacetylase activity toward α -tubulin (15). HDAC6 can also be phosphorylated by Aurora A kinase, a centrosomal kinase involved in regulating mitotic entry (16). Phosphorylation of HDAC6 by Aurora A enhances the ability of HDAC6 to deacetylate acetylated α -tubulin to promote ciliary disassembly, but the phosphorylation site for this kinase remains to be identified (16). Recently, the G protein-coupled receptor kinase 2 has also been shown to phosphorylate HDAC6 and stimulate its α -tubulin deacetylase activity (17). In addition to α -tubulin, phosphorylation of HDAC6 also alters its deacetylase activity toward other substrates, such as β -catenin. As reported by Zhu *et al.*, protein kinase C α (PKC α) enhances HDAC6 deacetylase activity toward β -catenin and regulates its nuclear translocation and promoter binding (18). Besides serine/threonine kinases, tyrosine kinases such as epidermal growth factor receptor (EGFR) kinase have been shown to phosphorylate tyrosine 570 within the HDAC6 C-terminal deacetylase domain, which results in a decrease in the deacetylase activity of HDAC6, an increase in α -tubulin acetylation, and an altered EGFR trafficking (19). Overall, numerous kinases could target HDAC6 and regulate its activity toward its substrate and ultimately influence cell motility or other cellular functions.

Extracellular signal-regulated kinase 1 and 2 (ERK1/2) are related protein-serine/threonine kinases that participate in the EGFR-Ras-Raf-MEK-ERK signal transduction cascade (20). Downstream targets of ERK1/2 are involved in many biological processes including cell migration. Migration of cells is shown to be influenced by ERK because inhibition of ERK by MEK inhibitor U0126 or PD98059 reduces cell migration in various cell types (21). It is believed that ERK regulates cell migration by targeting its nuclear or cytoplasmic substrates. One such ERK target is a nuclear protein, ELK-1, that is a member of the ternary complex factor subfamily of Ets (E-twenty six)-domain transcription factors. ELK-1 phosphorylation leads to prolonged *c-Fos* expression and results in increased expression of several genes including matrix metalloproteinase-9 (MMP-9), which promotes cell motility (22). ERK can also promote cell migration on extracellular matrix in a transcription-independent manner by directly impacting the migratory machinery in the cytosol. This occurs by enhancing the activity of myosin light chain kinase, which leads to increased myosin light chain phosphorylation and enhanced migration (23). Moreover, ERK has been shown to be associated with the microtubules (24). Here we have identified a novel ERK substrate, HDAC6. Our studies have suggested that phosphorylation of HDAC6 is

important for EGFR-Ras-Raf-MEK-ERK cascade-mediated cell motility.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, Reagents, and Cell Lines—HA-HD6-F is described in Ref. 13. HA refers to a hemagglutinin tag. F refers to the FLAG peptide. The point mutations on this construct were created by QuikChange[®] XL Site-directed Mutagenesis kit (Stratagene). For HA-HD6(S1035A)-F, the primers used were 5'-CCACCAGACCCCCCAACCGCACCTGTGCAG-3' and 5'-CTGCACAGGTGCGGTTGGGGGGTCTGGTGG-3'. For HA-HD6(S1035D)-F, the primers used were 5'-CCACCAGACCCCCCAACCGACCCTGTGCAG-3' and 5'-CTGCACAGGGTCGGTTGGGGGGTCTGGTGG-3'. GST-HD6(816–1215) plasmid was constructed by inserting the PCR-amplified HDAC6 sequence derived from HA-HD6-F into the Sall/NotI sites of pGEX-4T1 vector using the following primers: Sall/HDAC6, 5'-AGTCGACGGGCCCTGGCCTCAATCACTG-3' and NotI/HDAC6, 5'-AGCGGCCGCTTAGTGTGGGTGGGGCATATCC-3'. Point mutations of HDAC6 in this construct were created using the following primers: for GST-HD6(816–1215)S1045A, 5'-CACCCAGATAGCTCCAGTACACTGATTG-3' and 5'-CAATCAGTGTACTGGGAGCTATCTGGGGTG-3'; for GST-HD6(816–1215)T1031A/S1035A, 5'-CCACCAAGCCCCCAACCGCACCTGTGCAG-3' and 5'-CTGCACAGGTGCGGTTGGGGGGCCTGGTGG-3'; for GST-HD6(816–1215)T1031A, 5'-CCACCAGGCCCCCAACCTCACCTGTGCAG-3' and 5'-CTGCACAGGTGAGTTGGGGGGCCTGGTGG-3'; for GST-HD6(816–1215)S1035A, the primers are the same as used for generation of HA-HD6(S1035A)-F. The PCR cycle was as follows: 95 °C, 1 min; followed by 95 °C, 50 s; 60 °C, 50 s; 68 °C, 20 min for 25 cycles; and then 68 °C, 30 min. The GST-HD6, GST-HD6(1–503), GST-HD6(448–840), and GST-HD6(840–1215) plasmids were generated by inserting PCR products into pGEX-4T-1 vector. Detailed information is available upon request. The pLEX-GST-DN-ERK1 plasmid was made as follows. The human wild type ERK1 cDNA was isolated from pDONR223-MAPK3(Addgene Plasmid 23509) vector by PCR and then subcloned into pGEX to generate pGEX-ERK1. The resulting plasmid was then used as a template to generate dominant negative (DN) ERK1(T202A/Y204F) using the following primers: forward, 5'-GACCACACCGGCTTCCTGGCCGAGTTTGTGGCTACGCGCTGGTAC-3' and reverse, 5'-CAGGAAGCCGGTGTGGTCATGCTCAGG-3'. The mammalian expression vector pLEX-GST-DN-ERK1 was made by inserting the digested GST-DN-ERK1 between SpeI and XhoI sites in pLEX-MCS vector (Open Biosystem). C-RAF-BXB was described in Ref. 25; HA-Ras(G12V), HA-MEK(K97A), and HA-MEK(S218/222D) were kind gifts from Dr. Jie Wu. EGFR (vIII) plasmid was described by Shen *et al.* (26). pEF-BOS-GST-Braf(V600E) mammalian expression vector was a kind gift from Dr. Chuangui Wang.

Anti-HDAC6(H300) and anti-EGFR(1005) antibodies were purchased from Santa Cruz Biotechnology. Anti-phosphoserine/threonine antibody was purchased from BD Biosciences. Anti-HA antibody was purchased from Covance. Anti-acetylated α -tubulin antibody, anti- β -tubulin antibody, and Lipofectamine 2000 reagent were purchased from Invitrogen. Anti-FLAG antibody, collagen I (C7661), shRNA vectors against

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ERK1 (TRCN000006150) and ERK2 (TRCN000010040) were purchased from Sigma. Anti-ERK1/2 antibody (9102), anti-phospho-ERK1/2 (Thr-202/Tyr-204) antibody (9101), anti-phospho-MEK1/2 (Ser-217/Ser-221) antibody (9121), anti-GST (91G1) antibody (2625), anti-MEK1/2 antibody (9122), recombinant ERK1 kinase (7416), and human EGF (89165C) were purchased from Cell Signaling. U0126 and PD98059 were purchased from Calbiochem. Phosphorylated HDAC6 Ser-1035-specific polyclonal antibody, anti-pSer-1035(HDAC6), was produced by immunizing rabbits with keyhole limpet hemocyanin-conjugated peptide: DHQTPPT(pS)PVQG. The antibody was purified by phospho-peptide affinity column.

CHO, a Chinese hamster ovary cell line, H1299, and HDAC6 wild type and knock-out mouse embryonic fibroblasts (MEFs), 293T, and HeLa S3 cells were cultured in DMEM with penicillin (100 units/ml), streptomycin (100 μ g/ml), and 10% fetal bovine serum (FBS) and incubated at 37 °C with 5% CO₂. HeLa S3 suspension cells were cultured in Joklik medium (Sigma).

Generation of Baculoviruses—The baculoviruses expressing F-HD6, F-HD6(S1035A), and F-HD6(S1035D) were generated from modified pFastBac-HTb donor vector (Invitrogen) in which the His tag was changed to a FLAG tag. The bacmids containing the above cDNAs were generated by transposition in *Escherichia coli* cells according to the manual of Bac-to-Bac system (Invitrogen). Baculoviruses expressing wild type and A or D mutant of HDAC6 proteins were generated by transfection of recombinant bacmids into Sf9 cells using Cellfectin®II Reagent (Invitrogen). The P2 stocks of baculovirus were used to infect Sf9 cells. The overexpressed F-HD6, F-HD6(S1035A), and F-HD6(S1035D) in Sf9 cells were purified using anti-FLAG M2 agarose (Sigma). GST-HDAC6 baculoviruses were made as follows. HDAC6 was first inserted between Sall and NotI sites after a GST tag in pGEX-4T1 vector. Then GST-HDAC6 was amplified by PCR and inserted between SpeI and HindIII in pFastBac-1 vector (Invitrogen). The bacmid for GST-HDAC6 was made and used for baculovirus production followed by GST-HDAC6 protein expression and purification.

In Vitro Kinase Assay—GST fusion proteins containing C terminus of wild type or mutant of HDAC6 as shown in Fig. 2A were incubated with recombinant ERK1 (Cell Signaling) in the presence of 5 μ Ci of [γ -³²P]ATP, 10 μ M ATP, and 1 \times kinase buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM dithiothreitol (DTT)) for 30 min at 30 °C. Reactions were terminated by adding SDS loading buffer followed by heating at 100 °C for 5 min. Proteins were separated on 6% SDS-polyacrylamide gel, and the phosphorylated proteins were visualized by autoradiography.

Immunoprecipitation and Immunoblotting—For immunoprecipitations, cells were lysed in LS buffer (PBS, pH 7.5, 10% glycerol, 0.1% Nonidet P-40, and protease inhibitor mixture). Lysates were incubated with protein A- or protein G-agarose for 2 h for preclearing prior to incubation with the indicated primary antibodies for 12 h at 4 °C. Immunocomplexes were collected, washed four times in lysis buffer, and resolved by SDS-PAGE. For immunoblotting, samples were transferred to nitrocellulose membranes that were then probed with the indicated antibodies. Bound antibodies were detected using a Chemiluminescent Detection kit (Pierce).

HDAC Assay—Empty vector, HA-HD6-F, HA-HD6(S1035A)-F, or HA-HD6(S1035D)-F protein purified from 293T transfected cells lysate was precleared with protein A beads and immunoprecipitated with anti-FLAG beads before protein deacetylase activity was determined. Briefly, [³H]acetate-incorporated histones were isolated from butyrate-treated HeLa cells by acid extraction as described in Ref. 27. Purified core histones (12,000 cpm) were incubated with immunoprecipitates in 150 μ l of ice-cold histone deacetylase buffer (20 mM Tris, pH 8.0, 150 mM NaCl, and 10% glycerol) at 37 °C for 2 h. The reaction was terminated by the addition of an equal volume of stop solution (0.16 M acetic acid, 1.0 M HCl) and mixed well by vortexing. The released [³H]acetate was extracted with ethyl acetate and combined with scintillation mixture for analysis. Additionally, HDAC6 and its mutants (S1035A and S1035D) were also obtained from Sf9 insect cells. The purified proteins were used to performed HDAC assay as was done with 293T cells.

Tubulin Deacetylase (TDAC) Assay—Briefly, proteins were incubated with 100 μ l of 2 \times TDAC buffer (20 mM Tris-Cl, pH 8.0, 20 mM NaCl) and 50 μ g of reconstituted microtubules (Cytoskeleton) in a 200- μ l reaction at 37 °C for 2 h, then transferred to ice for 15 min. A fraction of the reactions were analyzed by Coomassie staining or Western blot analysis using anti- α -tubulin and anti-acetylated α -tubulin antibodies.

Establishment of Stable Clones—To generate the rescue clones in HDAC6 knock-out MEFs, empty vector, HA-HD6-F, HA-HD6(S1035A)-F, and HA-HD6(S1035D)-F were transfected into HDAC6 knock-out MEFs using Lipofectamine Plus reagent. One day after transfection, 500 μ g/ml G418 was added to the medium to select positive cells. Ten days later, stable cell lines were subcloned to 60-mm dishes, and 300 μ g/ml G418 was added to maintain the stable clones. The generated stable clones were used in migration experiments.

Cell Migration Assay—First, 24-well Transwell migratory cell inserts (8- μ m pores, Fisher Scientific) were coated with 5 μ g of collagen 1 and incubated at 4 °C overnight. Before use, liquid was aspirated out, and the inserts were rinsed with PBS and allowed to dry in sterile condition. Total 4 \times 10⁴ cells in serum-free medium were seeded into inserts in duplicates. 500 μ l of medium with 10% FBS was added to the bottom of the well and served as attractant. Cells were allowed to migrate through the inserts for 24 h at 37 °C. Cells that did not migrate were removed with a cotton swab, and migratory cells were fixed with 4% paraformaldehyde and stained with crystal violet in ethanol. Snapshots were taken of the migratory cells, and then cells were destained in 2% SDS, and absorbances were read at 560 nm.

Statistical Analysis—Results obtained from independent studies are shown with mean \pm S.D. Statistical analysis was performed using Student's *t* test. A *p* value < 0.05 was considered significant.

RESULTS

Thr-1031 and Ser-1035 of HDAC6 Are Phosphorylated by ERK1 in Vitro—To identify novel phosphorylation sites in HDAC6, 293T cells were transfected with adenovirus HA-HD6-F (13) for 36 h. The cells were then lysed, and HDAC6 was immunoprecipitated by anti-FLAG antibodies. The immunoprecipitates were resolved on SDS-PAGE followed by Coomassie Blue

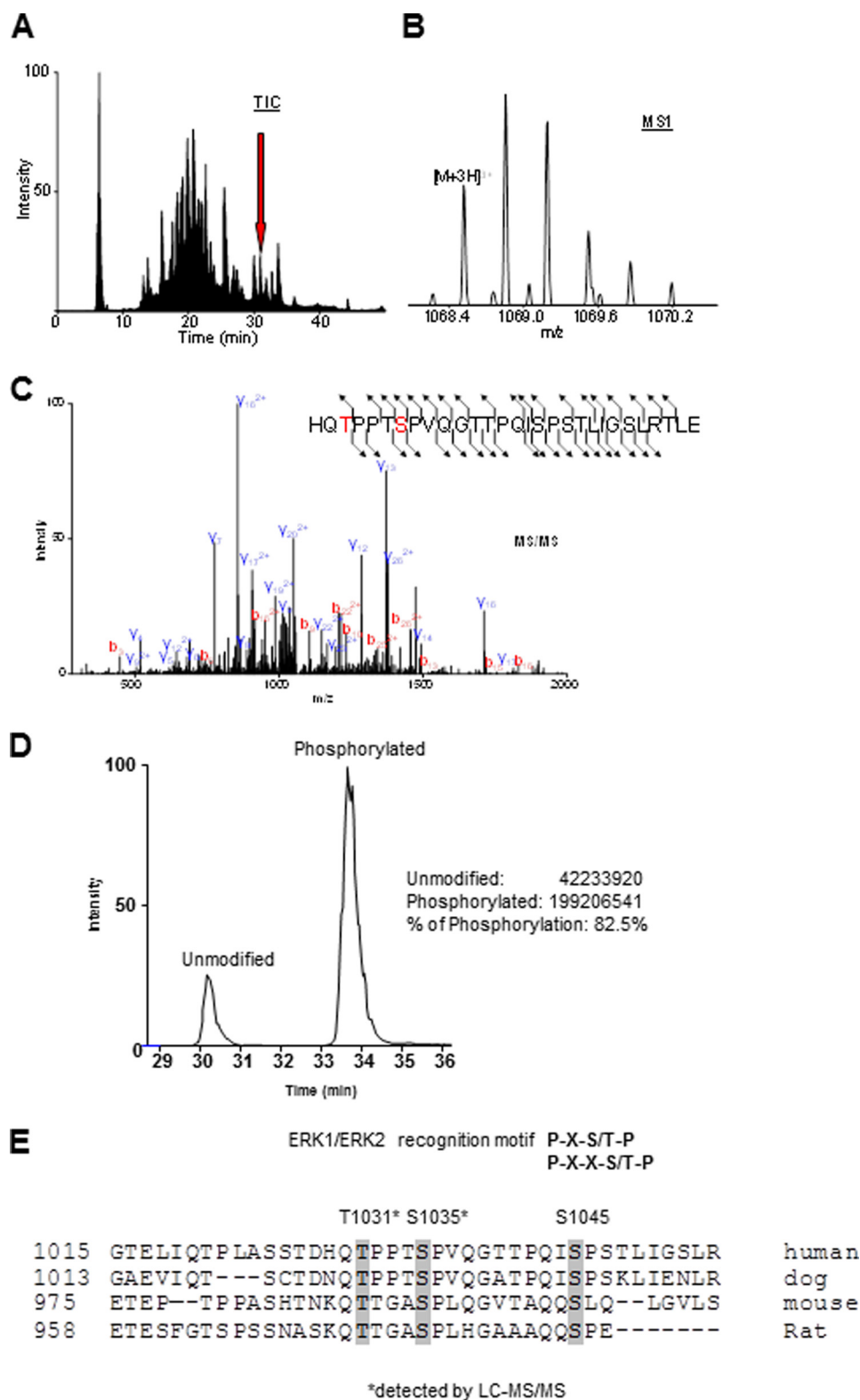


FIGURE 1. **Thr-1031 and Ser-1035 are phosphorylated in HDAC6.** *A* and *B*, the peptide was detected at 32.6 min in the total ion chromatogram (TIC) (*A*) with mass-to-charge ratio 1068.5189, triply charged, which represents an error of 6.8 ppm (*B*). *C*, the tandem mass spectrum (MS/MS) matched the following sequence, HQTPPTS^PVQGGTTPQIS^PSTLIGSLR^TLE, indicating that Thr-1031 and Ser-1035 (highlighted in red) were phosphorylated; the detection of b_3 , y_{22} , and y_{23} is consistent with this localization. The assignment was made with Mascot with a score of 50 and Sequest XCorr 5.62. *D*, ion chromatograms for peptides containing both phosphorylated Ser-1035 and unmodified Ser-1035 were extracted using Xcalibur 2.0 (Thermo Scientific). The peak areas under the curves were used to calculate the percentage of phosphorylation. *E*, upper panel, the consensus ERK1/ERK2 recognition motif is shown. Lower panel, a stretch of HDAC6 amino acid sequence showing the conservation of mass spectrometry analyses identified two ERK1/ERK2 sites (Thr-1031 and Ser-1035) and a putative ERK1/ERK2 site (Ser-1045) among rat, mouse, dog, and human.

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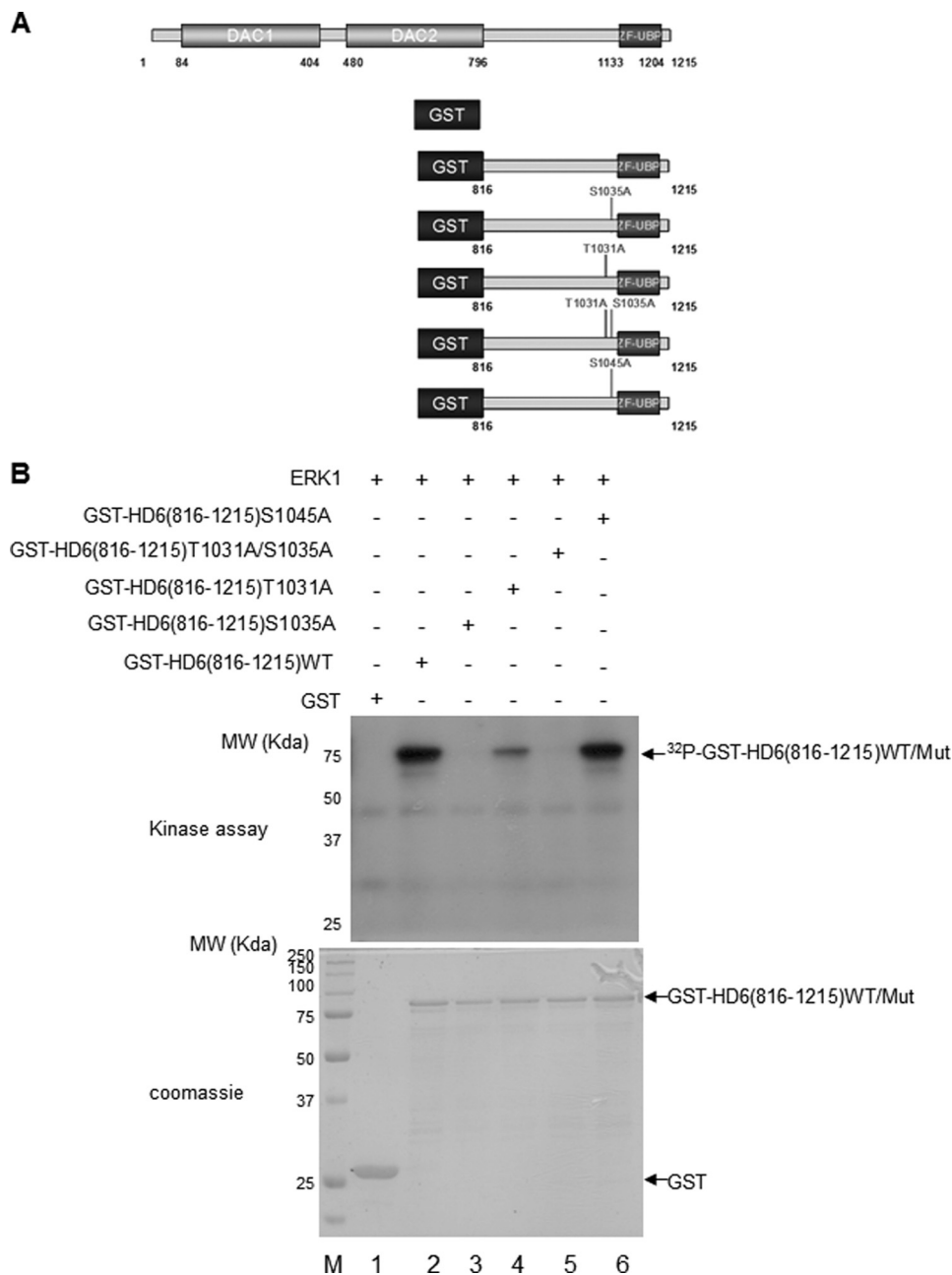


FIGURE 2. HDAC6 is phosphorylated by ERK1 *in vitro* at Thr-1031 and Ser-1035. *A*, diagrams show full-length and C terminus of HDAC6 with putative phosphorylation sites. *B*, upper panel, *in vitro* kinase assays were performed with the indicated substrates and recombinant ERK1 as described under "Experimental Procedures." The reactions were separated on 6% SDS-PAGE and analyzed by autoradiography. Lower panel, Coomassie Blue staining of the substrates shows the protein amount and purity for kinase assays.

staining. HDAC6 band was excised and subjected to LC-tandem MS analyses. As shown in Fig. 1, *A–C*, the mass spectrometry analyses identified two novel phosphorylation sites located in the C-terminal region of HDAC6, namely threonine 1031 and serine 1035. The extent of HDAC6 phosphorylation on these two sites was also examined by quantitative mass spectrometry. The majority of immunoprecipitated wild type HDAC6 (82.5%) was phosphorylated (Fig. 1*D*), suggesting the importance of Thr-1031 and Ser-1035 sites. Between these two sites, Ser-1035 is within the ERK consensus motif (P-X-S/T-P) (28), whereas Thr-1031 is not (Fig. 1*E*). In addition, Thr-1031 and Ser-1035 were conserved among rat, mouse, dog, and

human but not *Drosophila* and fish (Fig. 1*E* and data not shown), suggesting that these sites are conserved in mammals. Although Ser-1045 is also located within an ERK motif, the mass spectrometry analyses did not reveal it as a phosphorylation site. Based on this information, we set out to determine whether ERK is indeed involved in phosphorylating these sites. To this end, we used recombinant ERK1 and the C-terminal fragments of HDAC6 harboring wild type or mutants (T1031A, S1035A, T1031A/S1035A, S1045A) (Fig. 2*A*) to perform *in vitro* kinase assays. Our results showed that Ser-1035 was a major phosphorylation site for ERK1 as evidenced by the disappearance of the phosphorylation signal when this site was mutated

from serine to alanine (Fig. 2B, lane 3). Although the Thr-1031 site was mutated to alanine, there was a moderate decrease in the levels of phosphorylation (Fig. 2B, lane 4 versus lane 2), indicating that the Thr-1031 site is not a major ERK1 phosphorylation site. The results also showed that phosphorylation of Thr-1031 by ERK1 was dependent on Ser-1035 phosphorylation because in the double mutant, HD6(816–1215)T1031A/S1035A, no phosphorylation signal was observed (Fig. 2B, lane 5). Moreover, the phosphorylation signal of S1045A was comparable with that of the WT (Fig. 2B, lane 6 versus lane 2), showing that Ser-1045 was not phosphorylated by ERK1 *in vitro*. In addition, we also examined whether another MAPK, p38 α , can phosphorylate the C-terminal region of HDAC6. Our analyses showed that the p38 α kinase phosphorylated wild type as well as T1031A, S1035A, and S1045A mutants of HDAC6 to a similar extent, suggesting that Thr-1031, Ser-1035, and Ser-1045 are not p38 α phosphorylation sites (data not shown). Collectively, the mass spectrometry analyses and *in vitro* kinase assays have demonstrated that Ser-1035 and Thr-1031 can be phosphorylated by ERK1 *in vitro*, whereas Ser-1045 cannot.

Ser-1035 of HDAC6 Is Phosphorylated through EGF-EGFR-Ras-Raf-MEK-ERK Pathway *In Vivo*—To examine HDAC6 Ser-1035 phosphorylation, we raised the antibody against phosphorylated Ser-1035 of HDAC6, anti-pSer-1035(HDAC6) as described under “Experimental Procedures.” We then further characterized the antibody. As shown in Fig. 3A, this antibody only detects phosphorylated HDAC6 protein (lane 1) but not the one treated with the phosphatase (lane 2). As shown in Fig. 3B, this antibody only recognizes HDAC6 wild type (lane 2) but not HDAC6(S1035A) mutant (lane 3), suggesting that this antibody selectively detects phosphorylated Ser-1035 of HDAC6.

Because it was determined that ERK1 was a kinase that phosphorylated HDAC6 *in vitro*, we next asked whether phosphorylation by ERK1 also occurred *in vivo*. MEFs were treated with the MEK inhibitor U0126 or PD98059, and immunoprecipitation experiments using phosphoserine/threonine antibodies were performed to assess the phosphorylation status of HDAC6. Compared with the control, both U0126 (10 μ M) and PD98059 (100 μ M) caused a significant decrease in phosphorylation of HDAC6 when ERK was inhibited (Fig. 4A, lane 1 versus lanes 2 and 3; bar graph). To validate our results, active Ras was employed to stimulate the Raf-MEK-ERK signaling cascade to examine whether Ras is able to increase HDAC6 phosphorylation. Then DN-MEK, HA-MEK(K97A), or DN-ERK1, GST-DN-ERK1, was transfected into cells to block the MAPK pathway. As shown in Fig. 4B, active Ras dramatically increased HDAC6 phosphorylation (lane 3 versus 2), whereas either DN-MEK or GST-DN-ERK1 significantly decreased HDAC6 phosphorylation (compare lanes 4 and 5 with lane 3). The above results indicate that ERK is indeed involved in promoting phosphorylation of HDAC6 *in vivo*.

We next examined whether HDAC6 Ser-1035 phosphorylation is increased by upstream kinases of ERK. Active EGFR, EGFR(vIII), active Ras, Ras(G12V), active Raf, Braf(V600E), and c-RAF-BXB, and active MEK, MEK(S218/222D) (25, 29) were transfected into 293T cells to activate endogenous ERK. As shown in Fig. 4C, all active kinases increased the phosphorylation levels of HDAC6 wild type but not HDAC6(S1035A)

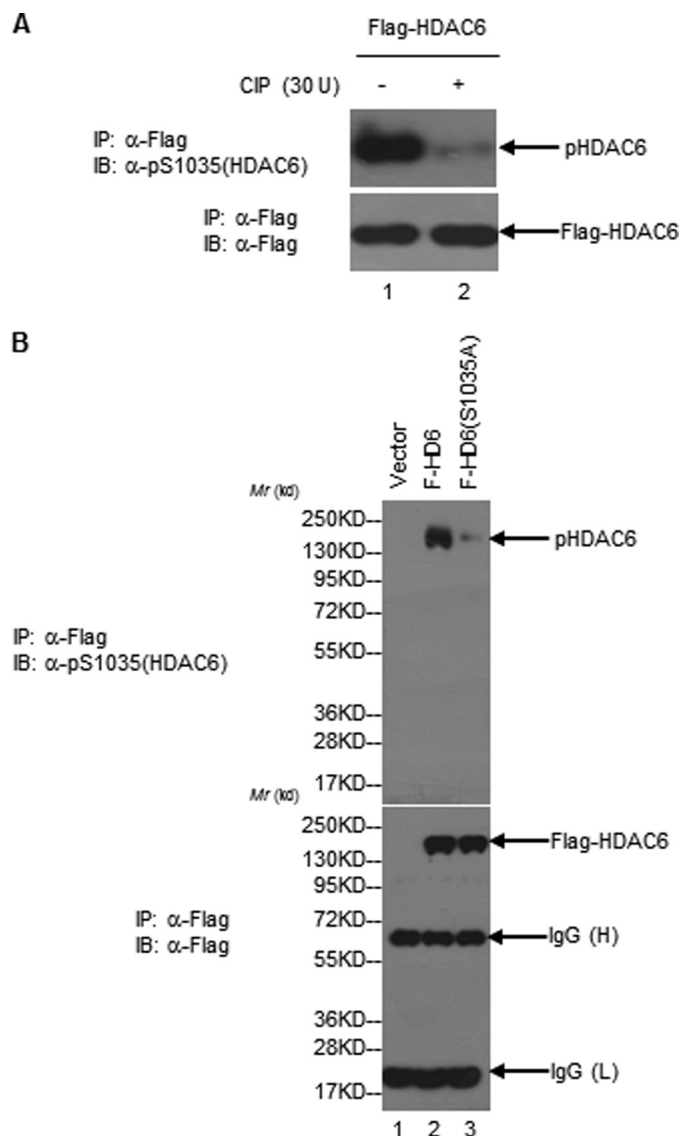


FIGURE 3. Characterization of anti-pSer-1035 (HDAC6) antibody. A, anti-FLAG-M2 bead-purified FLAG-HDAC6 protein from 293T cells was treated without (lane 1) or with (lane 2) calf-intestinal alkaline phosphatase (CIP) (30 units) (Takara) at 37 °C for 1.5 h. Western blot analysis (IB) was performed with anti-pSer-1035(HDAC6) antibodies (upper panel). The blot was stripped and reprobred with anti-FLAG antibodies (lower panel). B, ectopically expressed 5- μ g empty vector, FLAG-HDAC6 wild type, or S1035A was immunoprecipitated (IP) from 293T cells with anti-FLAG-M2 beads. Western blot analysis was carried out using rabbit anti-pSer-1035(HDAC6) antibodies (upper panel). The blot was then stripped and reprobred with anti-FLAG antibodies (lower panel).

mutant (Fig. 4C, compare lane 1 with lanes 2, 4, 6, and 8; compare lane 1 with lanes 3, 5, 7, and 9). Therefore, the HDAC6 Ser-1035 site is indeed phosphorylated by the EGFR-Ras-Raf-MEK-ERK signaling pathway *in vivo*. Both mass spectrometry analysis (Fig. 1D) and *in vitro* kinase assay (Fig. 2) indicate that Ser-1035 of HDAC6 is a predominant site targeted by ERK. Consistent with this assumption, the HDAC6(S1035A) or HDAC6(S1035D) mutant exhibits undetectable levels of phosphorylation in cells overexpressing active RAF, c-RAF-BXB (data not shown).

EGF is one of the ligands for EGFR. We next asked whether EGF-stimulated EGFR activation could lead to HDAC6 Ser-

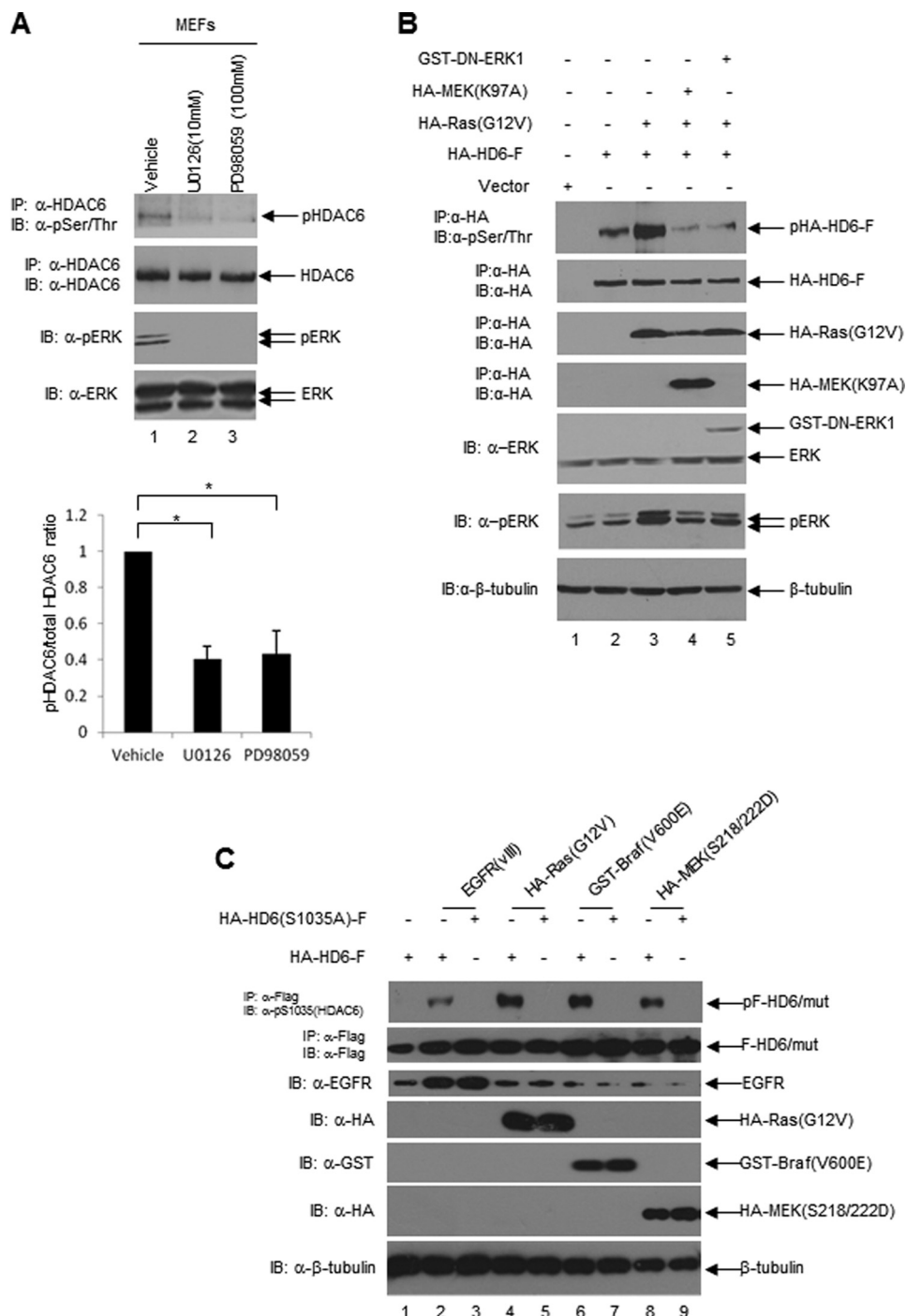


FIGURE 4. HDAC6(Ser-1035) phosphorylation is targeted by ERK pathway *in vivo*. *A*, HDAC6 phosphorylation is decreased by MEK inhibitor treatment. MEFs were treated with MEK inhibitor U0126 or PD98059 with the indicated concentration for 1 h. Endogenous HDAC6 was immunoprecipitated (IP) by anti-HDAC6 antibodies, and the phosphorylation status of HDAC6 was measured by immunoblotting (IB) using anti-phosphoserine/threonine antibodies. The blot was then stripped and reprobbed with anti-HDAC6 antibodies. Anti-pERK and anti-ERK Western blotting analyses were also performed as indicated. The bands of pHDAC6 were quantified by densitometry and are shown in the bar graph. Student's *t* test was performed with * indicating *p* < 0.05. Error bars, S.D. *B*, Ras-induced HDAC6 phosphorylation is attenuated by dominant negative MEK and ERK. 293T cells were transfected with the indicated plasmids. HA and FLAG double-tagged HDAC6 was immunoprecipitated by anti-HA-agarose beads, and phosphorylation of HDAC6 was examined by immunoblotting with anti-phosphoserine/Thr antibodies. The membrane was then stripped and reblotted with anti-HA antibodies to detect the immunoprecipitation efficiency of HDAC6, HA-MEK(K97A), and HA-Ras(G12V). The expression of GST-DN-ERK1 was examined by anti-ERK Western blotting. Anti-pERK, anti-ERK, and anti- β -tubulin Western blotting analyses were also performed. *C*, Ser-1035 site is a major phosphorylation site of HDAC6 targeted by the EGFR-Ras-Raf-MEK-ERK signaling pathway. 293T cells were transiently transfected with the indicated plasmids. Following transfection, cell lysate was immunoprecipitated using anti-FLAG M2 beads, and the phosphorylation status of HDAC6 (pHDAC6) was determined with anti-pSer-1035 (HDAC6) Western blotting analysis. The blot was then stripped and reprobbed with anti-FLAG antibody to examine the immunoprecipitation efficiency. The levels of EGFR, HA-Ras(G12V), GST-Braf(V600E), and HA-MEK(S218/222D) were examined by Western blotting analyses using the indicated antibodies. The anti- β -tubulin Western blotting analyses were also carried out.

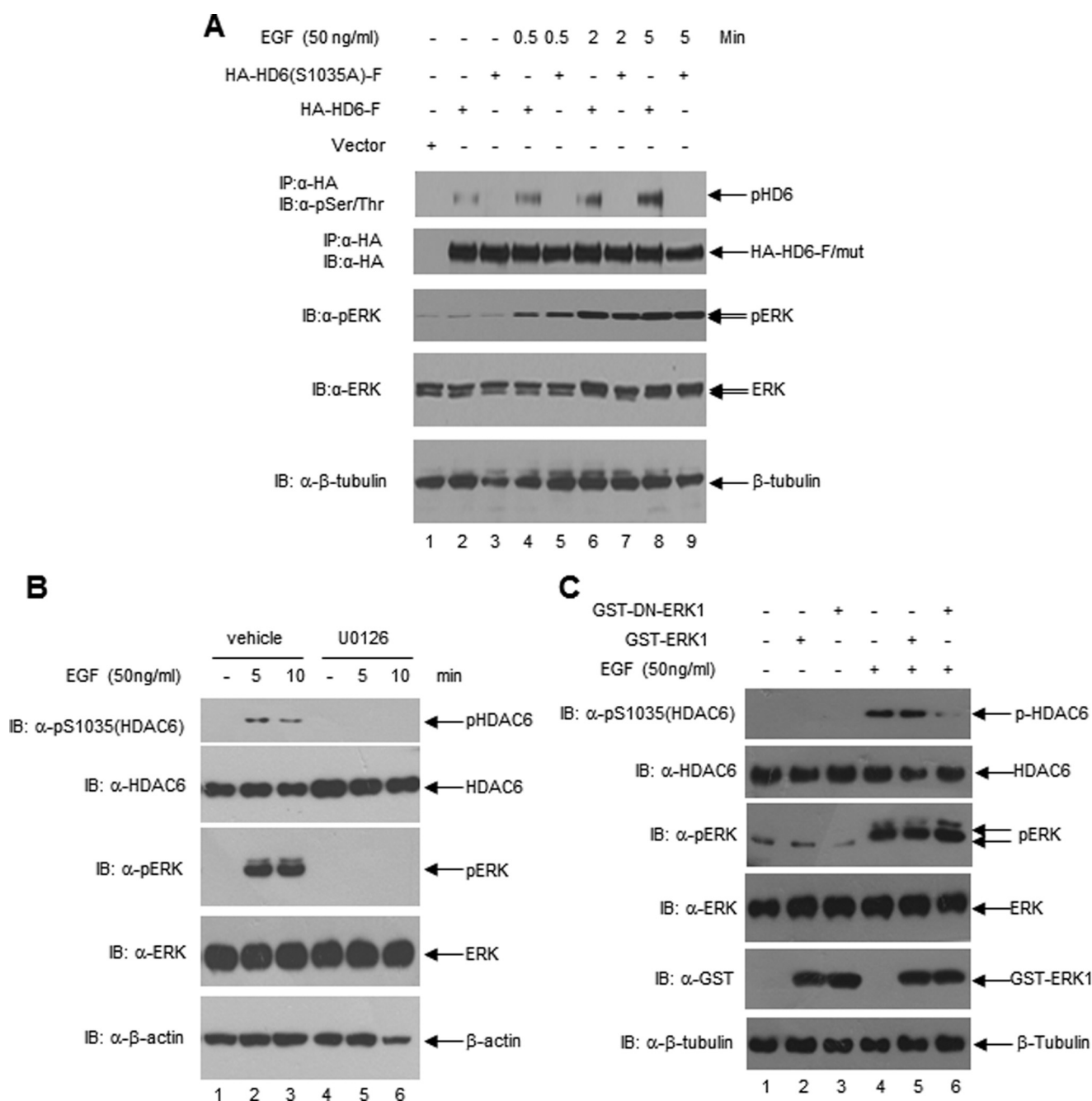


FIGURE 5. EGF promotes HDAC6(Ser-1035) phosphorylation via ERK1. *A*, EGF promotes HDAC6 Ser-1035 phosphorylation. 293T cells were transfected with indicated plasmids then serum-starved overnight followed by EGF treatment prior to harvest. Wild type or mutant HDAC6 was immunoprecipitated (IP) by anti-HA antibodies followed by anti-pSer/Thr Western blotting (IB) analyses. The blot was then stripped and reprobed by anti-HA antibodies. The total cell lysates were also subjected to anti-pERK, anti-ERK and anti- β -tubulin Western blotting analyses. *B*, HeLa cells were serum-starved for 24 h followed by pretreatment with vehicle or 10 μ M U0126 for 1 h prior to EGF treatment. The Western blotting analyses were performed with the indicated antibodies. *C*, 293T cells were transfected with the indicated plasmids. The cells were serum-starved for 24 h followed by 10-min EGF treatment prior to harvest. The Western blotting analyses were performed with the indicated antibodies.

1035 phosphorylation which is mediated by ERK. As shown in Fig. 5A, phosphorylation of wild type but not S1035A mutant of HDAC6 was dramatically increased upon EGF stimulation. EGF-mediated HDAC6 Ser-1035 phosphorylation was diminished either by MEK inhibitor, U0126 (Fig. 5B) or DN-ERK1 (Fig. 5C), suggesting that EGF-mediated HDAC6 Ser-1035 phosphorylation is via ERK1.

Some kinases and their substrates are known to interact. For example, G protein-coupled receptor kinase 2 binds to and

phosphorylates HDAC6 (17). To determine whether HDAC6 is associated with ERK, control anti-IgG, anti-HDAC6 antibody, bacterial expressed GST-tagged full-length or deletions of HDAC6 were used to pull down endogenous ERK1/2 in HeLa S3 nuclear and cytoplasmic fractions which were prepared as described by Dignam *et al.* (30). As shown in Fig. 6A, endogenous HDAC6 and ERK1/2 interact in both the nuclear and cytoplasmic fractions. In addition, HDAC6(1–503) and HDAC6(448–840) but not HDAC6(840–1215) interact with

ERK Phosphorylates HDAC6

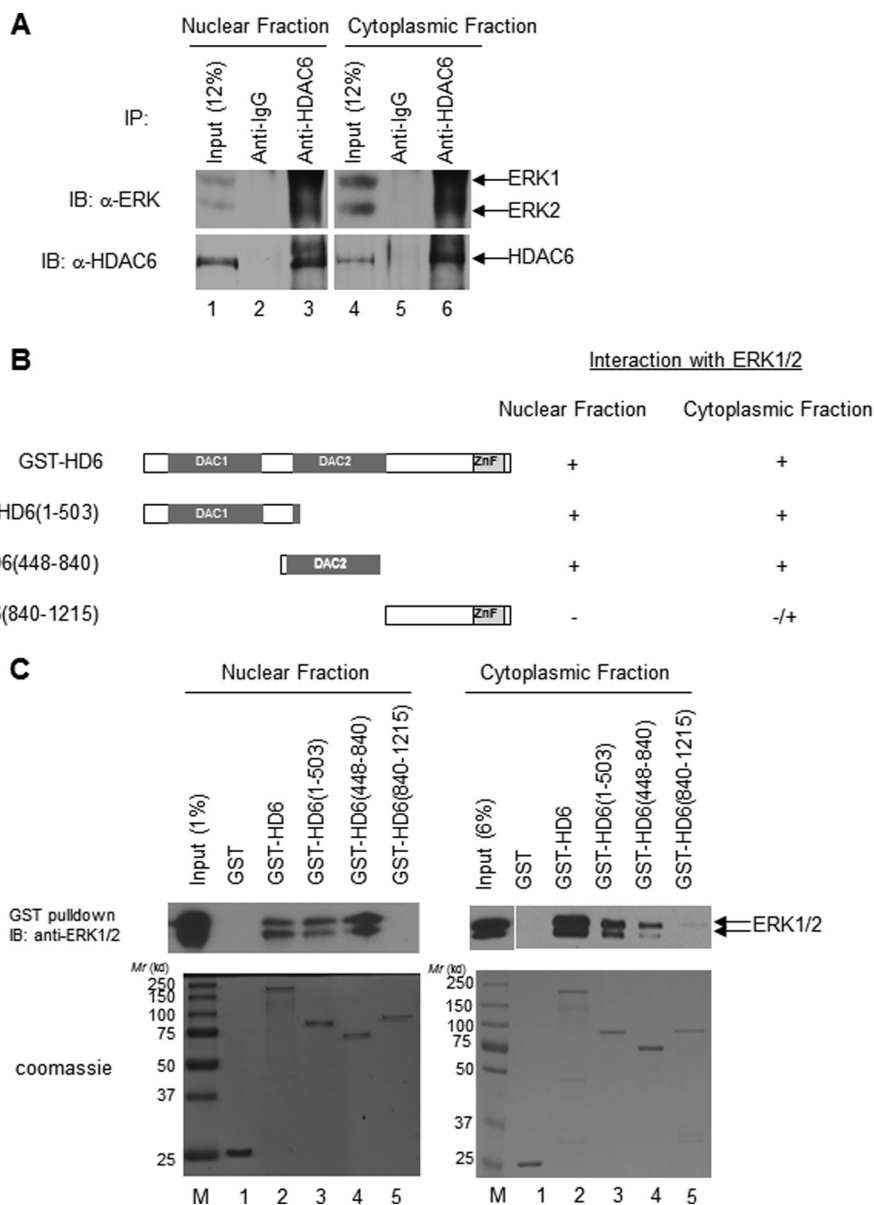


FIGURE 6. **ERK1/2 interacts with HDAC6.** *A*, HeLa S3 cells were fractionated into nuclear and cytoplasmic fractions as described by Dignam *et al.* (30). Immunoprecipitation (IP) was performed using control (anti-IgG) or anti-HDAC6 antibodies followed by anti-ERK and anti-HDAC6 Western blotting analyses. *B*, diagrams show HDAC6 full-length and HDAC6 deletion constructs. *C*, GST beads or GST bead-bound proteins generated from *B* were incubated with HeLa S3 nuclear or cytoplasmic fraction, and GST pull-down assays were performed followed by anti-ERK1/2 Western blotting (IB) analyses. Protein expression is indicated by Coomassie Blue staining.

ERK1/2 (Fig. 6, *B* and *C*), suggesting that ERK1/2 could bind to HDAC6 N-terminal catalytic domain 1 (DAC1) or domain 2 (DAC2) independently.

Mutation of HDAC6 at Ser-1035 Does Not Affect HDAC6 Histone Deacetylase Activity in Vitro—Histones are known substrates of HDACs. Therefore, we set out to determine whether HDAC6 phosphorylation at Ser-1035 is capable of affecting its deacetylase activity toward core histones. The Ser-1035 site of HDAC6 was mutated to alanine or aspartic acid to mimic unphosphorylated or phosphorylated forms of HDAC6, respectively. Empty vector, wild type, S1035A, or S1035D mutant was transfected into 293T cells. Wild type and mutant HDAC6 proteins were immunoprecipitated by anti-FLAG M2 agarose beads and subjected to HDAC assays as described under "Experimental Procedures." As shown in Fig. 7A, S1035A

or S1035D mutation of HDAC6 did not significantly change its enzymatic activity toward core histones compared with wild type. Additionally, wild type, S1035A, or S1035D mutant was also purified from Sf9 cells and subjected to HDAC assays. As presented in Fig. 7B, both S1035A and S1035D mutants exhibited enzymatic activities comparable with the wild type toward core histone. The expression of wild type and mutants of HDAC6 in 293T or Sf9 cells was approximately equal as evidenced by Coomassie Blue staining (Fig. 7, *A* and *B*). To validate whether the HDAC6(S1035D) mutant can faithfully mimic phosphorylation status, HA-HDAC6-F was either transfected alone or together with active Ras. HDAC6 was immunoprecipitated using anti-HA-agarose beads and then subjected to HDAC assay. As shown in Fig. 7C, HDAC activity was not significantly increased when compared with Ras-phosphorylated

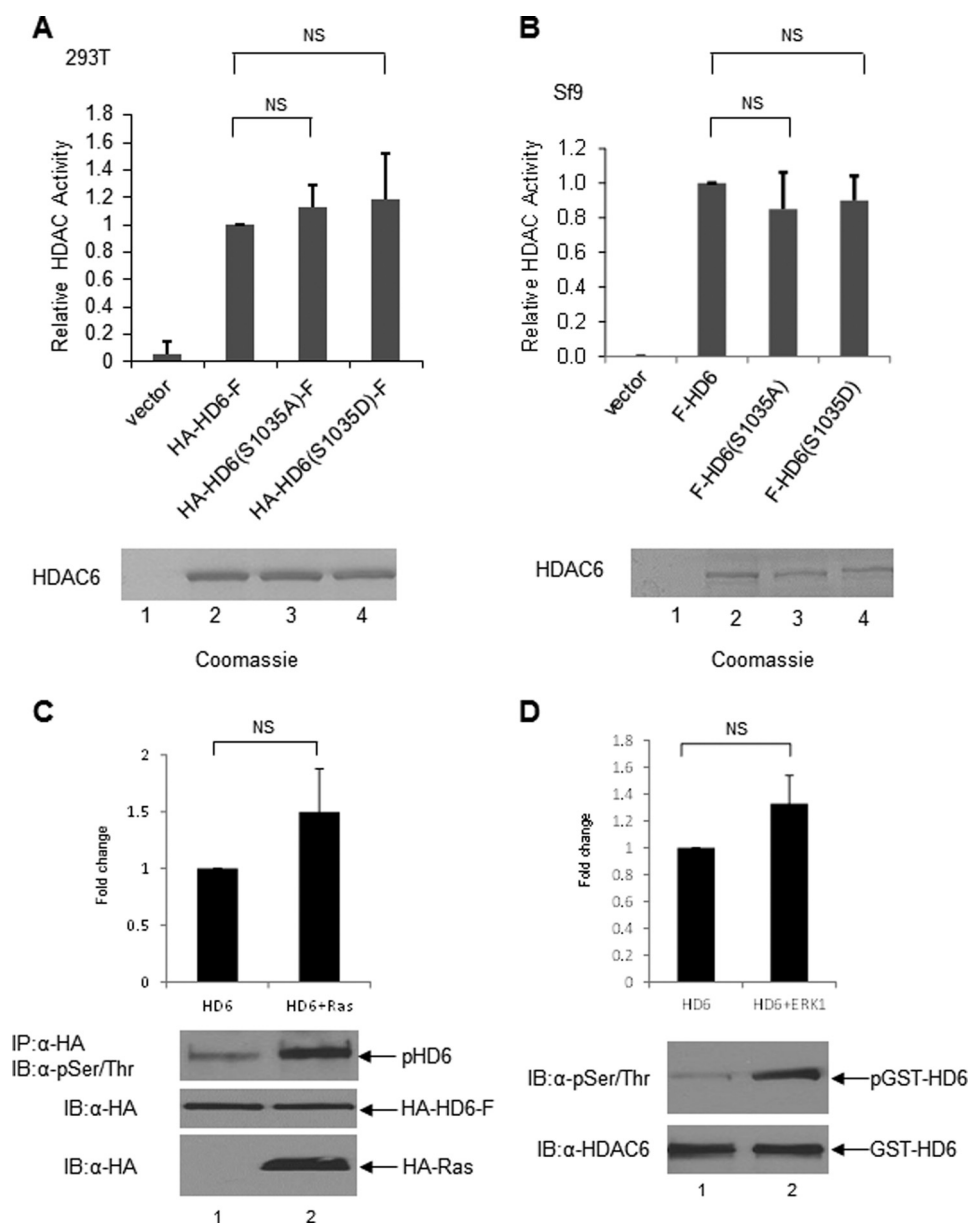


FIGURE 7. Substitution of Ser-1035 to alanine or aspartate does not affect HDAC6 enzymatic activity toward core histones. *A*, 293T cells were transiently transfected with the plasmids as indicated. HDAC6 wild type or mutant complexes were immunoprecipitated by anti-FLAG M2 agarose beads and then subjected to HDAC assay as described under "Experimental Procedures." The purified HDAC6 wild type or mutant complexes used in the HDAC assays are also shown by Coomassie Blue staining. *NS*, not significant. *B*, Sf9 cells were transfected by the indicated viruses. HDAC6 wild type or mutant proteins were purified by anti-FLAG M2 agarose and then subjected to HDAC assays as in *A*. The purified HDAC6 wild type or mutant complexes used in the HDAC assays are also shown by Coomassie Blue staining. Data shown are representative of at least three repeated experiments. *NS* stands for not significant. *C*, 293T cells were transiently transfected with HA-HD6-F alone or HA-HD6-F and HA-Ras(G12V). Anti-HA immunoprecipitated (IP) samples were subjected to HDAC assay as described in *A*. The phosphorylation status of HDAC6 was examined by anti-HA immunoprecipitation followed by anti-pSer/Thr Western blotting (IB) analyses. The expression of HA-HD6-F and HA-Ras(G12V) was also examined by anti-HA Western blotting analyses. *D*, GST-HD6 proteins were purified from Sf9 cells and subjected to a cold ERK1 assay (lane 2) or not (lane 1). The reactions were then subjected to HDAC assays as shown in the bar graph. The phosphorylation of GST-HD6 was examined by anti-pSer/Thr Western blotting analysis. The amount of HDAC6 was examined by anti-HDAC6 Western blotting analysis.

HDAC6 and control HDAC6. In addition, we used the recombinant ERK1 to phosphorylate HDAC6 proteins purified from Sf9 cells. As expected, ERK1 increased HDAC6 phosphorylation dramatically, but HDAC6 enzymatic activity toward core histone was not significantly elevated compared with the control HDAC6 (Fig. 7D). Overall, the above results suggest that phosphorylation/unphosphorylation of HDAC6 at Ser-1035 does not influence its deacetylase activity toward core histones.

HDAC6(S1035A) Mutant Displays Decreased TDAC Activity Compared with the Wild Type in Vivo— α -Tubulin is a known cytoplasmic substrate of HDAC6 (11). To determine whether the phosphorylation status of HDAC6 at its Ser-1035 site would affect its deacetylase activity toward α -tubulin, wild type HDAC6 or its mutant S1035A was transfected into CHO or H1299 cells. As shown in Fig. 8A, HDAC6(S1035A)-transfected cells possess higher levels of acetylated tubulin, suggesting that it harbors lower TDAC activity compared with the wild type.

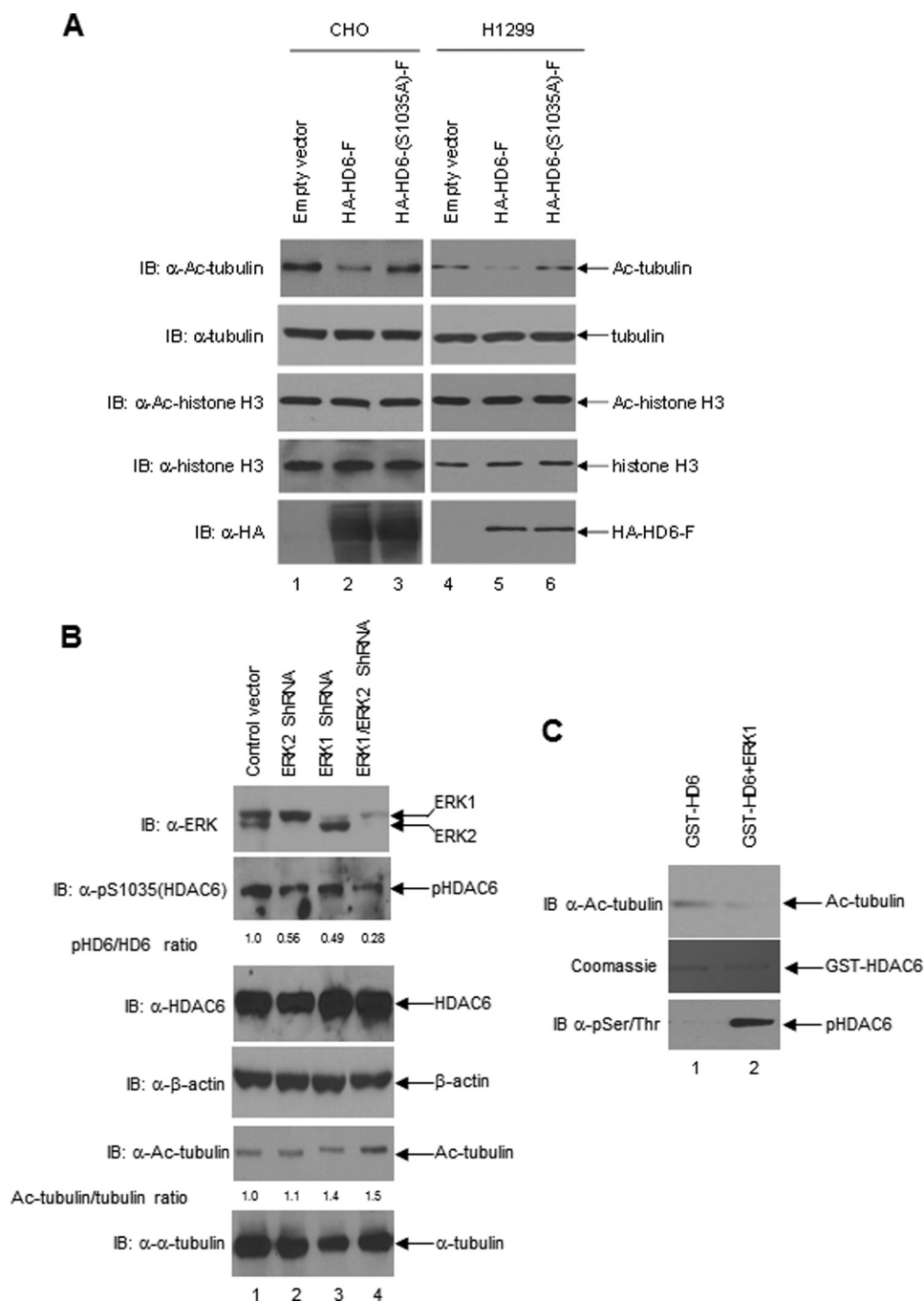


FIGURE 8. S1035A mutant of HDAC6 displays decreased TDAC activity *in vivo*. *A*, HDAC6(S1035A) mutant exhibits decreased TDAC activity *in vivo* compared with the wild type. CHO and H1299 cells were transfected with the indicated plasmids. After 36 h, cells were harvested and lysed. Western blotting (IB) analyses were performed using the indicated antibodies. *B*, 293T cells were transiently transfected with the indicated shRNA to knock down ERK1 or/and ERK2. shRNA vectors against ERK1 (TRCN000006150; Sigma) and ERK2 (TRCN0000010040; Sigma) were used to knock down ERK1 and ERK2, respectively. pLKO.1-puro empty vector was used as a control. These vectors were transfected individually or in combination as indicated into 293T cells for 24 h followed by the same transfections for another 24 h prior to harvesting. Western blot analyses were then performed with the indicated antibodies. *C*, GST-HDAC6 protein was purified from Sf9 cells. The proteins were either subjected to ERK1 kinase assay (*lane 2*) or not followed by TDAC assays as described under "Experimental Procedures." Ac-tubulin was detected by anti-Ac-tubulin Western blot analysis. The HDAC6 and pHDAC6 proteins were examined by Coomassie Blue staining. The anti-pSer/Thr Western blot analysis was carried out to examine the phosphorylation of HDAC6.

Conversely, HDAC6 wild type and HDAC6(S1035A) exhibit similar activity toward core histone H3 (Fig. 8A). To examine whether HDAC6(S1035A) indeed mimics the nonphosphorylation status of HDAC6, knockdown of ERK1, ERK2, or both was performed in 293T cells. As shown in Fig. 8B, depletion of either ERK2 or ERK1 reduced the levels of HDAC6 Ser-1035 phosphorylation approximately to 50% compared with the con-

rol. Depletion of both ERK2 and ERK1 additively reduced the phosphorylation levels of HDAC6 Ser-1035 to 28% compared with the control. These results suggest that both ERK1 and ERK2 are involved in HDAC6 Ser-1035 phosphorylation. Consistent with Fig. 8A, knockdown of both ERK1 and ERK2 significantly increased acetylated α -tubulin level compared with the control, indicating that reduction of HDAC6 Ser-1035 phos-

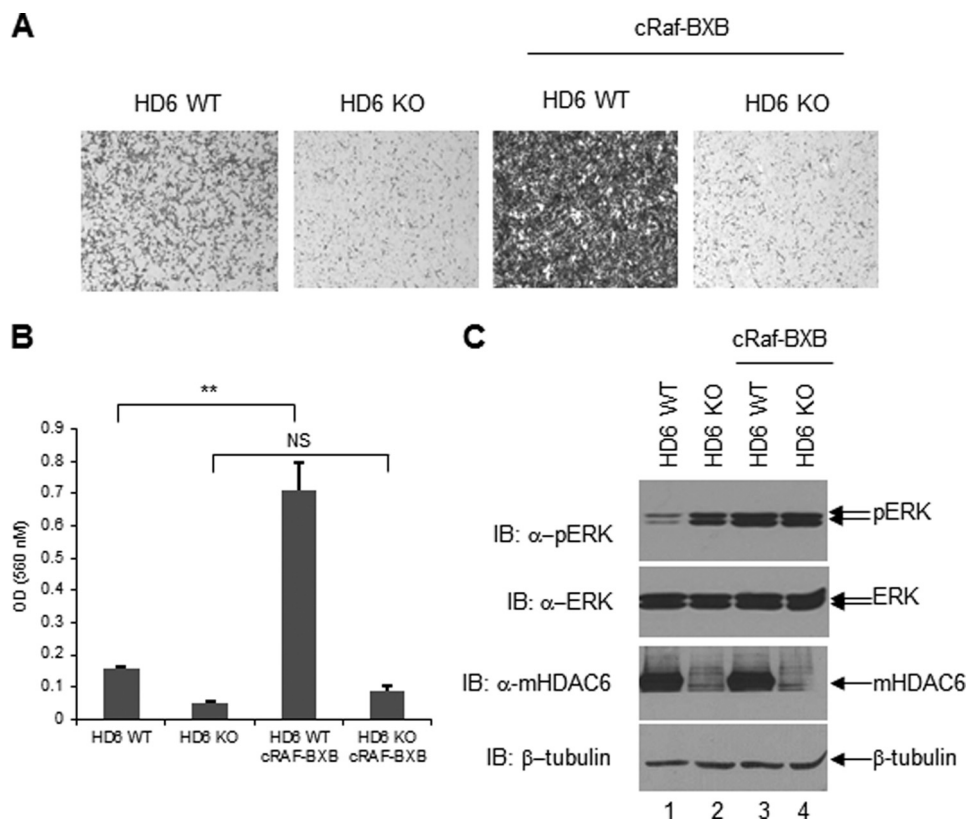


FIGURE 9. **Active Raf promotes cell migration via HDAC6.** *A*, c-RAF-BXB plasmid was transfected into HDAC6 WT or KO MEFs. Cell migration assays were performed as described under "Experimental Procedures." The snapshots of the migratory cells are shown as indicated. *B*, the stained migratory cells were de-stained in 2% SDS and then quantified at $A_{560\text{nm}}$. A bar graph was made after the quantification. *C*, anti-pERK, anti-ERK, anti-mHDAC6, and anti- β -tubulin Western blot (IB) analyses were performed using the cell lines described in *A* and *B*. NS refers to not significant. ** indicates $p < 0.01$.

phorylation decreased its TDAC activity. Intriguingly, knock-down of ERK2 did not alter the TDAC activity as did knock-down of ERK1. It seems that ERK1- but not ERK2-mediated phosphorylation regulates HDAC6 TDAC activity. In agreement with these data, phosphorylation of HDAC6 by recombinant ERK1 moderately increased HDAC6 TDAC activity (Fig. 8C). Furthermore, phosphorylation of HDAC6 *in vivo* with the active Ras also increased HDAC6 TDAC activity (data not shown). Overall, our data suggest that phosphorylation of HDAC6 increases its TDAC activity.

Active c-Raf-mediated Cell Migration Is via HDAC6—To investigate whether the Raf-MEK-ERK signaling cascade influences cell migration via HDAC6, we overexpressed active Raf in wild type or HDAC6-null MEFs. As shown in Fig. 9A and B, active Raf dramatically increased cell migration in wild type MEFs compared with HDAC6-null MEFs in Boyden chamber migration assays. Active Raf indeed elevated phospho-ERK levels in both wild type and HDAC6-null MEFs (Fig. 9C, lanes 3 and 4). Interestingly, HDAC6-null MEFs possess higher phospho-ERK levels compared with wild type (Fig. 9C, lane 2 versus lane 1). This observation raised the possibility that HDAC6 plays a role in suppressing ERK activity. Further examination will be warranted.

HDAC6-null MEFs Rescued by S1035A Displayed Decreased Migration Potential Compared with Those Rescued by Wild Type—Studies have implicated that HDAC6 promotes cell migration through the deacetylation of its cytoplasmic substrates α -tubulin or cortactin (11, 13). As shown in Fig. 8A,

HDAC6 (S1035A) harbors lower TDAC activity compared with wild type *in vivo*. We next determined whether HDAC6(S1035A) would decrease cell migration compared with wild type in a cell line model. As shown in Fig. 10, A and B, HDAC6-null MEFs rescued by S1035A exhibited significantly lower migration potential than those rescued by wild type. The levels of HDAC6 protein expression in those rescued clones were approximately equal (Fig. 10C). In agreement with Fig. 8A, the HDAC6(S1035A) rescued clone displayed lower TDAC activity compared with the wild type rescued one, whereas both clones exhibited similar HDAC activities for histone H3 (Fig. 10C). In conclusion, our results indicate that dephosphorylation of HDAC6 at the Ser-1035 site decreases its ability to induce cell migration.

DISCUSSION

In this study, we have demonstrated that HDAC6 is a novel ERK substrate. We have also identified Ser-1035 as a major ERK phosphorylation site in HDAC6. We have shown that HDAC6-null MEFs rescued by nonphosphorylation mimicking mutant displayed reduced migration potential compared with those rescued by wild type. Overall, our results indicate that the EGFR-Ras-Raf-MEK-ERK signaling pathway stimulates cell migration via HDAC6 phosphorylation at Ser-1035 (Fig. 10D).

Apart from the Ser-1035 site, we have also identified Thr-1031 as a phosphorylation site in HDAC6 (Figs. 1 and 2). However, as shown in Fig. 4C, HDAC6(S1035A) mutant could not be phosphorylated efficiently by upstream kinases of ERK, sug-

ERK Phosphorylates HDAC6

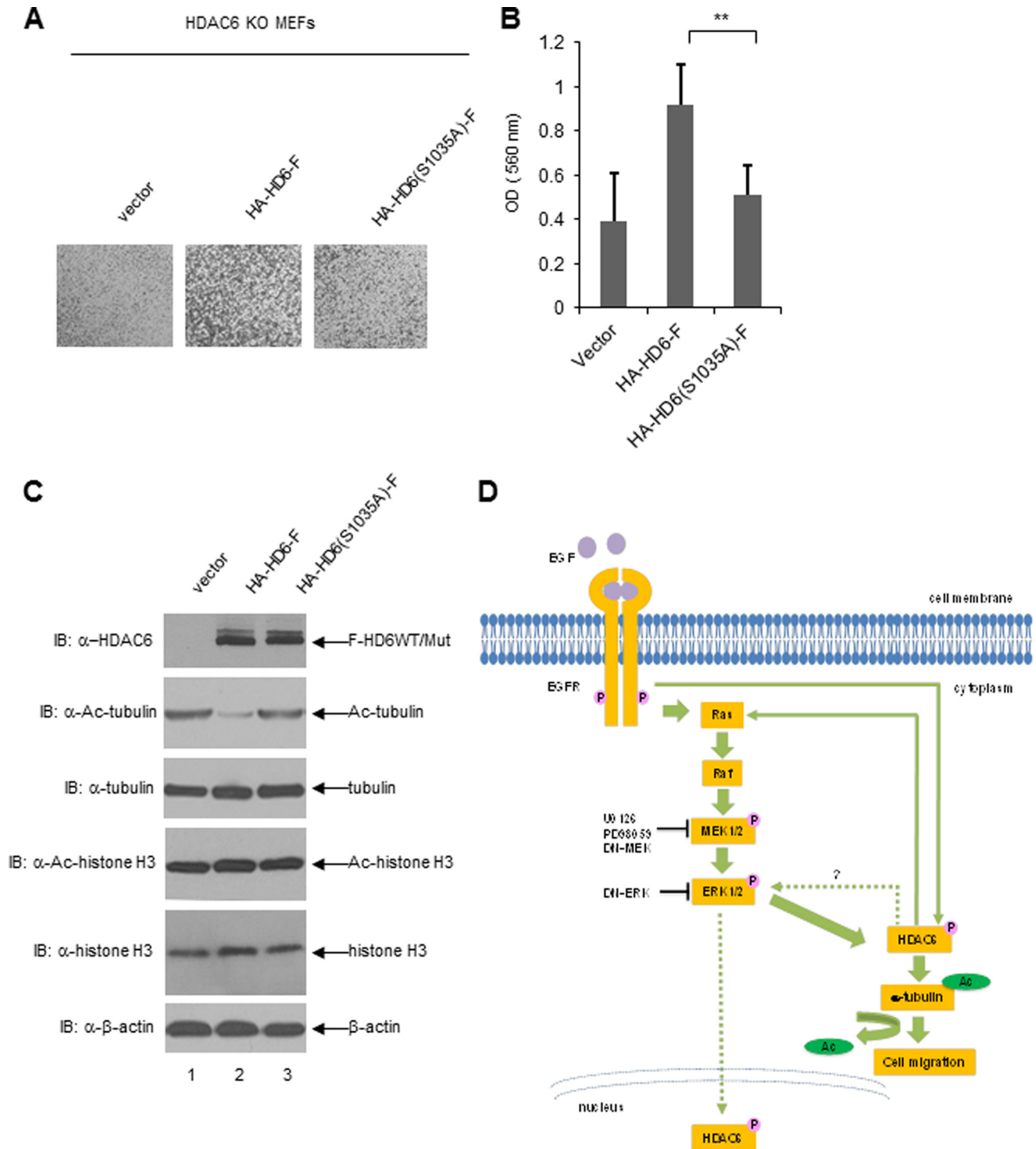


FIGURE 10. S1035A mutant of HDAC6 exerts decreased migration potential in MEFs. *A*, cell migration assays were carried out using HDAC6 KO MEFs cells stably expressing empty vector, HA-HD6-F, or HA-HD6(S1035A)-F as described under "Experimental Procedures." Snapshots of the migratory cells are shown as indicated. *B*, the stained migratory cells were de-stained in 2% SDS then quantified at $A_{560\text{nm}}$. The bar graph represents the quantification. *NS* refers to not significant. ** indicates $p < 0.01$. *C*, Western blot (IB) analyses were performed using the indicated antibodies and the cell lines described in *A*. *D*, the working model shows how the EGF-EGFR-Ras-Raf-MEK-ERK signaling cascade regulates cell migration via HDAC6.

gesting that Ser-1035 is the major site targeted by ERK and that Thr-1031 may not be important for ERK-mediated phosphorylation. Nevertheless, given the proximity of Thr-1031 and Ser-1035, we cannot rule out the possibility that Thr-1031 phosphorylation/dephosphorylation influences HDAC6 function. Future studies employing Thr-1031 phosphorylation or

dephosphorylation mimicking single mutant (T1031A and T1031D) or Thr-1031 and Ser-1035 double mutants (T1031A/S1035A and T1031D/S1035D) are warranted.

Early investigations have demonstrated that ERK1/2 is associated with microtubules in the cytoplasm (24). Upon mitogen stimulation ERK1/2 is translocated to the nucleus. As shown in

Fig. 6, HDAC6/ERK1/2 interaction was found in both nuclear and cytoplasmic fraction of HeLa S3 cells. This observation suggests that HDAC6 may also be one of the nuclear substrates of ERK1/2. Because EGF has pro-migratory function, we examined whether EGF affects HDAC6 phosphorylation. Our data indicate that EGF increases HDAC6 Ser-1035 phosphorylation (Fig. 5), suggesting that EGF induced cell migration via the EGFR-Ras-Raf-MEK-ERK signaling cascade. A previous report showed that EGFR phosphorylates HDAC6 at Tyr-570 and decreases TDAC activity of HDAC6 (19). It is conceivable to speculate that EGFR-mediated HDAC6 tyrosine phosphorylation increases α -tubulin acetylation. Thus, it remains to be determined how HDAC6 Ser-1035 and Tyr-570 in combination affect α -tubulin acetylation and cell migration.

As shown in Fig. 8, HDAC6(S1035A) displayed lower TDAC activity compared with the wild type *in vivo*. However, in an *in vitro* TDAC assay, HDAC6(S1035A) exhibited higher activity than the wild type (data not shown). This is very intriguing. It is possible that more factors are associated with HDAC6 as well as its substrate, microtubules, *in vivo* than *in vitro*. More investigations are certainly warranted. Although HDAC6 is also located in the nucleus (Fig. 6A), we did not observe any difference among empty vector, HDAC6 wild type, or S1035A mutant in terms of influencing core histone acetylation *in vivo* (Figs. 8A and 10C). This could be because neither HDAC6 wild type nor mutant interacts with core histones (data not shown). We also examined the affinity of HDAC6 wild type or HDAC6(S1035A) to tubulin by immunoprecipitation-Western blot analysis (data not shown). Both HDAC6 wild type and Ser-1035 mutant bind to tubulin, but we did not observe any difference in terms of binding between HDAC6 wild type or HDAC6(S1035A) to α -tubulin (data not shown). Therefore, the mechanisms by which HDAC6(S1035A) displays reduced TDAC activity compared with the wild type remain to be explored.

Cortactin is an F-actin-binding protein and plays an essential role in cell migration (13, 31, 32) and angiogenesis (32). It is also an HDAC6 substrate (32). However, we did not observe any differences in the deacetylation of cortactin between the unphosphorylation and phosphorylation mimicking mutants of HDAC6 at the Ser-1035 site (data not shown). This observation is consistent with the fact that ERK is associated with the microtubules but not with actin or intermediate filaments (24). This suggests that ERK-mediated HDAC6 phosphorylation modulates tubulin-dependent cell migration. Further studies in our laboratory are aiming to identify novel proteins that may be interacting with the phosphorylated form of HDAC6 to promote cell migration. Because HDAC6 interacts with ERK, it is conceivable to speculate that HDAC6 could also regulate ERK. In fact, we found that ERK activity is elevated in HDAC6-null MEFs (Fig. 9C). It is possible that HDAC6 modulates ERK acetylation, phosphorylation, and subsequently enzymatic activity.

It is well documented that HDAC6 is involved in transformation and tumorigenesis (33). Elevated expression of HDAC6 was observed in a panel of non-small cell lung cancer cell lines

compared with the nontransformed cells.⁴ HDAC6 overexpression leads to increased cell motility which is one of the key steps in tumor metastasis (34–37). The EGFR-Ras-Raf-MEK-ERK signaling pathway has attracted considerable attention as a target for anti-cancer therapy. It is plausible that the combination of HDAC6-selective inhibitors and inhibitors targeting the EGFR-Ras-Raf-MEK-ERK signaling cascade may represent an effective strategy for cancer treatment.

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