Histone Deacetylase 6 (HDAC6) Promotes the Pro-survival Activity of 14-3-3-ζ via Deacetylation of Lysines within the 14-3-3-ζ Binding Pocket*

Received for publication, August 26, 2014, and in revised form, March 11, 2015 Published, JBC Papers in Press, March 13, 2015, DOI 10.1074/jbc.M114.607580

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Background: 14-3-3-ζ promotes cell survival by interacting with a network of proteins. However, its regulation remains poorly understood.

Results: Proteomics identified several acetyl-lysines on 14-3-3-ζ, two of which are regulated by HDAC6 and impact 14-3-3-ζ activity.

Conclusion: HDAC6 controls 14-3-3-ζ binding via deacetylation of lysines within its binding pocket.

Significance: These findings provide a novel mechanism linking HDAC6 to the pro-survival activity of 14-3-3-ζ.

The phospho-binding protein 14-3-3-ζ acts as a signaling hub controlling a network of interacting partners and oncogenic pathways. We show here that lysines within the 14-3-3-ζ binding pocket and protein–protein interface can be modified by acetylation. The positive charge on two of these lysines, Lys49 and Lys120, is critical for coordinating 14-3-3-ζ phosphoprotein interactions. Through screening, we identified HDAC6 as the Lys49/Lys120 deacetylase. Inhibition of HDAC6 blocks 14-3-3-ζ interactions with two well described interacting partners, Bad and AS160, which triggers their dephosphorylation at Ser112 and Thr642, respectively. Expression of a deacetylation-refractory K49R/K120R mutant of 14-3-3-ζ rescues both the HDAC6 inhibitor-induced loss of interaction and Ser112/Thr642 phosphorylation. Furthermore, expression of the K49R/K120R mutant of 14-3-3-ζ inhibits the cytotoxicity of HDAC6 inhibition. These data demonstrate a novel role for HDAC6 in controlling 14-3-3-ζ binding activity.

The 14-3-3 protein family includes seven human isoforms that regulate diverse processes, including metabolism, cell cycle control, protein trafficking, cell motility, and apoptosis. Although these functions are somewhat dispersed between the different isoforms, the ζ isoform plays a prominent role in promoting cell growth and survival pathways (1). Like other 14-3-3 isoforms, 14-3-3-ζ functions by binding to and modulating the activity of a large network of serine/threonine-phosphorylated proteins. Depending on the phosphorylated protein in question, the effect of 14-3-3-ζ binding varies from activation, suppression, sequestration, or scaffold-like activity.

14-3-3-ζ coordinates the activation of several well characterized oncogenic, metabolic, and pro-survival pathways. For example, in response to insulin, 14-3-3-ζ binds to the AKT-phosphorylated GTPase protein AS160. This insulin-dependent binding event promotes translocation of the glucose transporter GLUT4 to the plasma membrane (2). Once inserted into the membrane, GLUT4 is active and supports Warburg metabolism and proliferation in breast cancer and multiple myeloma (3–5). As another example, in response to glucose, 14-3-3-ζ interacts with and suppresses Bad, a critical BH3-only protein required for chemotherapy-induced apoptosis (6–9). 14-3-3-ζ expression also promotes epithelial to mesenchymal transition by cooperating with HER2 in breast cancer (10), enhances anchorage-independent growth (11, 12), and suppresses anoikis (12). Many of these phenotypes have been attributed to 14-3-3-ζ-mediated regulation of the TGF-β/Smads, IGF1R, PI3K/AKT, and β-catenin pathways (reviewed in Ref. 13). Furthermore, 14-3-3-ζ interacts with and suppresses both Caspase-2 (14, 15) and the Forkhead family member FKHRL1, preventing the expression of the pro-apoptotic Bax and Fas proteins (16).

Clinical studies demonstrate a correlation between high 14-3-3-ζ expression and poor prognosis in several cancer types, including lung, head and neck, glioblastoma, and breast cancer (10–13, 17, 18). Furthermore, Bergamaschi et al. (19) demonstrated that high 14-3-3-ζ expression is positively correlated with breast tumor relapse, metastasis, tamoxifen treatment, and tamoxifen resistance. Moreover, we show here that 14-3-3-ζ expression is particularly high in the more aggressive breast cancer subtypes and in tumors with negative receptor status. In support of an oncogenic function for 14-3-3-ζ, others have shown that the depletion of 14-3-3-ζ suppresses proliferation and enhances chemosensitivity in xenografted breast tumors (10, 11). Based on this rationale, several groups have attempted to develop 14-3-3-ζ inhibitors (reviewed in Refs. 20 and 21), 2 but no 14-3-3-ζ-directed therapeutic strategies are currently being used in the clinic.

* This work was supported by funding from the Elsa U. Pardee Foundation (to J. L. A.) and a Simmons Center for Cancer Research Award (to L. N. H. and J. L. A.).

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The current paradigm of 14-3-3ζ regulation states that 14-3-3ζ interactions depend primarily on the serine/threonine phosphorylation of binding partners, thus, phosphorylation of binding partners is considered a major determinant of 14-3-3ζ binding activity. Comparatively little is known about other potential determinants of 14-3-3ζ activity (22–25). In search of post-translational modifications that regulate 14-3-3ζ directly, we identified several lysines on 14-3-3ζ that are modified by acetylation. Lysine-to-glutamine (Lys to Gln) mutations at two of these lysines, Lys49ζ and Lys120ζ, abolish 14-3-3ζ binding activity. In an effort to modulate 14-3-3ζ binding via acetylation, we developed site-specific antibodies to both acetyl-Lys49ζ and acetyl-Lys120ζ and identified HDAC6 as the 14-3-3ζ-targeted deacetylase.

Recent studies implicate HDAC6 as a therapeutic target in cancer (26–29), and our data suggest that HDAC6 inhibition may provide a means to inhibit 14-3-3ζ. Toward this end, we show that inhibition of HDAC6 triggers dissociation of 14-3-3ζ from AS160 and Bad, two well characterized binding partners. We also show that these dissociation events lead to decreased AS160 Thr642 and Bad Ser112 phosphorylation. Importantly, we also show that these dissociation events lead to decreased AS160 Thr642 and Bad Ser112 phosphorylation. Importantly, we show that inhibition of HDAC6 triggers dissociation of 14-3-3ζ from AS160 and Bad, two well characterized binding partners. We also show that these dissociation events lead to decreased AS160 Thr642 and Bad Ser112 phosphorylation. Importantly, we show that inhibition of HDAC6 triggers dissociation of 14-3-3ζ from AS160 and Bad, two well characterized binding partners. We also show that these dissociation events lead to decreased AS160 Thr642 and Bad Ser112 phosphorylation. Importantly, we show that inhibition of HDAC6 triggers dissociation of 14-3-3ζ from AS160 and Bad, two well characterized binding partners. We also show that these dissociation events lead to decreased AS160 Thr642 and Bad Ser112 phosphorylation. Importantly, we show that inhibition of HDAC6 triggers dissociation of 14-3-3ζ from AS160 and Bad, two well characterized binding partners. We also show that these dissociation events lead to decreased AS160 Thr642 and Bad Ser112 phosphorylation.
captoethanol, 0.15 M Tris, bromphenol blue). Samples were loaded and separated on 12% polyacrylamide gels run along side a Bluestain protein ladder (number P007, GoldBio, St. Louis, MO). Western blots were visualized on LI-COR Odyssey system (Lincoln, NE).

**Antibodies**—Primary antibodies included: AS160 (number 24475), Atg9 (number 13509S), Bad (number 9292S), FLAG (number 8146S), HDAC6 (number 7612S), phospho-14-3-3 binding motif (number 9601S), phospho-AS160 (Thr442, number 4288S), and phospho-Bad (Ser112, #9291S) purchased from Cell Signaling Technology (Beverly, MA); 14-3-3 (sc-1019), actin (sc-6161-R), HA (sc-7392), kinesin (sc-28538), and Liprin-β (sc-22876) purchased from Santa Cruz Biotechnology (Dallas, TX); polyhistidine was provided by Dr. Barry Willardson (Brigham Young University, Provo, UT). Secondary antibodies were purchased from LI-COR (Lincoln, NE) including: donkey anti-rabbit (number 926–32213), goat anti-mouse (number 926–68070), donkey anti-mouse (number 926–32212), and goat anti-rabbit (number 926–68071).

**Ac-Lys** and **Ac-Lys** Antibody Purification—Rabbit serum was prepared from animals immunized with an acetylated Lys peptide or Lys peptide sequence conjugated to keyhole limpet hemocyanin (Pocono Rabbit Farm and Laboratory, Inc.). Ac-Lys polyclonal antibody was immunopurified from serum using acetylated Lys antibody as previously described (30). Briefly, biotinylated Lys peptide was bound to streptavidin-agarose (number 20359, Thermo Scientific) and incubated overnight with rabbit serum diluted in buffer (150 mM Tris, pH 7.5, 20 mM NaCl) (1:1) to deplete serum of non-acetyl-specific antibody. After incubation, serum was removed and transferred to acetylated Lys peptide bound to streptavidin resin for overnight incubation. Acetylated Lys bound resin was then washed twice with buffer. Acetyl-specific antibody was eluted off resin using gravity fed elution (100 mM glycine, pH 2.8). Fractions were collected in neutralization buffer (1M Tris, pH 8.5) and tested for antibody titer. Ac-Lys antibody was produced as described above and purified on a protein-A resin, followed by elution with 100 mM glycine (pH 2.8) into neutralization buffer (above) to a final pH of 7.5.

**In Vitro Acetylation Assay**—BL21(DE3) cells were transformed with pGEX-His-14-3-3 and grown in LB broth with ampicillin (100 μg/ml). Once optical density reached 0.6, cells were induced with isopropyl 1-thio-β-d-galactopyranoside (1 mM) and allowed to express for 4 h. Bacterial cells were pelleted, lysed in buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) containing a protease inhibitor mixture (number 88665, Pierce). 2.5 mg of total protein lysate was loaded onto a Superdex 200 10/300 GL gel filtration column running on an AKTA FPLC system (GE Healthcare). Cell lysate was fractionated at a flow rate of 500 μl/min using hypotonic lysis buffer. 300 μl of elution fractions were collected and separated on a 12% SDS-PAGE gel. Following gel transfer, membranes were probed for HA epitope.

**Flow Cytometry**—HEK-293T cells were plated in 6-cm dishes at a confluence of 75%. Cells were transfected with HA-14-3-3 WT or RR mutant expression plasmid. HEK-293T cells were then trypsinized and plated in 6-well plates at 30% initial confluence and allowed to express for 36 h. Cells were treated with either vehicle or tubacin (10 or 40 μM) for 24 h. Following treatment, the 6-well plates were prepared for flow cytometry analysis. Briefly, cells were trypsinized and washed twice with cold PBS. Next, cells were resuspended in cold PBS containing 0.2% FBS and 1 μg/ml of propidium iodide. Cells were analyzed for cell death using BD FACS Canto system.

**RESULTS**

**14-3-3 Expression**—**Elevated in Receptor-negative Breast Tumors, and High Expression Correlates with Decreased Patient Survival**—Consistent with the role of 14-3-3 in driving multiple oncogenic pathways, previous studies have demonstrated that 14-3-3 expression is increased in several cancer types, including breast cancer (11, 13, 19). To acquire breast cancer subclass-specific information on 14-3-3, we analyzed 14-3-3 transcript levels from primary human breast tumors available through The Cancer Genome Atlas (TCGA) database. We found elevated 14-3-3 expression levels in the more aggressive basal, HER2-positive, and luminal B subtypes of breast cancer compared with lower expression levels in the typically less aggressive luminal A tumors (Fig. 1A). In addition, we found that breast tumors that lack estrogen and progesterone receptors show higher 14-3-3 expression levels (Fig. 1B). Interestingly, given a previous study showing that 14-3-3 acts cooperatively with HER2 to drive growth and metastasis in breast cancer (10), we found that 14-3-3 expression is higher in HER2-positive tumors (Fig. 1B). Finally, by comparing patients within the highest and lowest quintiles of 14-3-3 expression across all breast cancer subtypes in the TCGA database, we found that high 14-3-3 expression is correlated with decreased patient survival (Fig. 1C). Thus, both tumor expression data and our current understanding of 14-3-3-driven pro-growth and survival pathways support the notion of 14-3-3 as a therapeutic target to disrupt a hub of oncogenic signaling.
Acetylated Lysines within the 14-3-3/H9256 Binding Pocket and Protein-Protein Interface—Although several groups have worked to develop inhibitors of 14-3-3/H9256 (reviewed in Ref. 21), these efforts have proven challenging at least in part due to difficulty in developing high-throughput screens for 14-3-3/H9256 activity (although recent progress has been made in this area (31)). This challenge motivated us to look for other ways to manipulate 14-3-3/H9256 binding, potentially via 14-3-3/H9256 regulatory mechanisms. Using immunoprecipitation with acetyl-lysine antibodies followed by LC-MS/MS analysis, we found that several lysines within the 14-3-3/H9256 binding pocket and protein-protein interface were targets of acetylation. As shown in Fig. 2A, which is derived from a published crystal structure of 14-3-3 bound to c-Raf (2.2-Å resolution (32)), two of these lysines, Lys49 and Lys120, were previously identified in a large-scale proteomics screen (33) and are posited to interact with the negatively charged Ser/Thr phosphorylation of the binding partner. Lys49, in particular, is known to be important for 14-3-3/H9256 interactions (34, 35). Lys193 and Lys212 are located outside this binding pocket, but within regions of 14-3-3/H9256 that may interface with the interacting partner. Fig. 2B shows the high degree of

Acetylated Lysines within the 14-3-3ζ Binding Pocket and Protein-Protein Interface—Although several groups have worked to develop inhibitors of 14-3-3ζ (reviewed in Ref. 21), these efforts have proven challenging at least in part due to difficulty in developing high-throughput screens for 14-3-3ζ activity (although recent progress has been made in this area (31)). This challenge motivated us to look for other ways to manipulate 14-3-3ζ binding, potentially via 14-3-3ζ regulatory mechanisms. Using immunoprecipitation with acetyl-lysine antibodies followed by LC-MS/MS analysis, we found that several lysines within the 14-3-3ζ binding pocket and protein-protein interface were targets of acetylation. As shown in Fig. 2A, which is derived from a published crystal structure of 14-3-3ζ bound to c-Raf (2.2-Å resolution (32)), two of these lysines, Lys49 and Lys120, were previously identified in a large-scale proteomics screen (33) and are posited to interact with the negatively charged Ser/Thr phosphorylation of the binding partner. Lys49, in particular, is known to be important for 14-3-3ζ interactions (34, 35). Lys193 and Lys212 are located outside this binding pocket, but within regions of 14-3-3ζ that may interface with the interacting partner. Fig. 2B shows the high degree of
sequence conservation of these lysines (highlighted in red) from plants to humans.

**Acetylation-mimicking Mutations Abolish 14-3-3ζ Interactions**—To estimate the impact of each of these acetylations on 14-3-3ζ binding activity, we generated Lys to Gln mutations, which mimic the change in charge associated with acetylation. We assessed the binding activity of these mutants by HA-14-3-3ζ overexpression/co-immunoprecipitation and Western blotting for several readily detectable 14-3-3ζ interacting proteins, including known interactors such as AS160, kinesin, and Bad as well as Liprin-β, which we recently identified in an interactomics screen (36) (Fig. 3A). Both the K49Q and K120Q mutations blocked 14-3-3ζ interactions, with K49Q having the most potent inhibitory effect (Fig. 3A). In contrast, we were unable to see any consistent effect with the K193Q and K212Q mutations (Fig. 3A).

To examine the effect of the K49Q mutation on a larger scale, we performed gel filtration chromatography with HEK-293T lysates expressing either HA-tagged WT 14-3-3ζ or the K49Q mutant. Consistent with published reports showing 14-3-3ζ overexpression/co-immunoprecipitation and Western blotting for several readily detectable 14-3-3ζ interacting proteins, including known interactors such as AS160, kinesin, and Bad as well as Liprin-β, which we recently identified in an interactomics screen (36) (Fig. 3B). Strikingly, the Gln substitution at Lys⁴⁹ alone was sufficient to disrupt interactions with high molecular complexes, as this mutant was found shifted into the dimer/monomer range (Fig. 3B).

**Deacetylation-mimicking Mutations Rescue the Loss of 14-3-3ζ Interactions**—To determine whether the loss of 14-3-3ζ binding activity caused by the K49Q and K120Q substitutions was due simply to a disruption of the charge as opposed to some other alteration, such as blocking another lysine-targeted post-translational modification, we substituted arginine at Lys⁴⁹ and Lys¹²⁰ and measured 14-3-3ζ interactions as in Fig. 3A. In addition to the interacting partners mentioned above, we also measured 14-3-3ζ binding to Atg9, which we recently published as a new 14-3-3ζ binding partner (36). As shown in Fig. 4, A and B, the K49R/K120R double mutant effectively restores 14-3-3ζ binding activity to at or near WT levels.

As a measure of 14-3-3ζ binding, we assessed the activation status of Bad by monitoring its phosphorylation at Ser¹¹². The inhibitory phosphorylation of Bad at Ser¹¹² and Ser¹³⁶ leads to 14-3-3ζ binding (6), which maintains Bad inhibition likely by shielding the phosphorylations from phosphatase activity. We observed an increase in Ser¹¹² phosphorylation in the presence of WT 14-3-3ζ. Consistent with the interaction data, this increase in Ser¹¹² phosphorylation was lost in cells expressing the K49Q and K120Q mutants, but recovered by expression of
**HDAC6 Regulates 14-3-3ζ via Deacetylation of Lys**

K49R/K120R (Fig. 4A; see phosho-Bad/Bad overlay). Based on these data and the estimated distance between both Lys49 and Lys120 and the phosphate of the binding partner (~3 and ~6 Å, respectively; Fig. 2), we posit that acetylation at these sites disrupts a positive-negative electrostatic interaction between the primary amine of lysine and the phosphate that is important for stabilizing the 14-3-3ζ-protein complex (35).

**HDAC6 Deacetylates 14-3-3ζ at Lys49 and Lys120**—The strong inhibitory effect of charge disruption at Lys49 and Lys120 suggests that modulating the enzymes that govern acetylation at these sites may provide a means to inhibit 14-3-3ζ binding activity. Our attempts to detect acetylation at these sites with pan-acetyl-lysine antibodies yielded inconsistent results. Therefore, we generated site-specific acetyl antibodies to each lysine. Because the Gln substitution at Lys49 had a slightly stronger inhibitory effect on 14-3-3ζ than K120Q, we focused our initial efforts on Lys49. By treating HEK-293T cells expressing HA-tagged 14-3-3ζ with a panel of deacetylase inhibitors, we found that drugs targeting class I and II HDACs, including trichostatin A and SAHA, triggered an increase in Lys49 acetylation (Fig. 5A). Importantly, mutation of Lys49 to arginine abolished the tubastatin A-induced increase in acetylation, indicating the specificity of the antibody (Fig. 5B, last lane). Likewise, tubacin, a structurally distinct HDAC6 inhibitor (40), also triggered Lys49 acetylation in the osteosarcoma cell line U2OS (Fig. 5C). Furthermore, acetylation peaked at 6 h post-treatment for both drugs (Fig. 5D). Fig. 5E shows the quantified data of the HDAC6 inhibitor-induced increase in Lys49 acetylation from four experiments. To rule out potential off-target effects of drug treatment, we confirmed that siRNA against HDAC6 induced Lys49 acetylation (Fig. 5F, G and H) and recombinant HDAC6 was capable of deacetylating Lys49 *in vitro* (Fig. 5H).

Next, we asked whether we could detect acetylation of endogenous 14-3-3ζ. Consistent with our previous results, treatment of the triple negative breast cancer line MDA-MB-231 with tubacin triggered acetylation of endogenous 14-3-3ζ at Lys49 (Fig. 5I). Given the proximity of Lys49 and Lys120 in the three-dimensional structure of 14-3-3ζ, we suspected that both lysines were substrates of HDAC6. Indeed, HDAC6 inhibition resulted in acetylation of Lys120, as detected on endogenous 14-3-3ζ in MDA-MB-231 cells (Fig. 5J) and immunoprecipitated HA-tagged 14-3-3ζ from HEK-293T cells (Fig. 5K). As in Fig. 4B, the K120R mutant served as a specificity control for the acetyl-Lys120 antibody (Fig. 5K, last lane). Taken together, these data suggest that HDAC6 deacetylates Lys49 and Lys120 and thereby may regulate 14-3-3ζ binding activity.

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3 The abbreviations used are: HDAC, histone deacetylase; SAHA, suberanilohydroxamic acid.

**FIGURE 5.** HDAC6 deacetylates 14-3-3ζ at Lys49 and Lys120. A, HEK-293T cells were transfected with WT HA-tagged 14-3-3ζ expression vector and treated with various HDAC inhibitor drugs (SAHA, 10 μM; trichostatin A (TSA), 10 μM; tubastatin A (Tuba), 40 μM; salermide, 40 μM; and EX-527, 40 μM) or vehicle (dimethyl sulfoxide) under normal conditions for 9 h. Cell lysates were immunoprecipitated with HA resin and run on 12% SDS-PAGE immunoblot to detect Lys49 acetylation (40 μM) or vehicle for 9 h followed by HA immunoprecipitation and immunoblot as described in the legend to Fig. 4A (exception: HA is in the green channel, Ac-K49 is in the red channel). Representative data are of at least four replicate experiments. B, HEK-293T cells overexpressing WT or K49R mutant HA-tagged 14-3-3ζ were treated with tubastatin A (40 μM) or vehicle for 9 h followed by HA immunoprecipitation and immunoblot as described in the legend to Fig. 4A. Representative data are of at least three replicate experiments. C, U2OS cells overexpressing WT HA-tagged 14-3-3ζ were treated with tubacin (30 μM) for 8 h followed by HA immunoprecipitation and immunoblot as described in the legend to Fig. 4A. Representative data are of at least three replicate experiments. D, HEK-293T cells overexpressing WT HA-tagged 14-3-3ζ were treated with tubacin (35 μM) or tubastatin A (35 μM) in a 2-, 4-, 6-, and 8-h time course followed by HA immunoprecipitation and immunoblot as described in the legend to Fig. 4A. Representative data are of at least three replicate experiments. E, 8 h HDAC6 inhibitor time points from four replicate experiments of panel D were quantified. Relative acetylation signal is acetyl-Lys49 signal divided by HA signal. Bands were quantified by Li-COR infrared imaging. F, HEK-293T cells overexpressing WT HA-14-3-3ζ were transfected with HDAC6 siRNA (75 nM) or scrambled siRNA (75 nM). Cell lysates were HA immunoprecipitated and immunoblotted as in A. Representative data are of at least three replicate experiments. G, three replicate experiments from panel F were quantified as in panel E. H, recombinant His-tagged 14-3-3ζ was expressed in BL21-DE3 Escherichia coli and purified on a nickel resin. Resin-bound 14-3-3ζ was incubated with p300 acetyltransferase followed by vehicle, HDAC6 recombinant enzyme, or HDAC6 enzyme and tubacin (40 μM). 14-3-3ζ was analyzed by 12% SDS-PAGE and immunoblotted with Ac-Lys49 and His (14-3-3ζ) antibody. Representative data are of at least three replicate experiments. I, MDA-MB-231 breast cancer cells were treated with tubacin (30 μM) or vehicle for 8 h. Endogenous 14-3-3ζ was analyzed by resolving cell lysate (40 μg) using 12% SDS-PAGE and immunoblotting with Ac-Lys120 (green), 14-3-3ζ (red), and actin. Representative data are of at least three replicate experiments. J, MDA-MB-231 breast cancer cells were treated as described in the legend to Fig. 4G. Endogenous 14-3-3ζ was analyzed as described in the legend to Fig. 4G. Representative data are of at least three replicate experiments. K, HEK-293T cells overexpressing WT or K120R mutant HA-tagged 14-3-3ζ were treated with tubacin (40 μM) or vehicle for 9 h followed by HA immunoprecipitation and immunoblot with Ac-Lys120 (green) and HA (red). Representative data are of at least three replicate experiments.
Inhibition of HDAC6 Triggers a Loss of 14-3-3ζ Interactions and an Increase in Cell Death, Both of Which Are Rescued by the K49R/K120R Double Mutant—Our data suggest a model in which high HDAC6 activity promotes 14-3-3ζ binding activity by maintaining Lys49 and Lys120 in their deacetylated states. Conversely, as suggested by data in Fig. 5, HDAC6 inhibition allows a progressive build up of acetylation (presumably catalyzed by an unidentified acetyltransferase) at these residues. To test whether this increase in acetylation induces a loss of 14-3-3ζ binding activity, we chose to focus on the 14-3-3ζ-interacting proteins AS160 and Bad. AS160 is a Rab-GTase activating protein involved in GLUT4 translocation to cell membranes. Once on the membrane, GLUT4 serves as a major receptor for glucose uptake in breast, multiple myeloma, and other cancers (3–5). Phosphorylation of AS160 at Thr642 triggers the binding of 14-3-3 proteins (ζ and other isoforms), which leads to AS160 activation and GLUT4 translocation (2, 41). Bad is a pro-apoptotic BH3-only protein that transduces an apoptotic signal to mitochondria in response to various chemotherapeutics (6–9). Phosphorylation of Bad at Ser112, as well as Ser136, leads to 14-3-3 binding and Bad inhibition (6). Both of these proteins can be readily detected in overexpression/co-immunoprecipitation experiments with 14-3-3ζ.

Using HEK-293T cells and FLAG-AS160 immunoprecipitation, we found that HDAC6 inhibition decreases AS160-14-3-3ζ binding (Fig. 6A, second and third lanes). Importantly, the loss of AS160-14-3-3ζ interaction was rescued by the K49R/K120R 14-3-3ζ mutant (Fig. 6A, fourth and fifth lanes), suggesting that acetylation is the key event triggering disruption of the complex. We also performed the converse immunoprecipitation experiment with HA-14-3-3ζ and Bad. Consistent with data in Fig. 6A, inhibition of HDAC6 triggers a decrease in the 14-3-3ζ-Bad interaction, which is rescued by the K49R/K120R mutant (Fig. 6B). Furthermore, as shown in Fig. 6, C and D, HDAC6 inhibition effectively dissociates endogenous 14-3-3ζ from overexpressed interacting partners AS160 and Atg9.

A critical function of 14-3-3 proteins is their ability to protect phosphorylations from removal by phosphatases. For example, 14-3-3ζ binding to an inhibitory phosphorylation on Cdc25 protects it from protein phosphatase-1 activity, thereby preventing Cdc25 activation and entry into M phase (42). Thus, we reasoned that forcing disruption of 14-3-3ζ via Lys49/Lys120 acetylation should lead to dephosphorylation of the interacting partner. Indeed, phosphorylation of AS160 at Thr642 and Bad at Ser112 tracked with 14-3-3ζ binding, showing decreased phosphorylation in cells treated with HDAC6 inhibitor, and more importantly, a recovery of phosphorylation (albeit only a marginal recovery with AS160) in the presence of the K49R/K120R mutant despite HDAC6 inhibition (Fig. 6, A and B, see overlay images).

HDAC6 inhibition enhances stress-induced cell death, and HDAC6 inhibitors are currently being evaluated as potential therapeutics in cancer (28, 43). HDAC6 has only a few bona fide substrates, including its best characterized substrate tubulin, as well as HSP90, cortactin, and MSH2 (43, 44). To test the role of 14-3-3ζ acetylation in the toxicity of HDAC6 inhibition, we analyzed the cytoprotective effect of both WT 14-3-3ζ and the K49R/K120R mutant (compared with mock controls) against HDAC6 inhibitor-induced cell death. Although the non-acetylatable mutant was unable to completely suppress cell death (supporting the involvement of other HDAC6 substrates), it showed a significantly enhanced cytoprotective effect compared with WT 14-3-3ζ (Fig. 6, E–G). These data suggest that inhibitory acetylation of 14-3-3ζ contributes to HDAC6 inhibitor-induced cell death. Based on these results, we posit that the
exploitation of 14-3-3ζ pathways with combination therapies may enhance the anti-tumor effect of HDAC6 inhibitors.

**DISCUSSION**

As a phospho-binding protein, 14-3-3ζ has been implicated in numerous pro-growth pathways, and both in vitro and in vivo data point toward its function as an oncogene (reviewed in Ref. 13). We show here a new and potentially exploitable mechanism of 14-3-3ζ control by HDAC6. Specifically, we demonstrate that 14-3-3ζ can be suppressed by HDAC6 inhibition and consequent acetylation of Lys99 and Lys120, which sit within the 14-3-3ζ binding pocket. These data raise several questions addressed below.

The positions of Lys99 and Lys120 within the 14-3-3ζ binding pocket raise the question of how acetylation occurs if the residues are buried between 14-3-3ζ and the interacting partner. Because 14-3-3ζ exists in equilibrium between bound and unbound states, we favor the idea that the unbound “free” pool of 14-3-3ζ is subjected to deacetylation/acetylation. This idea is supported by our unpublished2 in vitro experiments in which we could only acetylate recombinant 14-3-3ζ (using the p300 catalytic domain as in Fig. 5H) when in the unbound form. Conversely, incubation of 14-3-3ζ with a purified interacting partner prevented acetylation, suggesting that lysines within the binding pocket may be inaccessible to KAT activity when 14-3-3ζ is in a bound complex. In this model, HDAC6 inhibition and acetylation of 14-3-3ζ simply shift the bound-to-unbound equilibrium, increasing the pool of unbound 14-3-3ζ (Fig. 7).

Another question raised by these data is whether HDAC6 inhibition and consequent acetylation of Lys99 result in the global loss of 14-3-3ζ interacting partners. We suspect that this is not the case given the vast array of 14-3-3ζ interacting partners (37) and the only partially overlapping localization of HDAC6 and 14-3-3ζ. Thus, it seems more likely that the various pools of 14-3-3ζ (e.g. nuclear, cytosolic, membrane-associated, etc.) may be regulated by different KATs and KDACs.

Notably, in a previous study using an unbiased biotin-switch proteomics approach in *Xenopus* eggs, we identified 14-3-3ζ as one of several putative substrates of Sirt1 (14). However, as shown in Fig. 5A, we observed a very minor increase in Lys99 acetylation with EX-527 (a Sirt1 inhibitor) when compared with HDAC6 inhibition, which had the most robust effect in human cell lines. In future studies, it will be important to identify the subset of 14-3-3ζ interactors affected by HDAC6 inhibition as well as other KDACs (and KATs) that might regulate acetylation at Lys99 in different cellular compartments.

One compartment in which HDAC6 and 14-3-3ζ are likely to be intertwined is in the cytoskeletal and microtubule network. A key pro-survival function of HDAC6 is to recruit misfolded proteins to microtubule-associated dynein motor complexes for transport to aggresomes, which alleviates the toxic accumulation of misfolded proteins in cells (45–48). Interestingly, 14-3-3ζ has recently been implicated in aggresome formation (49). Specifically, 14-3-3 proteins have been shown to interact with and promote the association of the dynein motor complex with the Bcl-2-associate athanogene 3 (BAG3), a co-chaperone protein that helps target misfolded proteins to aggresomes (49). Thus, the deacetylation of 14-3-3ζ and consequent maintenance of its binding activity may be part of the mechanism of HDAC6-mediated aggresome formation. Our observation that acetylation-refractory mutants of 14-3-3ζ protect cells from HDAC6 inhibitors is consistent with this idea (Fig. 6, E–G). HDAC6 and 14-3-3ζ also associate with Tau (50, 51), a microtubule-associated protein involved in neurodegeneration. 14-3-3ζ binding to Tau promotes Tau hyperphosphorylation and is implicated in the formation of toxic Tau lesions in Alzheimer-diseased brains (50, 52). In future studies, it will be important to determine the role that HDAC6 plays in regulating these and other functions of 14-3-3ζ.

The strong inhibitory effect of acetylation on 14-3-3ζ binding raises the question of why and when would these acetylations
occur physiologically. Under high growth conditions, HDAC6 activity appears to stay consistently high, which would lead to a low stoichiometry of acetylation. In turn, active 14-3-3ζ would reinforce pro-growth signaling. Indeed, our LC-MS/MS analysis of total 14-3-3ζ immunoprecipitated from cells indicates a low baseline level of acetylation under normal growth conditions. A study by Xiaohong Zhang and colleagues (53) found that HDAC6 is phosphorylated by ERK, which increases HDAC6 activity and promotes cell motility. Thus we speculate that the acetylation status of 14-3-3ζ is dictated by pro-growth signaling. For example, growth factor withdrawal may lead to increased 14-3-3ζ acetylation as a result of lower ERK-mediated HDAC6 activity. This seems likely given that 14-3-3ζ promotes numerous cell growth pathways and therefore would need to be shut off during quiescence.

In summary, our data highlight a novel role for HDAC6 in the regulation of 14-3-3ζ. Going forward, it will be critical to determine the full impact of HDAC6 inhibition on the vast network of 14-3-3ζ interactions, and whether other 14-3-3 family members are similarly regulated, as this may illuminate novel combination strategies to enhance the efficacy of HDAC6 inhibitors.

Acknowledgments—We thank members of the Kornbluth (Duke University) and Andersen laboratories for their assistance and thoughtful discussions. We thank members of the Willardson laboratory (Brigham Young University) for generously sharing reagents.

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