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HDAC6 Inhibitors Modulate Lys49 Acetylation and Membrane Localization of β-Catenin in Human iPSC-Derived Neuronal Cells

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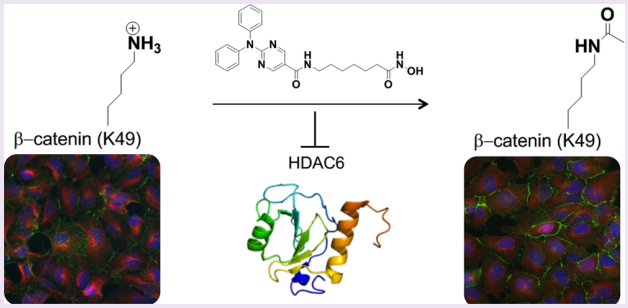
ABSTRACT: We examined the effects of isoform-specific histone deacetylase (HDAC) inhibitors on β-catenin post-translational modifications in neural progenitor cells (NPCs) derived from human induced pluripotent stem cells (iPSCs). β-catenin is a multifunctional protein with important roles in the developing and adult central nervous system. Activation of the Wnt pathway results in stabilization and nuclear translocation of β-catenin, resulting in activation of multiple target genes. In addition, β-catenin forms a complex with cadherins at the plasma membrane as part of the adherens junctions. The N-terminus of β-catenin has phosphorylation, ubiquitination, and acetylation sites that regulate its stability and signaling. In the absence of a Wnt signal, Ser33, Ser37, and Thr41 are constitutively phosphorylated by glycogen synthase kinase 3β (GSK3β). β-Catenin phosphorylated at these sites is recognized by β-transducin repeat-containing protein (βTrCP), which results in ubiquitination and degradation by the ubiquitin-proteasome pathway. The N-terminal regulatory domain of β-catenin also includes Ser45, a phosphorylation site for Casein Kinase 1α (CK1α) and Lys49, which is acetylated by the acetyltransferase p300/CBP-associated factor (PCAF). The relevance of Lys49 acetylation and Ser45 phosphorylation to the function of β-catenin is an active area of investigation. We find that HDAC6 inhibitors increase Lys49 acetylation and Ser45 phosphorylation but do not affect Ser33, Ser37, and Thr41 phosphorylation. Lys49 acetylation results in decreased ubiquitination of β-catenin in the presence of proteasome inhibition. While increased Lys49 acetylation does not affect total levels of β-catenin, it results in increased membrane localization of β-catenin.



β-Catenin is an evolutionarily conserved protein that is relevant in many different cellular contexts, including vital ones in the human central nervous system.[1−3] The canonical Wnt pathway plays an important role in the proliferation and differentiation of neural stem cells.[4] β-catenin is an integral component of the Wnt signaling pathway.[4,5] When the Wnt pathway is not active, β-catenin is retained in the cytoplasm in a "destruction complex" with Axin, adenomatous polyposis coli (APC) and GSK3β.[6,7] In this complex, β-catenin is constitutively phosphorylated by GSK3β. This phosphorylated version of β-catenin is recognized by βTrCP of the E3 ubiquitin ligase complex and degraded by the proteasome.[7] When the Wnt pathway is active, Wnt ligands bind to the Frizzled/LRP receptor complex, and Axin is recruited to the membrane by phosphorylated LRP, resulting in the dismantling of the destruction complex.[8] β-Catenin then accumulates and translocates to the nucleus, where it binds to the T-cell factor (TCF) family of transcription factors and increases transcription of a number of target genes.[9] In addition to the central role in the Wnt signaling pathway, β-catenin also forms a complex with cadherins at the plasma membrane as part of the adherens junctions, including with N-cadherin in neuronal cells.[2,3,10] The β-catenin/N-cadherin complex plays an important role in cell−cell adhesion in the nervous system and is present in both pre- and postsynaptic cells.[2,3,10,11]

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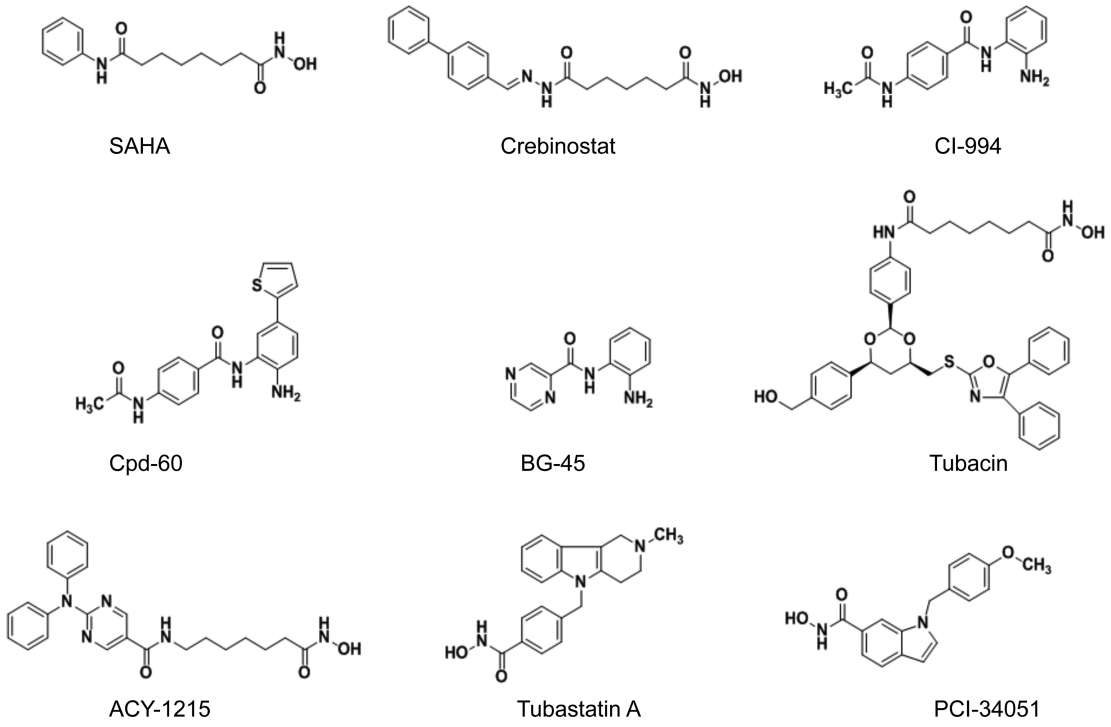

Figure 1. HDAC inhibitors used in the study. Structures of the small molecule inhibitors used in the study. The HDAC specificity of the small molecules are as follows: SAHA (HDAC 1, 2, 3, 6, 8), crebinostat (HDAC 1, 2, 3, 6), CI-994 (HDAC 1, 2, 3), Cpd-60 (HDAC 1, 2), BG-45 (HDAC3), tubacin (HDAC6), ACY-1215 (HDAC6), tubastatin A (HDAC6), PCI-34051 (HDAC8). IC_{50} values (μM) of the small molecules for inhibition of different HDAC isoforms are showed in Table 1.[31,36,37,44,55−57]

Human β-catenin consists of 781 amino acids that can be divided into three regions—a central region that comprises 12 Armadillo repeats, an N-terminal regulatory domain, and a C-terminal domain that binds transcription factors.[2] The structurally flexible N-terminal regulatory domain includes sites for posttranslational modification that regulate the stability and function of β-catenin. GSK3β phosphorylates Ser33, Ser37, and Thr41 and primes β-catenin for degradation by βTrCP-mediated ubiquitination.[1] CK1α phosphorylates Ser45 but the functional consequence of this phosphorylation is not well understood.[12] One model posits that phosphorylation of Ser45 primes β-catenin for GSK3β phosphorylation of Ser33, Ser37, and Thr41.[13−15] However, other reports note that Ser45 phosphorylation is uncoupled from phosphorylation of Ser33, Ser37, and Thr41.[16−19]

HDACs and HATs (histone acetyltransferases) have been studied extensively for their role in regulating chromatin function by modulating acetylation of histone proteins.[20,21] In addition, there is a growing recognition of cellular processes where HDACs play crucial roles by deacetylating nonhistone proteins.[22−24] One such instance is the acetylation of lysine residues on β-catenin. Lys49 is a posttranslational modification site on β-catenin that can be acetylated.[25−27] This acetylation site is adjacent to phosphorylation sites for CK1α and GSK3β in the N-terminal regulatory domain. Lys49 has been found to be mutated to arginine in anaplastic thyroid carcinomas, resulting in increased nuclear localization of β-catenin.[28] In the cancer cell lines SW480 and HCT116, it was shown that Lys49 deacetylation was necessary for epidermal growth factor (EGF)-induced nuclear localization of β-catenin.[29] However, outside of these tumor cell lines, the function and regulation of this acetylation site remains poorly understood.

HDACs have important roles in the central nervous system, especially in learning and memory and in the regulation of synaptic plasticity.[30−33] Most HDAC inhibitors studied to date in neuronal cells have broad selectivity and target multiple HDAC isoforms.[34] Recent advances in chemical biology approaches have led to a better understanding of different HDAC isoforms and development of small-molecule probes that target specific HDAC isoforms.[35,36] While many of these studies on HDACs have focused on class I HDACs (HDACs 1,2,3) located in the nucleus, there have been significant advances in the design of novel inhibitors that target the cytoplasmic IIb HDAC, HDAC6.[36−42] These efforts have led to the development of multiple small molecules with different chemical scaffolds that are highly selective for HDAC6 and allow for specific inhibition of HDAC6 at the cellular level.[42−49]

Here, we report on studies designed to investigate the nature of Lys49 acetylation in human iPSC-derived NPCs. We tested a set of annotated isoform-specific HDAC inhibitors to identify the HDAC isoform that deacetylates Lys49. We show that only small molecules that can inhibit HDAC6 result in increased Lys49 acetylation. We find that Lys49 acetylation is accompanied by increase in Ser45 phosphorylation without affecting the phosphorylation of the Ser33, Ser37, Thr41 sites. Lys49 acetylation is also accompanied by decreased ubiquitination of β-catenin in the presence of proteasomal inhibition. We find that Lys49 acetylation does not alter overall levels of β-catenin in these human NPCs but results in increased membrane localization of β-catenin pointing to a novel mechanism for differentially regulating the function of β-catenin in distinct subcellular compartments.

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Table 1. IC_{50} Values (μM) of the Small Molecules for Inhibition of Different HDAC Isoforms[31,36,37,44,55−57]

	HDAC1	HDAC2	HDAC3	HDAC4	HDAC5	HDAC6	HDAC7	HDAC8	HDAC9	HDAC selectivity
SAHA	0.0013	0.0016	0.005		3.6	0.0016		0.48		1, 2, 3, 6, 8
crebinostat	0.0007	0.001	0.002			0.009				1, 2, 3, 6
CI-994	0.05	0.19	0.55							1, 2, 3
Cpd-60	0.001	0.008	0.458							1, 2
BG-45	2	2.2	0.289			>20				3
tubacin	0.028	0.042	0.275	17	1.5	0.016	8.5	0.17		6
ACY-1215	0.058	0.048	0.051	7	5	0.004	1.4	0.1	10	6
tubastatin A	3.2	3.5	4.9			0.018				6
PCI-34051	>50	>50	6.8			2.9		0.01	>50	8

RESULTS AND DISCUSSION

Effect of Different HDAC Inhibitors on Lys49 Acetylation on β-Catenin. We undertook experiments to dissect the nature of Lys49 acetylation in the human neuronal context by undertaking experiments in human iPSC-derived NPCs. In order to identify the HDAC isoform that deacetylates Lys49 in human NPCs, we used a set of annotated HDAC inhibitors that are selective for specific HDAC isoforms (Figure 1, Table 1).[35,36] Under basal conditions, these human NPCs had minimal Ac-Lys49 β-catenin present, as measured by Western blotting of whole-cell extracts (Figure 2A). Treatment with the pan-HDAC inhibitor SAHA led to a marked increase in the level of Ac-Lys49 β-catenin (Figure 2A). We observed similar results with crebinostat, which inhibits HDACs 1, 2, 3, and 6.[31] However, inhibitors specific for class I HDACs, such as CI-994, which inhibits HDACs 1, 2, and 3, Cpd-60, which inhibits HDACs 1 and 2, and BG-45, which preferentially inhibits HDAC3, did not increase levels of Ac-Lys49 β-catenin. In contrast, treatment with three structurally distinct HDAC6 inhibitors—tubacin, tubastatin A, and ACY-1215—resulted in marked increase in Ac-Lys49 β-catenin levels (Figure 2A). We repeated the experiment in a second human iPSC-derived NPC line 8330 and again found that only compounds that inhibit HDAC6 increased Ac-Lys49 β-catenin levels (Figure 2B).[50] The increase in Ac-Lys49 β-catenin in response to HDAC6 inhibition could be observed within 2 h of treatment (Figure 2C). While HDAC6 inhibitors increased levels of Ac-Lys49 β-catenin, there was no change in overall levels of β-catenin in the NPCs (Figure 2).

Effect of HDAC6 Inhibitor-Mediated β-Catenin Lys49 Acetylation on N-Terminal Posttranslational Modification Sites. We investigated how Lys49 acetylation on β-catenin affected the adjacent posttranslational modification sites in the N-terminal regulatory domain. At baseline, there was minimal Ser45 phosphorylation on β-catenin in the NPCs (Figure 3A). We found that the increase in Lys49 acetylation in response to HDAC6 inhibitors was mirrored by an increase in Ser45 phosphorylation that followed a similar time course (Figure 3A, B). We also carried out functional genomic studies with siRNA knockdown to confirm these findings. In the presence of HDAC6 knockdown, we again observed an increase in Lys49 acetylation and Ser45 phosphorylation. (Figure 3C, D). We next determined how Lys49 acetylation affected the GSK3β phosphorylation sites on β-catenin by examining levels of β-catenin phosphorylated at Ser33, Ser37, and Thr41. In the presence of the HDAC6 inhibitor ACY-1215, the increased levels of Ac-Lys49 β-catenin had no effect on the levels for β-catenin phosphorylated at Ser33, Ser37, and Thr41 (Figure 3A, B). These results suggest that Lys49 acetylation increases the ability of CK1α to bind and phosphorylate Ser45 on β-catenin while preventing GSK3β from phosphorylating its target sites Ser33, Ser37, and Thr41. The exact mechanisms behind these effects on the phosphorylation sites remain to be elucidated. Our results show that Lys49 acetylation is

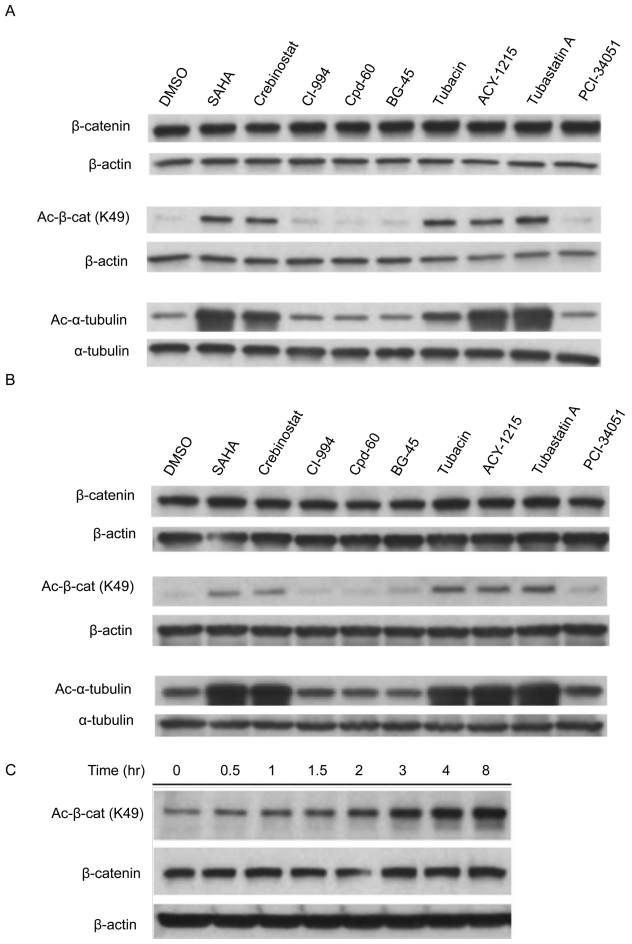

Figure 2. Effect of HDAC inhibitors on β-catenin Lys49 acetylation. (A) Immunoblot analysis of lysates from human NPCs treated with different HDAC inhibitors at 5 μM for 18 h, with Western blot using antibodies against β-catenin and Ac-Lys49-β-catenin. β-Actin is shown as loading control. Antibodies against Ac-α-tubulin (Lys40) are shown to reflect the level of HDAC6 inhibitory activity of each small molecule. (B) Immunoblot analysis of lysates from a second human iPSC-derived NPC line (8330NPC) treated similarly with HDAC inhibitors at 5 μM for 18 h, with Western blot using antibodies against β-catenin and Ac-Lys49-β-catenin. (C) Time course of increase in Ac-Lys49-β-catenin treated with the HDAC6-specific inhibitor ACY-1215 at 10 μM.

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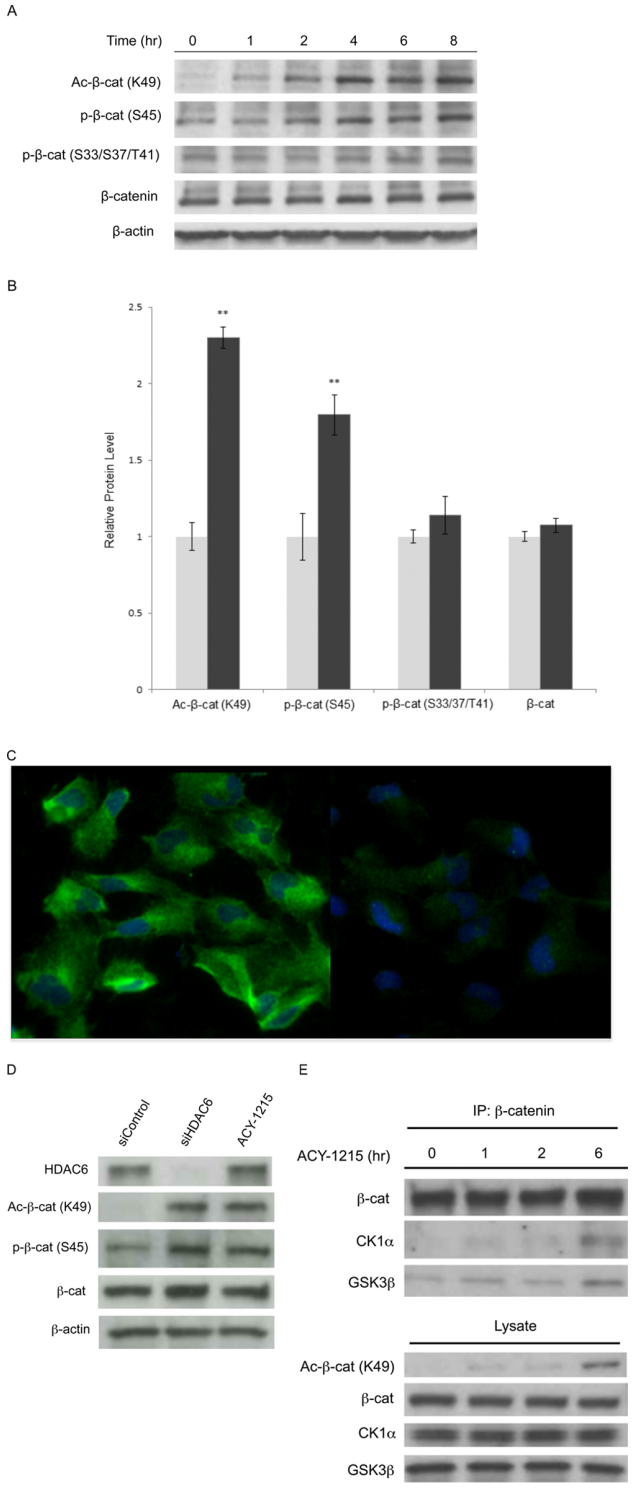

Figure 3. Effect of HDAC6 inhibitor-mediated increase in acetylation of β-catenin Lys49 on N-terminal posttranslational modification sites. (A) Time-course immunoblot analysis of human NPCs treated with an HDAC6 inhibitor. Human NPCs were treated with HDAC6 inhibitor ACY-1215 at 5 μM for the times points indicated and the lysates immunoblotted with antibodies against β-catenin, Ac-Lys49-β-catenin, phos-Ser45 and phos-Ser33, Ser37, and Thr41. β-actin is using shown as loading control. (B) Quantification of the intensity of Western blot band signals from *A.* in indicated antigens normalized to total β-catenin before and after treatment with ACY-1215 at 5 μM for 8 h. Results are representative of three independent experiments. Error bars indicate standard deviation. ** and *** denote significance with $p < 0.01$ and $p < 0.001$, respectively (paired t test). (C) Immunofluorescence staining for HDAC6 in human NPCs when treated with control siRNA (left) and HDAC6 siRNA (right). (D) Immunoblot analysis of lysates from human NPCs treated with HDAC6 siRNA, control siRNA, and ACY-1215, using antibodies against HDAC6, β-catenin, Ac-Lys49-β-catenin, and phos-Ser45-β-catenin. β-actin is shown as loading control. (E) Time course of CK1α and GSK3β bound to β-catenin bound to in human NPCs treated with 5 μM ACY-1215. Cell lysates were immunoprecipitated with anti-β-catenin antibody and levels of CK1α and GSK3β were measured by immunoblot analysis.

accompanied by both increased CK1α and GSK3β binding, even though only CK1α is able to phosphorylate its target residue (Figure 3E).

Ser45 phosphorylation has been hypothesized to be a priming step for phosphorylation at Ser33, Ser37, and Thr41 and subsequent ubiquitination and proteasomal degradation.[13−15] In the human NPCs, Ac-Lys49 β-catenin was accompanied by increase in Ser45 but not in Ser33, Ser37 and Thr41 phosphorylation (Figure 3A,B). We also did not find any changes in overall levels of β-catenin. Lys49 is a known site for ubiquitination, which primes β-catenin for degradation by the proteasome.[51] There have been suggestions of cross-talk between acetylation and ubiquitination on β-catenin.[26] To explore this possibility, we studied the effect of the proteasome inhibitor MG-132 in NPCs in the presence or absence of ACY-1215. When the NPCs were treated with the proteasome inhibitor MG-132, there was significant accumulation of ubiquitinated β-catenin, along with increase in overall level of β-catenin (Figure 4). However, when NPCs were pretreated with the HDAC6 inhibitor ACY-1215, there was minimal change in levels of ubiquitinated β-catenin or in overall level of β-catenin in the presence of MG-132. In our experiments, the lack of increase in overall levels of β-catenin in the presence of ACY-1215 and MG-132 was surprising. It is possible that the increased β-catenin may be in the insoluble fractions and hence not seen in the Western blot. Total ubiquitination levels in cell lysates was unaffected by ACY-1215 pretreatment suggesting that the effects of ACY-1215 on ubiquitination are specific rather than global (Figure 4). Our findings suggest that HDAC6 inhibitor-mediated increase in Lys49 acetylation has a major impact on β-catenin ubiquitination.

Effect of HDAC6 Inhibition on Subcellular Localization of β-Catenin. While increases in Lys49 acetylation did not result in gross changes in β-catenin levels, we investigated if it affected subcellular localization of β-catenin in human NPCs. Acetylation has been shown to modulate subcellular localization of nonhistone proteins.[23] Using immunofluorescence, we found that both ACY-1215 and siRNA knockdown of HDAC6 induced pronounced increases in β-catenin at the plasma membrane (Figure 5A, B). To corroborate the results of these imaging assays, we carried out cell fractionation of NPCs treated with ACY-1215. These cell fractionation studies showed that the increase in Ac-Lys49 β-catenin was primarily in the membrane/cytoplasmic fraction and not in the nuclear fraction (Figure 5C). We observed that HDAC6 was localized in the cytoplasmic/membrane fraction and not in the nuclear fraction (Figure 5C). We also carried out immunoprecipitation experiments using antibodies against N-cadherin in cells treated

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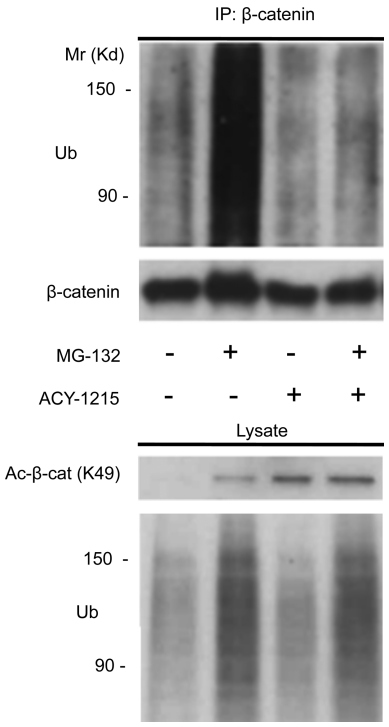

Figure 4. Effect of HDAC6 inhibition on ubiquitination of β-catenin. Immunoblot analysis of ubiquitination in human NPCs. Human NPCs were treated with 1 μM of the proteasome inhibitor MG-132 for 4 h, the cell lysates were immunoprecipitated with anti-β-catenin antibody, and the level of ubiquitination was measured in a Western blot using an antiubiquitin antibody. Results are also shown for human NPCs preincubated with 5 μM ACY-1215 for 6 h prior to MG-132 treatment.

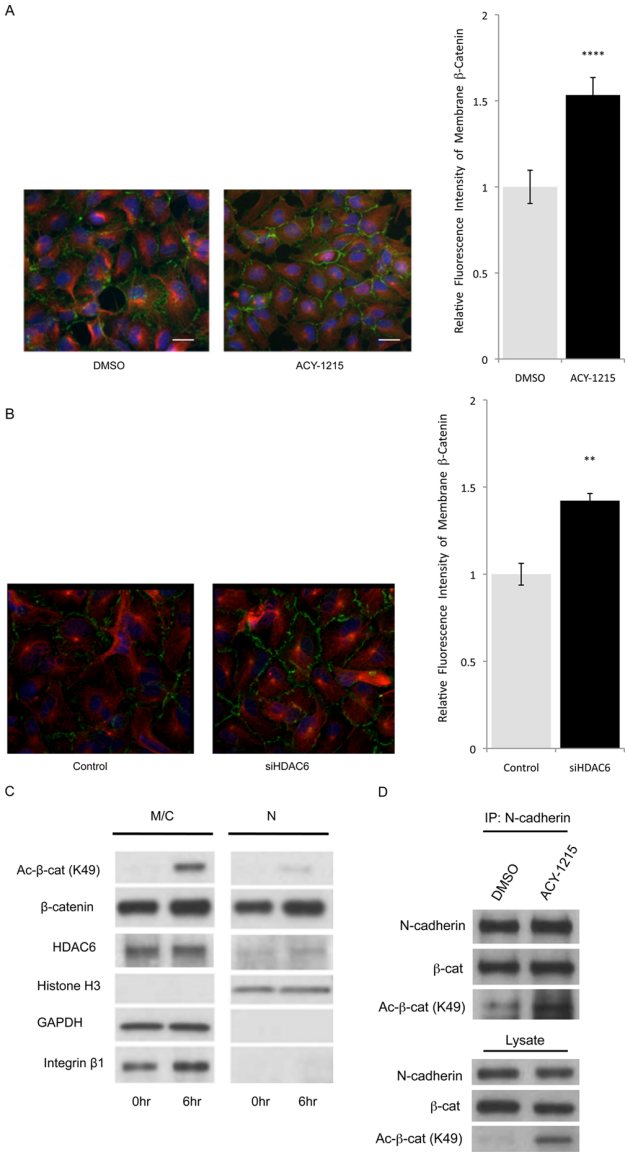

Figure 5. Effect of HDAC6 inhibition on membrane localization of β-catenin. (A, B) Immunofluorescence staining for β-catenin treated with HDAC6 inhibitor (A) and HDAC6 siRNA (B) in human NPCs. Cells were treated with 5 μM ACY-1215 or DMSO for 18 h (A) or with HDAC6 siRNA and control siRNA for 72 h (B) and imaged with anti-β-catenin antibody (green), anti-β-tubulin antibody (red), and Hoechst 33342 (blue). Scale bar, 20 μm. Quantification of β-catenin levels at the membrane represent two independent experiments with three fields of view each at 20× magnification. Error bars indicate standard deviation. ** and **** denote significance at $p < 0.01$ and $p < 0.0001$, respectively (unpaired t test). (C) Immunoblot analysis for nuclear (N) and membrane/cytoplasmic (M/C) fractions for NPCs treated with HDAC6 inhibitor ACY-1215 (5 μM) or DMSO for 6 h. Histone H3 is shown as a nuclear marker, GAPDH as a cytoplasmic marker and integrin β1 as a membrane marker. (D) Immunoblot analysis for Ac-Lys49-β-catenin bound to N-cadherin. Human NPCs were treated with ACY-1215 (5 μM) or DMSO for 24 h, lysates immunoprecipitated with anti-N-cadherin antibody and immunoblotted with antibodies against β-catenin and Ac-Lys49-β-catenin.

with the HDAC6 inhibitor ACY-1215. While overall levels of β-catenin bound to N-cadherin did not appear to change in NPCs treated with ACY-1215, the pool of β-catenin bound to N-cadherin was enriched for Lys49 acetylation (Figure 5D).

β-Catenin plays important roles in multiple cellular and disease contexts but much remains to be learned about the regulation of β-catenin in the different cellular contexts, including pivotal ones in the human central nervous system.[1−3]

Given the central role of β-catenin in the biology of neural stem cells, we had carried out studies to dissect the role of posttranslational modifications of β-catenin in human iPSC-derived NPCs, focusing on the Lys49 acetylation site.[52] After systematically testing a set of annotated isoform-selective HDAC inhibitors and siRNA knockdown, we found that HDAC6 inhibition alone was sufficient to induce Lys49 acetylation whereas selective class I HDAC inhibitors targeting HDAC1/2/3/8 were ineffective. This is consistent with earlier studies in cancer cell lines where it was shown that HDAC6 activity was necessary for EGF-mediated β-catenin nuclear localization.[29] Lys49 deacetylation was shown to be necessary for β-catenin nuclear localization in that cancer context, but the role played by β-catenin acetylated at Lys49 was not known. We show here that increased β-catenin Lys49 acetylation in human neuronal cells correlates with an increased localization of β-catenin at the plasma membrane.

There are a few differences to note in the HCT116 and SW480 cancer cell lines that had been used to study the EGF-mediated nuclear translocation of β-catenin and the human iPSC-derived NPCs used in this study. In the two cancer cell lines, there was an abundance of Ac-Lys49 at baseline.[29] Activation of the EGF pathway resulted in *decreased* levels of

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Ac-Lys49, along with increased nuclear staining of β-catenin.[29] In the human NPCs we studied, Ac-Lys49 levels were undetectable at baseline, and there was no noticeable β-catenin staining in the nucleus suggesting that either p300/CBP acetylase activity is low or HDAC6 activity is high in NPCs relative to the cancer cell lines. Upon HDAC6 inhibition, Ac-Lys49 β-catenin levels *increased* in NPCs, which was accompanied by increased membrane localization of β-catenin. These data emphasize the importance of cellular context for studying β-catenin function and localization.

The interaction between the different posttranslational modification sites in the β-catenin N-terminal regulatory domain has been an active area of investigation. It is known that Ser33, Ser37, and Thr41 are phosphorylated by GSK3β, which creates the binding site for β-TrCP and subsequent ubiquitination by the E3 ubiquitin ligase system.[6,7] What has not been clear is the interaction between Ser45 and Lys49 with the GSK3β phosphorylation sites. Ser45 phosphorylation by CK1α has been described as a priming site for GSK3β phosphorylation.[13−15] However, there have been other reports that indicated that Ser45 phosphorylation was uncoupled from the GSK3β phosphorylation sites Ser33, Ser37, and Thr41.[16−19] Our results in the human NPCs are supportive of this latter model as HDAC6 inhibition-induced increase in Lys49 acetylation was accompanied by increase in Ser45 phosphorylation but increased Ser45 phosphorylation was not accompanied by increased phosphorylation of Ser33, Ser37, and Thr41. We also found that HDAC6 inhibitor-mediated increase in Lys49 acetylation and Ser45 phosphorylation led to decreased ubiquitination in the presence of proteasomal inhibition. This suggests that the presence of acetylated Lys49 interferes with ubiquitination of β-catenin.

In summary, we report that multiple structurally distinct HDAC6 inhibitors, as well as HDAC6 knockdown with siRNA, led to increased acetylation of Lys49 on β-catenin in human NPCs. This acetylation increase was accompanied by increased phosphorylation of the CK1α phosphorylation site but not of the GSK3β phosphorylation sites. We found that HDAC6 inhibition mediated increase in acetylation of Lys49 on β-catenin did not change overall levels of β-catenin. However, increased Lys49 acetylation on β-catenin resulted in increased membrane localization. We hypothesize that increased membrane localization of β-catenin will affect cadherin−catenin interactions between neuronal cells, and modulate intercellular interactions.

METHODS

Cell Culture. NPCs derived from human iPSCs (HIP) were obtained from GlobalStem. All experiments were carried out in these NPCs unless described otherwise. 8330 NPCs derived from a control human iPSC line as described previously were used to show reproducibility in a second human NPC line.[50] NPCs were cultured in media containing 70% DMEM with high glucose (Life Technologies), 30% Ham's F12 with L-glutamine (Cellgro/Mediatech), penicillin/streptomycin, and B27 supplement (Life Technologies) as well as 20 ng/mL epidermal growth factor (EGF) (Sigma-Aldrich), 20 ng/mL basic fibroblast growth factor (bFGF) (Stemgent), and 5 μg/mL heparin (Sigma-Aldrich). Plates were coated with 20 μg/mL poly-L-ornithine (Sigma-Aldrich) solution in ddH_2O followed by 5 μg/mL laminin (Sigma-Aldrich) in phosphate-buffered saline (PBS) (Life Technologies).

Western Blots and Immunoprecipitation. Cells were lysed by boiling in Laemmli sample buffer (Bio-Rad) with 5% 2-mercaptoethanol (Sigma-Aldrich). Proteins in the lysate were separated by SDS-PAGE and transferred to PVDF membrane. The blots were probed with antibodies against β-catenin (carboxy-terminal antigen) (Cat. No. 9587), phospho-β-catenin (Ser45) (Cat. No. 9564), phospho-β-catenin (Ser33/Ser37/Thr41) (Cat. No. 9561), Ac-Lys49-β-catenin (Cat. No. 9030), GAPDH (Cat. No. 2118), and integrin β1 (Cat. No. 4706), which were all obtained from Cell Signaling. The N-cadherin (Cat. No. 610921) and GSK3β (Cat. No. 61020) antibodies were obtained from BD Bioscience. The CK1α antibody (Cat. No. CTX50020) was obtained from GeneTex. Antiubiquitin antibody (Cat. No. ST1200) and anti-histone-H3 antibody (Cat. No. 06-755) were obtained from Millipore. Antibodies against Ac-Lys40-α-tubulin (Cat. No. T7451), α-tubulin (Cat. No. T9026), β-tubulin (Cat. No. 8328), and β-actin (Cat. No. A5441) were obtained from Sigma-Aldrich. Anti-HDAC6 antibody (Cat. No. SC-11420) was obtained from Santa Cruz. siRNA reagents were obtained from Thermo Scientific. Accell HDAC6 siRNA (Cat. No. EQ-003499-00-0005) and nontargeting siRNA (D-001910-01-05) were initially diluted in buffer (Cat. No. B-002000-UB-100) to prepare 100 μM solutions and subsequently diluted to a 1 μM solution in delivery media (Cat. No. B-005000) supplemented with 20 ng/mL EGF, 20 ng/mL bFGF, and 5 μg/mL heparin. For the knockdown experiments, NPCs were plated at 25% confluency in 96-well plates precoated with poly-L-ornithine and laminin and incubated overnight. NPC media was then replaced with100 μL of the HDAC6 siRNA solution incubated for 72 h. HDAC6 knockdown was assessed by Western blot as well as immunostaining. For immunoprecipitation experiments, lysates were incubated with primary antibody and A/G Plus-Agarose beads (Cat. No. sc-2003, Santa Cruz) overnight at 4 °C. Cell fractionation was performed using standard protocols as described in the Cell Fractionation Kit obtained from Cell Signaling. Western blot quantification was performed using ImageJ analysis software.[53]

Imaging. For immunofluorescence experiments, cells were grown on glass coverslips coated with 20 μg/mL poly-L-ornithine in ddH_2O followed by 5 μg/mL laminin in PBS, fixed in 4% formalin in PBS for 20 min and permeabilized for 20 min in 0.1% Triton-PBS. Cells were then blocked with 2% bovine serum albumin (BSA) in PBS for 1 h at room temperature (RT). Cultures were first incubated with primary antibodies in 2% BSA overnight at 4 °C and then with secondary antibodies for 1 h at RT in 2% BSA. After washing in PBS, the glass coverslips were mounted on to glass slides. Images were captured with AxioPlan microscope (Zeiss) equipped with AxioVision software (Zeiss). Quantification of β-catenin at the plasma membrane was carried out using CellProfiler image analysis software, by identifying and spatially binning the cytoplasm into concentric regions, then measuring the β-catenin distribution from the outermost bin.[54]

Sources of Chemicals. HDAC inhibitors CI-994, Cpd-60, suberoylanilide hydroxamic acid (SAHA), tubacin, tubastatin A, ACY-1215, and PCI-34051 were purchased from commercial vendors. Crebinostat and BG-45 were synthesized in house, as described previously.[31,55]

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Notes

The authors declare the following competing financial interest(s): R.M. has financial interests in SHAPE Pharmaceuticals and Acetylon Pharmaceuticals. He is also the inventor on IP licensed to these two entities. R.M.'s interests were reviewed and are managed by Massachusetts General Hospital and Partners HealthCare in accordance with their conflict of interest policies.

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