Histone deacetylase 6 (HDAC6) is a multidomain cytosolic enzyme having tubulin deacetylase activity that has been unequivocally assigned to the second of the tandem catalytic domains. However, virtually no information exists on the contribution of other HDAC6 domains on tubulin recognition. Here, using recombinant protein expression, site-directed mutagenesis, fluorimetric and biochemical assays, microscale thermophoresis, and total internal reflection fluorescence microscopy, we identified the N-terminal, disordered region of HDAC6 as a microtubule-binding domain and functionally characterized it to the single-molecule level. We show that the microtubule-binding motif spans two positively charged patches comprising residues Lys-32 to Lys-58. We found that HDAC6-microtubule interactions are entirely independent of the catalytic domains and are mediated by ionic interactions with the negatively charged microtubule surface. Importantly, a crosstalk between the microtubule-binding domain and the deacetylase domain was critical for recognition and efficient deacetylation of free tubulin dimers both in vitro and in vivo. Overall, our results reveal that recognition of substrates by HDAC6 is more complex than previously appreciated and that domains outside the tandem catalytic core are essential for proficient substrate deacetylation.

Acetylation at the Nε group of lysines is a major posttranslational modification found on most proteins of the human proteome (1). Both histones and nonhistone proteins are targets of the acetylation/deacetylation machinery. Lysine acetylation has been implicated in diverse biological functions, including metabolic stress response, inflammation, chromatin assembly, DNA repair and recombination, circadian rhythm, as well as immune surveillance, neurological development, and brain function (2–7). At the molecular level, the protein acetylation status is regulated by opposing activities of histone acetyltransferases (writers) and histone deacetylases (HDACs) (erasers). In addition to enzymatic acetylation by histone acetyltransferases, nonenzymatic acetylation by metabolic intermediates/end products (such as acetyl-CoA) has been reported (8). In contrast, the removal of acetyl groups is more tightly regulated by substrate specificities of individual HDACs and their spatiotemporal distribution within the cell. Among 11 zinc-dependent HDACs, class I and IV enzymes are primarily located in the nucleus, class IIa enzymes are shuttling between the nucleus and cytosol, and class IIb enzymes, including HDAC6, are mainly localized to the cytosol (9).

HDAC6 stands out as a structurally and functionally unique lysine deacetylase, and it represents an attractive target for therapeutic interventions in cancer (10) and neuropathologies (11, 12). Structurally, human HDAC6 consists of five domains: (i) the N-terminal domain (amino acids 1–87), (ii) tandem deacetylase domains DD1 (amino acids 88–448), (iii) DD2 (amino acids 480–855) connected by a glutamate-rich linker, (iv) the Ser-Glu–containing repeated tetrapeptide domain (SE) (amino acids 856–1109), and (v) the C-terminal ubiquitin-binding zinc finger domain (ZnF) (amino acids 1109–1215) (see Fig. 2A). Recently, X-ray structures of both catalytic domains of zebrafish HDAC6 as well as the ZnF and DD2 domains of human HDAC6 have been reported (13, 14). Functionally, DD2 and ZnF are also characterized in the greatest detail. The DD2 is primarily responsible for the
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Figure 1. Ensemble and single-molecule interactions between HDAC6 and stabilized MTs. A, saturation binding curve representing HDAC6 binding affinity to MTs. MTs were immobilized on the glass surface and the dilution series of GFP-HDAC6 FL (FL), GFP-HDAC6 FL H611A (FL H611A), and GFP-HDAC6 FL in the presence of 1.6 μM Nexturastat A, an HDAC6-specific inhibitor (FL + NexA), was added to the channel. The fluorescence signal of the MT-bound HDAC6 was quantified using TIRF microscopy. Apparent dissociation constants (appKd), calculated from binding isotherms, are 135 ± 9.2 nM, 233 ± 15.2 nM, and 141 ± 19.5 nM, respectively. Data represent mean values ± S.D.; n = 3. B, the kymograph representing the mode of interactions between GFP-HDAC6 FL fusion and stabilized MTs at the single-molecule level, visualized by TIRF microscopy. Both static binding-unbinding events (straight lines) and fast nondirectional diffusion interactions are observed.

Results

Expression and characterization of HDAC6 mutants

All HDAC6 mutants used in this study were expressed in HEK293T cells and purified to apparent homogeneity by the combination of Strept-Tactin affinity chromatography and size-exclusion chromatography. Relative molecular weight and purity of the final preparations were verified by SDS-PAGE (Fig. S1). For total internal reflection fluorescence (TIRF) microscopy experiments, GFP was fused at the N terminus of HDAC6 mutants, and the presence of the tag did not have any effect on HDAC6 deacetylase activity (37). Additionally, the CD spectra for each mutant correlated well with the predicted secondary structure content (Fig. S2). Overall, all proteins used in this report are thus correctly folded and enzymatically active (when harboring the DD1-DD2 catalytic core that comprises the tandem catalytic domains DD1 (amino acids 75–440) and DD2 (amino acids 480–855)).

HDAC6 binds microtubules with nanomolar affinity

Our recent study showed that HDAC6 full length (FL) binds double stabilized MTs uniformly along their entire length with fast kinetics (37). To obtain more detailed and quantitative insights into HDAC6/MT interactions, we used TIRF microscopy to determine HDAC6/MT affinity as well as the mode of HDAC6/MT interactions at the single-molecule level. We first determined HDAC6 FL affinity for MTs by incubating immobilized MTs with increasing concentrations of GFP-HDAC6 fusions (ranging from 12.5 nM–1 μM) and quantifying fluorescence signal of the bound fusion. The resulting binding isotherm reveals high affinity with an apparent dissociation constant of appKd = 135 ± 9.2 nM (Fig. 1A).

We next examined the mode of HDAC6 binding to MTs using single-molecule measurements. Kymograph analyses revealed two predominant interaction modes: static binding (binding/unbinding) and one-dimensional diffusion (Fig. 1B;
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see Video S1). At the same time, the HDAC6 diffusion rate along MTs is too fast to be precisely quantified in our experimental setup with the shortest exposure time of 30 ms, thus preventing the assessment of the proportion of molecules in each interaction mode class.

The above-mentioned measurements (and all additional experiments dealing with MT/HDAC6 interactions) were carried out in BRB50, unless stated otherwise. Although the ionic strength of BRB50 is somewhat lower compared with "physiological ionic strength" of the BRB80 buffer, the use of low ionic strength buffers is a standard practice in studies investigating interactions between MTs and MAPs and molecular motors, where buffers down to BRB12 (12 mM PIPES) are typically used (38–40).

HDAC6 deacetylase activity is not required for MT binding

To determine whether HDAC6 binding to MTs depends on its deacetylase activity, we repeated the binding experiment (i) using the catalytically inactive H611A mutant and (ii) in the presence of Nexuturast A (NexA), a nanomolar HDAC6 inhibitor. Although the use of the H611A catalytically inactive mutant directly uncouples the HDAC6 deacetylation activity from MT binding, it does not block potential interactions between the αK40 loop and HDAC6 DD2. On the other hand, NexA binding to the active-site of DD2 (and DD1) would sterically block the αK40 side-chain insertion into the HDAC6 active-site tunnel. Consequently, if HDAC6/αK40 interactions were critical for MT binding, one could expect more pronounced effects of the latter. Using both experimental approaches, however, we did not observe any significant effect of DD2 inhibition/inactivation on HDAC6 microtubule-binding affinity and the mode of interaction (Fig. S3). This observation reveals that HDAC6 binding to MTs is independent of its catalytic function, and there is no/negligible contribution from the αK40 motif toward HDAC6 interactions (Fig. 1A).

The N-terminal part of HDAC6 is a microtubule-binding domain

To identify domain(s) responsible for interactions with stabilized MTs, we performed an extensive deletion mutagenesis study using a panel of truncated HDAC6 mutants (Fig. 2A; Fig. S1). Surprisingly, the construct comprising the tandem deacetylation domains that are sufficient to effectively deacetylate a wide variety of substrates (14, 22, 41–45), including tubulin, did not interact with stabilized MTs. Instead, only mutants harboring the N-terminal domain (amino acids 1–87), either isolated or in combination with other HDAC6 domains, were capable of MT binding (Fig. 2B). Clearly, the N-terminal part of HDAC6, but not the tandem catalytic core (DD1-DD2), is necessary and sufficient to bind MTs and henceforth denoted the microtubule-binding domain (MBD).

Using TIRF microscopy, we compared the affinity and the interaction mode of the isolated catalytic core of HDAC6 and two other constructs harboring the N terminus: the HDAC6 MBD and the HDAC6 MBD-DD2 mutant (Fig. 2C). These experiments yielded virtually identical apparent dissociation constants for two constructs 98 ± 9.3 nm and 140 ± 2.7 nm, respectively. It reveals that the N-terminal MBD is primarily responsible for HDAC6 affinity toward MTs without any obvious contribution of other domains.

Because the pelleting assay is a gold standard in the field, this method was used independently to corroborate direct HDAC6 binding to MTs. To this end, stabilized MTs were incubated with various HDAC6 mutants. Both the supernatant and pelleted fraction were analyzed for the presence of MTs and/or HDAC6 mutants by SDS-PAGE. Overall, the pelleting assay fully reproduced major findings from the TIRF microscopy that (i) the duplicated catalytic core does not interact appreciably with MTs and (ii) the N-terminal domain is necessary and sufficient for MT binding (Fig. 2D).

HDAC6 microtubule-binding motifs span amino acids Lys-32 to Lys-37 and Lys-51 to Lys-58

The sequence analysis of the microtubule-binding domain of human HDAC6 revealed the presence of two lysine/arginine-rich patches (patch A and B comprising amino acids Lys-32 to Lys-37 and Lys-51 to Lys-58, respectively) (Fig. 3A) that in principle can be involved in ionic interactions with the negatively charged MTs' surface.

The lysine residues at both patches were shown to be acetylated in vivo by acetyltransferase p300, and their acetylation status is linked to HDAC6 nucleocytoplasmic shuttling (18). We hypothesized that N-acetylation at the two patches could, in addition to its control over the HDAC6 nuclear import, also influence HDAC6/MT interactions. To test this notion, lysines at both patches were mutated to glutamines to mimic lysine acetylation. Mutants harboring the mutated MBD were purified, and their affinity for stabilized MTs was determined. Compared with the WT sequence, mutants mimicking lysine acetylation at patches A and B showed 40- and >150-fold weaker affinity for stabilized MTs, respectively, pointing toward more pronounced involvement of patch B in mediating HDAC6 interactions with MTs. Additionally, the combined patch AB acetylation mimic mutant failed to bind MTs altogether (Fig. 3B).

Using the synthetic peptide corresponding to amino acids Ser-31 to Leu-59 of HDAC6 (P-WT) and its acetylation mimics (P-KQ PA, P-KQ PB, P-KQ PAB) (Fig. S13), we corroborated and extended principal findings of the above TIRF-binding experiments where recombinant proteins were used (Fig. 3A; Figs. S4 and S12). The P-WT peptide derived from the native N terminus bound to stabilized MTs with an apparent dissociation constant of 131 ± 5.1 nm, whereas the marked decrease in binding affinity was observed for each of patch A (P-KQ PA) and B (P-KQ PB) mutants. Finally, no binding was observed for the doubly mutated P-KQ PAB variant (Fig. 3C). Peptide-based data pinpointed the Ser-31 to Leu-59 sequence as a key MT-interacting motif with a submicromolar affinity for stabilized MTs and confirmed the dominant contribution of patch B for MTs binding (Fig. 3C).

Furthermore, in competition experiments, the Ser-31 to Leu-59 peptide, but not its acetylation mimic variant, was able to completely abolish interactions between MTs and the GFP-HDAC6 fusion, thus verifying the specificity of HDAC6/MT interactions (Fig. 4).
Tubulin-negative charges are essential for MT recognition by HDAC6

The fast, one-dimensional diffusion kinetics observed at the single-molecule level suggest that HDAC6/MT interactions can be mediated by electrostatic forces between the negatively charged MT surface and positively charged motif(s) of HDAC6, as reported for several MAPs (31, 46, 47). To test this hypothesis, we first examined the influence of salt concentration on HDAC6 binding to MTs by performing the TIRF-binding assay in buffers of different ionic strengths ranging from BRB80 (80 mM PIPES) to BRB10 (10 mM PIPES). The fluorescence signal quantification of HDAC6 bound to MTs revealed the strong dependence of HDAC6/MTs interactions on salt concentration, with the highest signal observed for BRB10 and binding virtually absent in BRB80 at the HDAC6 concentration used (100 nM) (Fig. 5A).

We next examined whether the presence of C-terminal unstructured acidic tails on MTs is required for HDAC6 binding. The C-terminal tails were removed by treatment with the nonspecific protease subtilisin and their absence verified by MS (Fig. 5B; Fig. S5). The mixture of untreated and subtilisin-treated MTs was then immobilized on coverslips and binding of GFP-HDAC6 fusions assayed by TIRF microscopy. Although HDAC6 avidly bound to native, nontreated MTs, there was a...
complete absence of the GFP signal from MTs lacking C-terminal tails (Fig. 5C). Collectively, these findings show that HDAC6/MTs interactions are mediated by ionic interactions between negatively charged MT surface and lysine patches of HDAC6 N-terminal domain.

MBD is critical for efficient tubulin deacetylation in vitro

Based on the above experimental data, it is apparent that the MBD endows HDAC6 with MAP-like properties. At the same time, as free tubulin dimers are the physiological substrate of HDAC6 (14, 37), we set out to examine the importance of the MBD on HDAC6 deacetylase activity in vitro and in vivo. Using microscale thermophoresis (MST), we first quantified interactions between free tubulin dimers and HDAC6 mutants and HDAC6-derived MBD peptides, because to the best of our knowledge no such direct quantification has ever been reported. All tested mutants comprising the WT MBD interacted with free tubulin dimers, whereas no HDAC6/tubulin interactions were observed for mutants lacking MBD. Unfortunately, as we were not able to reach the upper plateau of the signal, the corresponding values of dissociation constants could not be calculated (Fig. S6). At the same time, quantification of interactions between tubulin and HDAC6-derived MBD peptides revealed appKD values of 577 ± 102, 665 ± 181, and 589 ± 800 nM for P-WT, P-KQ PA, and P-KQ PB peptides, respectively, with no interaction signal observed for the KQ PAB peptide (Fig. 6).

Identification of the MBD interaction with free tubulin motivated us to examine whether the presence of the domain also influences the HDAC6 deacetylase activity. To this end, we assessed the deacetylation activity of the HDAC6 FL and its truncated/mutated constructs against several substrates, including a fluorescent Ac-GAK-AMC peptide, the αk40 loop-derived peptide, and free tubulin dimers, a preferred physiological substrate in vitro (Fig. 7). When short peptidic substrates were used, we did not observe any substantial difference in kinetic parameters between the constructs (Fig. S7), suggesting that domains beyond the catalytic tandem deacetylase core (and likely only the C-terminal deacetylation domain) do not
MBD were 50- to 120-fold (or 20-fold, respectively) lower compared with constructs having the WT MBD (Fig. 7A and B).

To support the kinetic data and to underscore the importance of the MBD interactions with tubulin, but not small peptidic substrates, we carried out competition experiments in which deacetylation of tubulin or short peptidic substrates was assayed in the presence of HDAC6-derived MBD peptides (for examples of Western blots and HPLC chromatograms used for calculations/quantifications, see Fig. S8). Although the WT peptide inhibited tubulin deacetylation by HDAC6 FL in a concentration-dependent manner, it had no effect on either tubulin deacetylation by the DD1-DD2 variant or on deacetylation of short peptidic substrates by both HDAC6 FL and DD1-DD2 (Fig. 7C).

Taken together, our kinetic data thus parallel binding experiments with both free tubulin dimers and MTs, revealing that the presence of the MBD is also critical for efficient deacetylation of tubulin, but not of short peptidic substrates.

**MBD is required for efficient tubulin deacetylation in the cellular environment**

In the final set of experiments, we extended our *in vitro* findings into more complex cellular environment. To this end, we prepared a set of HDAC6 mutants with a truncated/modified MBD (Fig. 8A), and their activity was tested in HEK293T-HDAC6 KO cells (20). The catalytically inactive HDAC6 (H216A/H611A) double mutant was used as an additional control.

Although HDAC6 FL and its mutants were equally expressed in cells, tubulin αK40 acetylation levels were different (Fig. 8B; Fig. S14). In line with our *in vitro* observations, HDAC6 mutants FL_Δ_PA and FL_Δ_PB showed less deacetylase activity on tubulin (Fig. 8B, lanes 2, 4, and 5). The patch B is likely to have more significant contribution to the activity (Fig. 8B, lanes 4 and 5), and furthermore, the FL_PB(K/Q) mutant (the patch B acetyl mimic neutralizing the positive charge) shows decreased activity, similar to its deletion (Fig. 8B, lanes 5 and 6). In contrast, the FL_PB(K/R) mutant (nonacetylated but retaining the overall positive charge) still has catalytic activity, which is comparable with that of HDAC6 FL (Fig. 8B, lanes 2 and 7). Thus, it appears that the positive charge of patch B plays an important role in tubulin αK40 deacetylation in cells. Two N-terminal domain deletion mutants (amino acids 45–1215 and amino acids 62–1215 designated HD6 Δ2–44 and HD6 Δ2–61, respectively), which are the human versions of the *Danio rerio* constructs used for crystallization (14), also exhibit less activity, in good agreement with the observations above (Fig. 8B, lanes 9 and 10). Previous reports showed that the HDAC6 N-terminal region contains a nuclear localization signal (amino acids 14–58) and an NES (amino acids 67–76) (21); we therefore tested the cellular localization of all HDAC6 mutants to rule out that the differential deacetylase activity observed might be because of changes in subcellular localization. As shown in Fig. 8C, all mutants equally localize to the cytosol, and thus it is unlikely that mutation leads unintended localization of HDAC6 to the nucleus and a subsequent decrease of tubulin deacetylation (Fig. 8C). In summary, we revealed that these two N-terminal lysine/arginine-rich patches (patch A and patch B) are

**Recognition and deacetylation of tubulin by HDAC6**

Figure 5. HDAC6/MT interactions are mediated by electrostatic interactions with negatively charged tubulin surface. A, influence of salt concentration on HDAC6 FL binding to MTs. The GFP-HDAC6 FL fusion (100 nM) was incubated with immobilized MTs in buffers of increasing ionic strength (10 mM PIPES (BRB10) through 80 mM PIPES (BRB80)) and the amount of the GFP-HDAC6 FL binding was quantified using TIRF microscopy and normalized to the mean fluorescence signal in the BRB10 buffer. The HDAC6 FL binding to MTs is strongly modulated by ionic strength with the GFP-HDAC6 FL fusion (100 nM) being quantitatively bound to MTs in the BRB10 buffer, while the GFP-HDAC6 FL fusion was not bound to MTs in the BRB80 buffer. B, schematic representation of subtilisin treatment of MTs. C, tubulin C-terminal tails are required for HDAC6 FL binding. Native (rhodamine-labeled; violet) and subtilisin-treated (Alexa Fluor 647–labeled; red) MTs were simultaneously attached to a glass coverslip and probed with GFP-labeled HDAC6 FL (250 nM) in the BRB50 buffer. Although the GFP-HDAC6 FL fusion co-localizes with native MTs (red arrow), interactions are absent in the case of MTs missing C-terminal tails (yellow arrow). Color images are shown in the top row; the bottom row displays the same images in greyscale.

Figure 6. The MBD is essential for HDAC6 interactions with free tubulin dimers. Binding affinity between HDAC6 MBD–derived peptides and free tubulin dimers was quantified using MST. FITC-labeled peptides (100 nM) were titrated by a dilution series of tubulin dimers (100 μM–3 nM), MST curves were fitted using the MO.Affinity software (NanoTemper, Munich, Germany) and calculated apparent $K_a$ values were $577 \pm 102$ nM, $665 \pm 181$ nM and $5895 \pm 800$ nM for the WT (P WT), patch A mutant (P KQ PA), and patch B mutant (P KQ PB), respectively. No binding was observed for the patch A/B double mutant (P KQ PB). Experimental data points represent mean values ± S.D.; n = 3.

Figure 7. In the final set of experiments, we extended our *in vitro* findings into more complex cellular environment. To this end, we prepared a set of HDAC6 mutants with a truncated/modified MBD (Fig. 8A), and their activity was tested in HEK293T-HDAC6 KO cells (20). The catalytically inactive HDAC6 (H216A/H611A) double mutant was used as an additional control.

Although HDAC6 FL and its mutants were equally expressed in cells, tubulin αK40 acetylation levels were different (Fig. 8B; Fig. S14). In line with our *in vitro* observations, HDAC6 mutants FL_Δ_PA and FL_Δ_PB showed less deacetylase activity on tubulin (Fig. 8B, lanes 2, 4, and 5). The patch B is likely to have more significant contribution to the activity (Fig. 8B, lanes 4 and 5), and furthermore, the FL_PB(K/Q) mutant (the patch B acetyl mimic neutralizing the positive charge) shows decreased activity, similar to its deletion (Fig. 8B, lanes 5 and 6). In contrast, the FL_PB(K/R) mutant (nonacetylated but retaining the overall positive charge) still has catalytic activity, which is comparable with that of HDAC6 FL (Fig. 8B, lanes 2 and 7). Thus, it appears that the positive charge of patch B plays an important role in tubulin αK40 deacetylation in cells. Two N-terminal domain deletion mutants (amino acids 45–1215 and amino acids 62–1215 designated HD6 Δ2–44 and HD6 Δ2–61, respectively), which are the human versions of the *Danio rerio* constructs used for crystallization (14), also exhibit less activity, in good agreement with the observations above (Fig. 8B, lanes 9 and 10). Previous reports showed that the HDAC6 N-terminal region contains a nuclear localization signal (amino acids 14–58) and an NES (amino acids 67–76) (21); we therefore tested the cellular localization of all HDAC6 mutants to rule out that the differential deacetylase activity observed might be because of changes in subcellular localization. As shown in Fig. 8C, all mutants equally localize to the cytosol, and thus it is unlikely that mutation leads unintended localization of HDAC6 to the nucleus and a subsequent decrease of tubulin deacetylation (Fig. 8C). In summary, we revealed that these two N-terminal lysine/arginine-rich patches (patch A and patch B) are
important for the catalytic activity on tubulin, and also confirmed that positively charged residues are necessary for the patch B function.

Discussion

Within the past decade, our comprehension of structural and functional aspects of HDAC6 has expanded rapidly. Understandably, major efforts have been invested into studying the DD2 and ZnF domains, which have a compact three-dimensional fold and fairly well-defined physiological functions (13, 14, 26, 48). Much less is known about the contribution(s) of the DD1 domain toward HDAC6 function (20), and there is virtually no information concerning the physiological role(s) of intrinsically disordered N-terminal and SE domains. In this report, we identified the N-terminal part of HDAC6 as an MBD that is critical for tubulin recognition and efficient deacetylation by HDAC6.

The N-terminal domain/extension of HDAC6 is present in orthologs across various species. It shows a high degree of phylogenetic conservation and pronounced cationic character (Fig. S9) with the calculated pI of the human sequence to be pI = 10.5 (amino acids 1–75). Because the electrostatic potential of tubulin/MT surface is highly negative (Fig. S10), it is plausible that ionic attraction forces are critical to high-affinity interactions between HDAC6 (the MBD) and tubulin/MTs. Several lines of evidence presented here support this conclusion. In addition to human HDAC6, interactions between MTs and HDAC6 orthologs from zebrafish and mouse were evaluated. In line with human HDAC6 data, both full-length orthologs avidly bind Taxol/GMPCPP double-stabilized MTs (Fig. S11). It is also interesting to note that in eukaryotic cells, the HDAC6 mutant in which the patch B lysines were replaced by arginines (FL_PB(K/R)) has deacetylase activity virtually identical to the WT protein (Fig. 8). The findings above suggest that the overall charge, rather than the exact sequence, is critical for tubulin/MT interactions. This notion is further supported by the use of the scrambled 31–59 peptide (a scrambled sequence of the MBD), which binds to MTs with the same affinity as the parent WT peptide (Fig. S12). Finally, it shall be noted that in addition to patches A and B analyzed here, three additional clusters of positively charged lysines (Lys-553, Lys-555; Lys-849, Lys-853, Lys-854; and Lys-872, Lys-873), which are subject to acetylation by p300, have been identified in the HDAC6 sequence (18). However, as these additional sites are located in the DD2 and SE domains of HDAC6 and neither of these domains is capable of mediating HDAC6/MTs interactions, their acetylation can play a role in HDAC6 physiological functions other than MT/tubulin binding/deacetylation. As disordered regions in various proteins have been associated with phase separation or condensate formation (49), it is possible to speculate that this property might also contribute to the enhanced affinity of HDAC6 for MTs.

Note that although the presence of the unmodified MBD significantly increases the deacetylation rate of tubulin, the domain is not essential for HDAC6 deacetylase activity on all
substrates tested so far, including tubulin and microtubules (13, 14, 26, 37). Additionally, other HDAC6 structural features outside the MBD, such as the helix H25 and the loop H20–H21 of the DD2, are critical for deacetylation of αK40 on α-tubulin, but not in the context of short peptides (14). These findings demonstrate the recognition complexity of physiological substrates by HDAC6 that warrant further studies. Another distinction shall be made between binding to and deacetylation of tubulin/MTs by HDAC6 N-terminal mutants. For example, using stabilized MTs as a substrate, we observed a weak deacetylase activity of HDAC6 mutants lacking the MBD (14, 37), yet not its binding to the MT surface. At the same time, it is clear that for the deacetylation reaction to proceed, the enzyme must recognize (and interact with) its cognate substrate. This apparent discrepancy can be explained by temporal resolution and sensitivity of experimental techniques used. The MT deacetylation in cells is easily followed with the anti-AcK40 antibody by Western blotting or fluorescent microscopy as the end point signal at various time intervals. On the other hand, in the case of real-time TIRF microscopy, longer residence time, and ideally also higher concentration (more molecules) of fluorophores, is needed to obtain a detectable signal. We believe that the presence of the MBD allows GFP-HDAC6 fusions to stay attached to the MT surface for a longer period that can be easily moni-

Figure 8. In vivo deacetylase activity and localization of HDAC6 mutants in HEK293T HDAC6 KO cells. A, panel of HDAC6 mutants used for transfection of HEK293T-KO cells. HDAC6 harbors two lysine/arginine rich patches (patch A and patch B) in its N-terminal domain (amino acids 1–86). FL_PB(K/Q) is a neutralized mutant of patch B, whereas FL_PB(K/R) is a positive-charge retention mutant. FL_H216A/H611A is a catalytic-dead mutant. HD6_Δ2–44 and HD6_Δ2–61 are human constructs corresponding to D. rerio HDAC6 constructs (drHD6_Δ1–24 and drHD6_Δ1–39, respectively) previously used for crystallization and tubulin deacetylation assays (Miyake et al. (14)). B, analysis and quantification of tubulin acetylation levels in transfected HEK293T-HDAC6 KO cells using Western blotting. HDAC6 FL or mutants in pcDNA3 vector were transiently expressed in HEK293T-HDAC6 KO cells, and tubulin αK40 acetylation level was tested by immunoblotting. Although the expression of all variants was approximately similar, the acetylation level significantly decreased in the case of HDAC6 mutants with the native patch B and the K/R mutant (FL_PB(K/R). The result is normalized by the expression levels of α-tubulin and HDAC6. *, p < 0.05; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001 by one-way analysis of variance. C, subcellular localization of HDAC6 FL and mutants. The indicated HDAC6 constructs were transiently expressed in HEK293T-HDAC6 KO cells. Localization of HDAC6 was assessed by immunofluorescence microscopy. All HDAC6 mutants localize to the cytoplasm and no re-localization to the nucleus was observed. Scale bar, 10 μm.
HDAC6 are predicted to exist (30). Thirty-three alternatively spliced mRNA variants of human protein kinase CK2 (Ser-458) (31). The local concentration of the binary HDAC6/tubulin complex, which results in a 100-fold faster tubulin deacetylation.

Apart from acetylation, HDAC6 is a target of additional post-translational modifications including ubiquitinylation and phosphorylation and also a subject to alternative splicing (50, 51). Thirty-three alternatively spliced mRNA variants of human HDAC6 are predicted to exist (52) although the evidence for the existence of the translated protein species in vitro is missing. In fact, only one of the splice variants, denoted hHDAC6bApr07, was partially characterized at the protein level. This variant starts at the position 152 of the HDAC6 FL, lacking thus the MBD and a part of DD1, and retains a weak tubulin deacetylase activity. Moreover, it has been suggested that the truncated variant could differ from the full-length HDAC6 in the activation of gene expression, although the mechanism has not been studied (51). Apparently, in addition to lowered tubulin deacetylase activity, HDAC6 variants with the missing or posttranslationally modified N terminus might have other disparate physiological functions and be differentially regulated under various (patho)physiological conditions.

Numerous kinases target HDAC6 and regulate its deacetylase activity toward physiological substrate(s), ultimately influencing diverse cellular functions. G protein–coupled receptor kinase 2 (phosphorylates HDAC6 at positions Ser-1060, Ser-1062, and Ser-1069) (53), extracellular signal-regulated kinase (Thr-1031, Ser-1035) (54), p38α (54), protein kinase Cζ (50), protein kinase CK2 (Ser-458) (55), Aurora protein kinase A (56), and glycogen synthase kinase 3β (Ser-22) (57) were all shown to positively modulate HDAC6 tubulin deacetylase activity in vitro/in vivo, whereas HDAC6 phosphorylation by epidermal growth factor receptor kinase (Tyr-570) (58) has the opposite effect. Ser-22, the target residue of glycogen synthase kinase 3β, is the only characterized phosphorylation site residing within the N-terminal domain of HDAC6, whereas the other phosphorylated residues are distributed throughout DD1, DD2, and SE domains. Seen through the prism of findings presented here, it is not clear how the introduction of a negatively charged phosphate group at the MBD could contribute to increased HDAC6 deacetylase activity against tubulin. In general, mechanisms of HDAC6 activation/inhibition by phosphorylation events are not well-characterized and warrant further studies in the future.

The SE domain, together with the NES, is responsible for the cytoplasmic retention of HDAC6 and it has been reported to bind MTs with low affinity in cellulo (21). Our in vitro reconstitution experiments, however, do not support the notion of direct SE/tubulin interactions (Fig. 2). It is thus likely that observed binding of SE to MTs in the cellular milieu can either be mediated by an unknown MAP or, alternatively, dependent on unidentified PTM(s) within the SE domain that is missing from our recombinant enzyme.

Based on our findings reported here, we proposed a molecular mechanism of tubulin deacetylation by HDAC6 and compared it to the deacetylation of short peptides (Fig. 9). For short peptides with internal lysine, the target sequence is directly recognized and deacetylated by the DD2 in a single step. Because the recognition process (limited complementary interfaces) is not very effective, high concentrations of a peptidic substrate, reflected in high millimolar $K_m$ values, are needed for efficient deacetylation. On the other hand, in the case of tubulin deacetylation, HDAC6 is first attracted to tubulin via nonspecific ionic interactions of its disordered MBD, increasing thus the local concentration of the binary HDAC6/tubulin complex that is then deacetylated much more efficiently.

Collectively, we identified the N-terminal domain of HDAC6, denoted the MBD, as a component critical for facilitating HDAC6 interactions with MTs as well as enhancing HDAC6 deacetylase activity against free tubulin by more than 100-fold. From the data reported here, it is obvious that substrate recognition by HDAC6 is more complex than previously appreciated, and these findings shall be considered when mutated/truncated constructs are used in biological experi-

Figure 9. Mechanism of substrate deacetylation by HDAC6. A, short peptides, such as the 11-mer peptide derived from the αK40 loop of α-tubulin (acetylated lysine K40 denoted by a star), are directly recognized and deacetylated by the catalytic domain DD2 in a single step. However, the overall catalytic efficiency of the deacetylation is low due to suboptimal recognition/binding of the peptide by HDAC6 (high $K_m$ values). β, HDAC6 mutants missing the MBD bind and deacetylate tubulin dimers in a single step and with similar efficacy to that for short peptides. C, tubulin deacetylation by HDAC6 FL is enhanced ~100-fold compared with mutants missing the MBD. Our data suggest that the MBD of HDAC6 first interacts with tubulin via ionic interactions, thus increasing the local concentration of the binary HDAC6/tubulin complex, which results in a 100-fold faster tubulin deacetylation.
ments. It will also be interesting to see whether the MBD and/or domains outside the catalytic core can modulate deacetylation of other HDAC6 physiological substrates. Finally, our findings open new avenues in the design of HDAC6-specific compounds that could selectively block deacetylation of only a single (or a limited set) of its physiological substrates.

**Experimental procedures**

**Chemicals**

If not stated otherwise, all chemicals were purchased from Sigma-Aldrich. Alexa Fluor 647 NHS Ester (A37573) and NHS-Rhodamine (46406) were purchased from Thermo Fisher Scientific. Nexturastat A was a kind gift from Alan Kozikowski, StarWise Therapeutics LLC (Madison, WI).

**Cloning and site-directed mutagenesis**

Full-length WT human HDAC6 (UniProtKB: NP_006035.2, Q9UBN7) was used as a template for the cloning of all HDAC6 truncated/mutated constructs. Truncated mutants were PCR amplified using corresponding sets of gene-specific primers (Table S1) and cloned into the pDONR221 donor vector via the BP recombination reaction according to the manufacturer protocol (Invitrogen). The identity of each donor clone was verified by Sanger sequencing and the sequence encoding a given identity of all HDAC6 expression clones was verified using Sanger sequencing, and their schematic representations are shown in Fig. 2.

**HDAC6 expression and purification**

All HDAC6 mutants were expressed in HEK293T17 cells and purified to near homogeneity by the combination of Strep-Tactin affinity and size-exclusion chromatography essentially as described previously (19, 37). Briefly, HEK293T17 cells were grown in Gibco FreeStyle F17 Expression Medium (Thermo Fisher Scientific) supplemented with 0.1% Pluronic F-68 (Invitrogen) and 2 mM L-glutamine at 110 rpm under a humidified 5% CO2 atmosphere at 37 °C. Linear polyethyleneimine (Polysciences Inc., Warrington, PA) was used to mediate transient transfection. Four h post transfection, the cell suspension was diluted by the addition of an equal volume of ExCell293 Serum-Free Medium and cells were harvested 72 h post transfection by centrifugation at 500 × g for 5 min. The cell pellet was snap-frozen in liquid nitrogen and stored at −80 °C.

For purification, cell pellets were lysed in 50 mM Tris, 150 mM NaCl, 10 mM KCl, 10% glycerol, 0.2% Nonidet P-40, pH 8.0, supplemented with a mixture of protease inhibitors (Roche) followed by incubation for 30 min on ice. Cell lysate was cleaved by centrifugation at 40,000 × g for 30 min at 4 °C, the supernatant loaded on a Strep-Tactin column (IBA, Göttingen, Germany) and then eluted with the lysis buffer containing 2 mM desthiobiotin. If desired, the N-terminal tag was cleaved by tobacco etch virus protease (1:20 ratio (w/w)) overnight at 4 °C.

The final purification step included size-exclusion chromatography on Superose 6 column (GE Healthcare Life Sciences). Purified proteins were concentrated to 1 mg/ml and flash-frozen in liquid nitrogen until further use.

**Fluorimetric assay**

The deacetylation activity of HDAC6 constructs was determined as described previously (37). Briefly, HDAC6 constructs were incubated with 2-fold serial dilutions of the substrate acetyl-Gly-Ala-(acetyl-Lys)-AMC (Bachem, Bubendorf, Switzerland) in the reaction buffer (50 mM HEPES, 140 mM NaCl, 10 mM KCl, 0.2 mg/ml BSA, 1 mM TCEP, pH 7.4) in 384-well plates for 30 min at 37 °C in total volume of 20 μl. The reaction was quenched by adding 10 μl trypsin solution (4 mg/ml trypsin) and incubating 15 min at 37 °C. Subsequently, the aminomethylcoumarin (AMC) fluorescence signal was detected by a CLARIOstar fluorimeter (BMG Labtech GmbH, Ortenberg, Germany) (λex = 365 nm, λem = 440 nm). The GraphPad Prism software (GraphPad Software, San Diego, CA) was used for data analysis and fitting.

**HPLC-based deacetylation assay**

A 2-aminobenzoyl–labeled 11-mer peptide derived from the α-tubulin sequence (Abz-QMPSDK(Ac)TIGGG-NH2) was used as a substrate in the RP-HPLC setup. Reaction mixtures comprised the peptide in the concentration range of 0.8–400 μM and 20 nM HDAC6 construct in an assay buffer comprising 50 mM HEPES, 140 mM NaCl, 10 mM KCl, 0.2 mg/ml BSA, 1 mM TCEP, pH 7.4. Reactions were incubated for 60 min at 37 °C, quenched by the addition of 0.5% acetic acid, and centrifuged at 2000 × g at 37 °C for 15 min to remove precipitated BSA and HDAC6. Reactions were analyzed by RP-HPLC (Shimadzu, HPLC Prominence system) using a Kinetex 2.6 μm XB-C18 100 Å column (100 × 3 mm; Phenomenex, Torrance, CA). The mobile phase A was 5% acetonitrile (ACN) with 0.1% (v/v) trifluoroacetic acid (TFA), and the mobile phase B was 95% ACN with 0.1% (v/v) TFA. The separation of the reaction product from the acetylated substrate was performed in a 12-min linear gradient from 10 to 35% of eluent B at a flow rate of 0.6 ml/min. The excitation/emission wavelengths were set to 320/420 nm, respectively, to monitor the fluorescence of the reaction product. The amount of the reaction product was quantified from the peptide calibration curve. The data were fitted using the GraphPad Prism software (GraphPad Software, San Diego, CA), and kinetic values were calculated through nonlinear regression analysis.

**Pelleting assay**

A pelleting assay was run in a similar way to already-published co-sedimentation assays (59) with several modifications, described below. Working stocks of HDAC6 constructs were precleared by centrifugation at 30,000 × g for 45 min at 30 °C prior to combining the binding reaction with MTs. Binding reactions were kept at 32 °C throughout the whole procedure. In a 40-μl reaction mix, Taxol-stabilized MTs in a final concentration of 0.5 μM were combined with HDAC6 and its constructs of final concentration 1 μM in BRB50 supplemented with 0.01% C12E8 detergent, 10 μM Taxol, and 0.5 mM TCEP.
Recognition and deacetylation of tubulin by HDAC6

After a 5-min incubation, the reaction was centrifuged at 30,000 \( \times g \) for 45 min without using any cushion. The whole supernatant was taken and mixed with 5 \( \mu l \) of 4 \( \times \) concentrated Laemmli sample buffer, whereas the pellet was directly dissolved in 20 \( \mu l \) of Laemmli sample buffer. Samples were heated at 95 °C for 5 min, and protein separation by SDS-PAGE in 13% Tris-glycine gels followed. Proteins were visualized in gel by Coomassie Brilliant Blue G-250 staining. Photograph of the gel was processed in Adobe Photoshop software.

**Tubulin isolation from porcine brains**

Tubulin isolation from pig brain tissue was carried out according to the established protocol (60) as described previously (37). The final tubulin stock was stored in the BRB80 buffer at the 20 mg/ml concentration as determined from absorbance readings at \( \lambda_{280} \) using NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific).

**Tubulin labeling**

Tubulin was diluted in a polymerization buffer (1 mM GTP, 3.5 mM MgCl\(_2\), 30% glycerol, BRB80 buffer, pH 6.9) at a final concentration of 30 \( \mu M \) and left to polymerize for 1 h at 37 °C. MTs were centrifuged through pre-warmed high-pH cushion (0.1 mM HEPES, 1 mM EGTA, 1 mM MgCl\(_2\), 60% glycerol, pH 8.6) at 150,000 \( \times g \) for 30 min. Resuspended MTs were labeled through NHS coupling with Alexa Fluor 647 NHS ester or rhodamine NHS ester at a 10-molar excess of the dye to tubulin for 1 h at 37 °C. Two volumes of a stop solution (2 \times BRB80 buffer, 40% glycerol, 100 mM glutamate) were added and incubated for 5 min to terminate the labeling. The labeled MTs were pelleted through a low pH cushion (BRB80, 60% glycerol, pH 6.9), resuspended in an ice-cold BRB80 buffer, and left for 70 min on ice to induce depolymerization. Following the two cycles of polymerization and depolymerization tubulin concentration was determined from the absorbance at \( \lambda_{280} \) nm, the labeled tubulin ali-quoted at 4 mg/ml and snap-frozen in liquid nitrogen. For TIRF experiments, the Alexa Fluor 647– or rhodamine-labeled tubulin was mixed with unlabeled tubulin at 1:30 ratio.

**Microtubule polymerization**

Tubulin (2.5 \( \mu M \)) in the polymerization buffer (BRB80 buffer, 1 mM GMPCPP (Jena Bioscience, Jena, Germany), 1 mM MgCl\(_2\)) was incubated on ice for 5 min and then for 2 h at 37 °C. The sample was centrifuged at 14,000 \( \times g \) for 30 min at RT, the supernatant carefully discarded and the pelleted MTs dissolved in the BRB80 buffer supplemented with 1 \( \mu M \) Taxol (total volume 100 \( \mu l \)). The double-stabilized MTs were stored at room temperature until further use.

**Microtubule subtilisin treatment**

Double-stabilized MTs were digested by incubating with subtilisin (1:50 w/w ratio) for 60 min at 30 °C (61). The reaction was quenched by the addition of 5 \( \mu l \) phenylmethylsulfonyl fluoride (PMSF) and incubation 1 h at RT. The sample was then centrifuged at 14,000 \( \times g \) for 30 min at RT, and pelleted MTs were dissolved in BRB80 buffer (supplemented with 1 \( \mu M \) Taxol) and stored at RT. The removal of the C-terminal tails was verified by the MS analysis (Fig. S5).

**Total internal reflection fluorescence microscopy**

TIRF microscopy was performed using a Nikon Ti-E microscope equipped with an H-TIRF System (Nikon, Japan), a 60× oil immersion 1.49 NA TIRF objective and an Andor iXon Ultra EMCCD camera (Andor Technology, Belfast, UK) controlled by the NIS Elements software (Nikon, Japan). Visualization was done by sequential dual-color imaging (switching between 488 nm and 640 nm excitation lasers to detect the GFP-HDAC6 fusion and Alexa Fluor 647–labeled MTs, respectively). Additionally, rhodamine-labeled MTs (detected at 561 nm) were used as nontreated controls in the experiments with subtilisin-treated MTs. The image acquisition rate was 1 frame per 0.4–0.5 s. For the determination of HDAC6 single-molecule movement mode, we used Nikon Ti-E microscope with N-SIM and N-STORM modules (Nikon, Japan), Nikon CFI HP Apo TIRF 100× oil objective, NA 1.49, an Andor iXon Ultra DU897 camera (Andor Technology, Oxon, UK) controlled by the NIS Elements software (Nikon, Japan). The image acquisition rate was increased one frame per 30 ms. Image analysis was carried out using Fiji (62, 63).

**TIRF-based evaluation of HDAC6/MTs affinity**

MTs and flow cells were prepared as described previously (37, 64). Briefly, microscope chambers were built from silanized coverslips (Corning Cover Glass Product) prepared as described previously (65). Parafilm was used to space glasses to form channels of ~0.1-mm thickness, 3-mm width, and 18-mm length. MTs were attached to the glass surface in each chamber via anti-β-tubulin antibodies (Sigma-Aldrich, T7816, 20 \( \mu g/ml \) in PBS). Subsequently, various concentrations of GFP-HDAC6 fusion constructs were added to the chamber in the binding buffer (BRB50, 0.5 mM TCEP, AMRESCO, Solon, OH), 0.5 mg/ml casein, 10 \( \mu M \) paclitaxel, 0.001% C\(_{12}\)E\(_{8}\) (dodecylglactoclycol), 20 mM d-glucose, 110 \( \mu g/ml \) glucose oxidase, and 20 \( \mu g/ml \) catalase. The fluorescent signal of the GFP-HDAC6 fusion was determined by completely co-localizing with the signal of labeled MTs. Experimental data were processed with Fiji and binding curves fitted with the GraphPad Prism software using the nonlinear regression model. Experimental data points represent mean values ± S.D.; \( n = 3 \). Details of data processing and fitting, including calculations of apparent \( appK_d \) values, are described in the supporting information (see method description S15).

**Deacetylation of tubulin dimers**

Deacetylation reactions were performed essentially as described previously (37). Briefly, tubulin dimers (1 \( \mu M \)) were incubated with optimized concentrations of HDAC6 constructs in a reaction buffer (BRB80, 1 mM TCEP, 0.2 mg/ml BSA, 80 \( \mu M \) colchicine) for 30 min at 37 °C in the total volume of 20 \( \mu l \). Five microliters of TFA (5%) was used to quench the reaction. The deacetylation reaction was stopped by the addition of 20 \( \mu l \) of 5 \( \times \) Laemmli buffer and boiling samples at 95 °C for 5 min.

**SDS-PAGE, Western blotting, and data analysis**

Protein samples were resolved by SDS-PAGE in a 10% polyacrylamide gel at 150 V for 90 min. For immunoblotting, the gel...
was transferred to a PVDF membrane using the Bio-Rad Trans-Blot Turbo Transfer System (Hercules, CA) under standard conditions. The membrane was blocked with 5% BSA dissolved in TBS. Monoclonal anti-acetylated tubulin antibody (Sigma, T7451, 0.3 μg/ml) with the combination of secondary antibody goat anti-mouse antibody conjugated to Alexa Fluor 488 were used for the detection of acetylated αK40 tubulin. Total tubulin was detected by rabbit polyclonal anti-α-tubulin antibody (Abcam, Cambridge, MA; ab18251; 1 μg/ml) and the secondary donkey anti-rabbit antibody conjugated to Alexa Fluor 568. The fluorescent signal was detected on a Typhoon FLA9500 imager (GE Healthcare Life Sciences) and quantified using Quantity One 1-D Analysis Software (Bio-Rad).

Microscale thermophoresis

Two-fold dilution series in the final volume of 10 μl were prepared from nonlabeled tubulin in the BRB50 buffer with 0.1% (v/v) Tween 20. A GFP-HDAC6 fusion was diluted in the same buffer, and an equal amount was added to the dilution series (100 μM–3 nm) of tubulin to the final concentration of 100 nM. The reactions were incubated at room temperature for 5 min. Reactions were centrifuged at 13,000 × g for 5 min at 4 °C. Monolith NT.115 Capillaries standard (NanoTemper, Munchen, Germany) were used for the measurement. Excitation power was set to 30 and 40% and MST power was set to 40 and 60% for FITC-labeled synthetic peptides and GFP-fusions, respectively. The results were processed with MO.Afinity Analysis and GraphPad Prism. For data analysis, the change in sample fluorescence upon IR-laser activation is monitored by calculating the ratio between the fluorescence after a given MST-on time (F1) and the fluorescence prior to IR laser activation (F0). A model for calculating apparent $K_D$, value is implemented in the control software of the MST instrument and is derived from the law of mass action:

$$f(\text{concentration}) = U + (B - U) \frac{(C + Tc + appK_D - \text{Sqrt}((C + Tc + appK_D)^2/2*Tc))}{2*Tc},$$

where $U$ is response value of unbound state (F0), $B$ is response value of bound state (F1), $appK_D$ is dissociation constant, $Tc$ is final concentration of a fluorescent molecule, $C$ is concentration of the ligand molecule.

Cell-based HDAC6 localization and activity test

To construct expression vectors for expression of HDAC6 functional mutants, pcDNA3-hsHDAC6 was amplified with appropriate sets of primers, and the PCR product was self-ligated to generate internal deletions and obtain a mutated plasmid. Each mutated plasmid was transfected into HEK293T-HDAC6 KO cells, which were established in the previous work (Saito et al. 20) with Lipofectamine 2000 (Life Technologies). After 2 days, cells were subjected to immunoblotting or immunofluorescence.

For immunoblotting, cells were washed by ice-cold PBS and lysed in Triton lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100 and Complete EDTA-free Protease Inhibitors (Roche)) for analysis. The extracted cytosolic fraction was boiled for 10 min in SDS-PAGE sample buffer and separated in 4–12% NuPAGE gels (Invitrogen). Proteins were transferred onto PVDF membranes (Immobilon-P, Millipore), proved with specific primary antibody for overnight and secondary antibody for 1 h under 5% nonfat dry milk in TBS blocking conditions and detected with Amersham Biosciences ECL Western blotting reagent (GE Healthcare). Primary antibodies were as follows: anti-α-tubulin (DM1A) (Sigma-Aldrich, T9026), anti-(KAc)4-α-tubulin (Enzo, NY; BML, SA452–0100), and anti-human-HDAC6 was developed in Barinka Laboratory, Institute of Biotechnology CAS (clone 2H3G1). Secondary antibodies were as follows: Amersham Biosciences ECL Mouse IgG, HRP-linked whole Ab from sheep (GE Healthcare, NA931V), Amersham Biosciences ECL Rabbit IgG, HRP-linked whole Ab from donkey (GE Healthcare, NA934V).

For immunofluorescence, cells on a coverglass were washed with ice-cold PBS, then fixed with 4% paraformaldehyde. After permeabilization with 0.5% Triton X-100 in PBS for 10 min, the cells were incubated with specific primary and secondary antibodies in 10% goat serum blocking buffer, then mounted with ProLong Gold Antifade Reagent with DAPI (Cell Signaling Technology, 8961). Images were captured by Axio Imager Z1 microscope (Zeiss, Germany). Primary antibody was as follows: anti-human-HDAC6 was developed in Barinka Laboratory (clone 2H3G1). Secondary antibody was as follows: Alexa Fluor 488 Goat anti-Mouse IgG (H+L) Secondary Antibody (Invitrogen, A11001).

Peptide synthesis

Chemicals and general methods—Most of the Fmoc-protected amino acids were purchased from GL Biochem Ltd (Shanghai, China). N,N-dimethylformamide (DMF), piperidine, ethyl (hydroxyimino)cyanoacetate (OxymaPure), TentaGel R RAM resin (0.18 mmol/g), and N-α-(9-fluorenylmethoxycarbonyl)-N-α-(2,4-dimethoxybenzyl)-glycine were purchased from Iris Biotech (Marktredwitz, Germany). N,N-diisopropylethylamine (DIEPA), TFA, and dichloromethane (DCM) were purchased from Carl Roth (Karlsruhe, Germany). For HPLC separations solvents consisting of water (solvent A) and ACN (solvent B), both containing 0.1% TFA, were used.

UPLC-MS analysis was performed using Waters ACQUITY UPLC-MS system (Milford, MA) with a Waters ACQUITY-UPLC-MS-BEH C18; 1.7 μm (2.1 × 50 mm; 30 Å) column. As a mobile phase, 0.1% formic acid in H2O (solvent A) and 0.1% formic acid in ACN (solvent B) solutions were used. A typical gradient from 95:5 (v/v) of H2O:ACN to 5:95 (v/v) of H2O:ACN in 6 min was used for most of the runs. Data analysis was performed using Waters MassLynx software. Purification of peptides was done on Shimadzu LC System with a Phenomenex Kinetex 1.7 μm 5 μm XB-C18 (250 × 21.1 mm, 100 Å) column using different gradients of 0.1% TFA in H2O (solvent A) and 0.1% TFA in ACN (solvent B) solutions.

Solid-phase peptide synthesis—The peptides were synthesized using Fmoc-based solid-phase peptide synthesis with automated microwave peptide synthesizer Liberty Blue (CEM Corporation, Matthews, NC). The coupling of amino acids was performed with DIC/OxymaPure at 90 °C for 2 min. All coupling steps were performed twice. Fmoc deprotection was accomplished with 10% piperazine solution in ethanol: N-methyl-2-pyrrolidone (1:9, v/v) containing 0.1 M of N-hydroxysuccinimide at 90 °C for 1 min. To avoid side reactions
and improve synthesis efficiency, all methionine residues were replaced by norleucine and some glycine residues were incorporated as N-2,4-dimethoxybenzyl protected derivatives.

**FITC labeling**

FITC labeling was performed on the resin using FITC (3 eq) and DIPEA (6 eq) in DMF for 3 h.

**Global deprotection**

After the synthesis, the resin was washed several times with DCM, MeOH, DCM before TFA treatment. Then it was incubated with TFA-H₂O-TIPS (92.5:5:2.5, v/v/v) solution for 2–4 h, filtered and the volatiles were removed in vacuo. The residue was dissolved in ACN-H₂O solution and purified with HPLC. Fractions containing pure peptide (as judged by UPLC-MS) were frozen and lyophilized affording pure peptide.

N-terminal modification was performed with 2-aminobenzoic acid (4 eq), O-(benzotriazol-1-yl)-N,N,N′,N″-tetramethyluronium hexafluorophosphate (4 eq) and DIPEA (6 eq) in DMF for 3 h. Alternatively, acetylation was performed using acetic anhydride/DIPEA/DMF (1:2:7, v/v/v) mixture.

**Data availability**

All data generated or analyzed during this study are included in this published article (and its supporting information files).

**Author contributions**—K. U. and C. B. conceptualization; K. U., M. Saito, M. M., J. M., P. B., M. Schutkowski, P. M., and C. B. resources; K. U. and C. B. data curation; K. U., M. Saito, M. M., J. M., P. B., B. H., and C. B. formal analysis; K. U., M. Saito, J. M., P. B., M. Schutkowski, P. M., and C. B. supervision; K. U., M. Saito, J. M., P. B., M. Schutkowski, P. M., and C. B. funding acquisition; K. U. and C. B. validation; K. U., Z. N., M. Saito, M. M., J. M., Z. K., P. B., B. H., M. Schutkowski, P. M., and C. B. investigation; K. U., M. Saito, M. M., J. M., P. B., M. Schutkowski, P. M., and C. B. methodology; K. U., M. Saito, M. M., J. M., P. B., B. H., M. Schutkowski, P. M., and C. B. writing—original draft; K. U. and C. B. project administration; K. U., M. Saito, M. M., J. M., P. B., M. Schutkowski, P. M., and C. B. writing—review and editing.

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