

Profiling of Substrates for Zinc-dependent Lysine Deacetylase Enzymes: HDAC3 Exhibits Decrotonylase Activity In Vitro**

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The lysine deacetylase (KDAC) enzymes are hydrolases that catalyze the removal of acetyl functionalities from the ϵ -amino group of lysine residues in a variety of proteins including the tail regions of histones in chromatin complexes.^[1] Recently, protein acetylation has been recognized as an important general posttranslational modification (PTM), thus considerably extending the possible targets of the KDAC enzymes;^[2] however, the N-terminal segments of core histones (H2A, H2B, H3, and H4) are the most extensively studied proteins among those that have been shown to contain ϵ -N-acetyllysine (Kac) modifications.^[3] Histone acetylation affects both chromatin packing and recruitment of transcription factors and thus indirectly affects gene expression in the cell.^[3,4] Several potent histone deacetylase inhibitors that lead to an increase in global histone acetylation (primarily through inhibition of class-I KDACs) have entered clinical trials for treatment of various types of cancer.^[5] Two compounds have been approved for treatment of cutaneous T-cell lymphoma, that is, suberoylanilide hydroxamic acid (SAHA, vorinostat)^[6] and depsipeptide FK-228 (romidepsin)^[7] formulated as the drugs Zolinza and Istodax, respectively. Individual KDACs have also been linked to a variety of noncancerous diseases including neurodegenerative disorders,^[8] chronic pain,^[9] and cystic fibrosis,^[10] as well as to learning and memory;^[11] therefore reliable tools (substrates and inhibitors) for efficient and accurate profiling of KDAC subtypes are highly desirable. Furthermore, ϵ -N-crotonyllysine (Kcr) has been identified as a PTM of histone proteins,^[12] and both ϵ -N-succinyllysine (Ksuc) and ϵ -N-malonyllysine (Kmal) have been observed in various proteins.^[13]

Since the first discovery of a mammalian lysine deacetylase enzyme,^[14] eleven zinc-dependent human KDACs have been identified and are classified according to sequence similarity; class I (HDAC1, 2, 3, and 8), class IIa (HDAC4, 5, 7, and 9), class IIb (HDAC6 and 10), and class IV

(HDAC11).^[15] Members of class I, IIb, and IV are all believed to be functional deacetylases, whereas the class-IIa isoforms exhibit considerably lower intrinsic enzymatic activity and have been shown to associate with HDAC3 in multiprotein complexes that harbor deacetylase activity.^[16] The physiological role of the class-IIa isoforms has therefore been the subject of debate.^[17,18] The fact that only limited information has been reported to date on the pharmacology of HDAC10^[19] and HDAC11^[20] might at least in part be due to a lack of efficient substrates for these two isoforms.^[18,21]

Catalytic efficiencies and inhibitor selectivity profiling data obtained from in vitro experiments with recombinant enzymes may not always reflect the behavior of these enzymes in their native environments, which often involve multiprotein complexes in vivo.^[22] However, in vitro screening protocols using recombinant enzymes remain important for discovery and evaluation of new inhibitors as well as for mechanistic investigations.^[23] With all eleven different zinc-dependent KDAC isoforms now commercially available, more selectivity profiling studies have started to appear in the literature.^[21,24,25] To ensure the best possible foundation for the performance of these types of studies and to complement the excellent recent profiling of a diverse selection of inhibitors against HDACs 1–9,^[18] we decided to evaluate the activities of the complete panel of recombinant human KDAC isoforms against a collection of fluorogenic substrates. The selection of substrates encompasses the most commonly used commercial chemotypes as well as candidates designed to address the recently discovered lysine PTMs.^[26] To our knowledge, such a systematic investigation of substrates with respect to lysine acylation has not been reported to date for the full panel of zinc-dependent lysine deacetylase enzymes (HDAC1–11), although several important studies on substrate specificity of KDACs have been reported.^[27]

Recently, fluorogenic substrates containing ϵ -N-trifluoroacetyllysine (Ktfa) have been shown to enable efficient profiling of class-IIa KDAC isoforms that are otherwise inactive towards common acetylated substrates. In the first report describing this discovery, the Boc-Ktfa-AMC (**1b**; for structure see Figure 1a) substrate was used,^[17] and an optimized trifluoroacetylated substrate based on amino acids 10–12 of core histone H4 (**3b**) was later reported in an impressive profiling of a large series of histone deacetylase inhibitors (including several drug candidates currently in clinical trials) against HDACs 1–9.^[18] We therefore prepared **1b** and **3b** as well as their acetylated counterparts **1a** and **3a**, and since **2a** is also a known fluorogenic deacetylase substrate we included substrates **2a** and **2b** in our panel (Figure 1a). Furthermore, two commercially available substrates (**4a** and **4b**), which are based on amino acids 379–382 of pro-apoptotic

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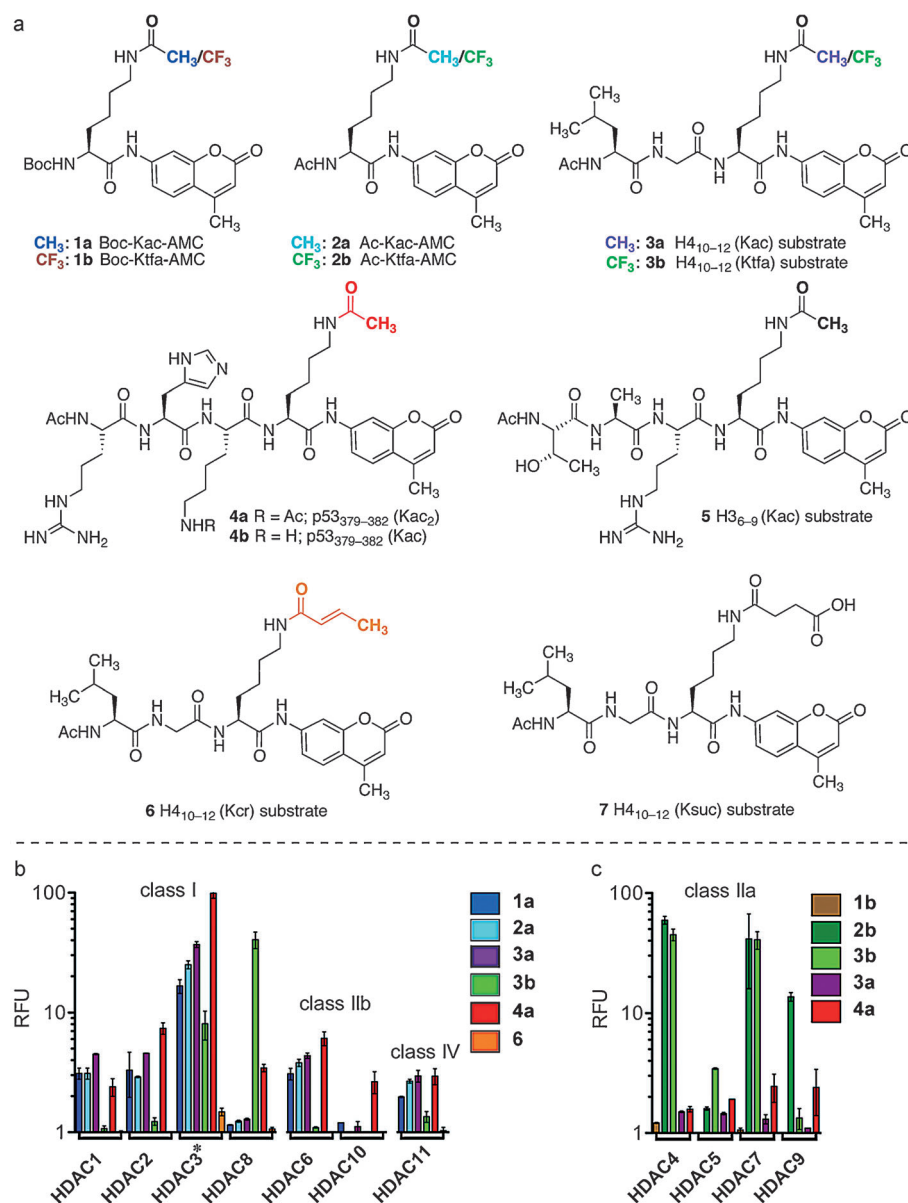


Figure 1. Substrate structures and selected deacylation data. a) Structures of the analyzed collection of substrates. b) Selected deacylation data (*HDAC3 was obtained as a complex with nuclear receptor corepressor 1 (NCoR1)). c) Selected deacylation data of the class-IIa KDACs. In the assay, the enzyme trypsin cleaves the AMC group of substrates with a deacetylated lysine residue, thereby resulting in a fluorescent signal. Consult the Supporting Information for full profiling data. The data were obtained as end-point readings after incubation for one hour with each substrate at 37 °C, and represent at least two individual experiments performed in duplicate. The amount of recombinant enzyme added to each reaction was 50 ng as determined from stock solution concentrations and purities given by the respective vendors. The relative fluorescence unit (RFU) values were normalized to control wells without KDAC present. Notice that although we find the KDAC abbreviation to be more meaningful for this class of enzymes, we use the common names of individual isozymes (i.e., HDAC1, HDAC2 etc.). AMC = 7-amino-4-methylcoumarin, Boc = *tert*-butoxycarbonyl.

protein p53, were included in our collection along with **5**, which contains amino acids 6–9 of core histone H3.^[28] To address the recent work describing Kcr^[12] and Ksuc^[13a,b] functionalization as novel protein modifications,^[26] we finally included substrates **6** and **7** (Figure 1a), the syntheses of which we have described recently.^[29]

To obtain a comparative data set, the initial screening was performed with a fixed concentration (10 μ M) of each substrate and equal amounts of enzyme per reaction regardless of expectations with respect to the inherent deacetylase activity. Importantly, this initial comparison does not take into account the possible differences in enzyme and/or substrate concentrations required to reach an optimal range of linearity for each enzyme. Thus, the data obtained from our systematic screening enables a comparison of activities under these specific conditions exclusively, whereas a comparison of kinetically determined catalytic efficiencies ($k_{\text{cat}} \times K_{\text{m}}^{-1}$) is provided below for selected enzyme–substrate combinations.

Not surprisingly based on literature precedents,^[18] HDAC1–3 and HDAC6 were generally active against acetylated substrates, while HDAC4, 5, 7, 8, and 9 exhibited activity with trifluoroacetylated substrate **3b** (Figure 1b,c and Figure S1 in the Supporting Information). Substrate **3b** was hydrolyzed more efficiently than **1b**, as reported for class-IIa KDACs, whereas substrate **2b** (Ac-Ktfa-AMC) gave rise to enzyme activities that were similar or better than those observed with tripeptide **3b** for HDAC4, 7, and 9 (Figure 1c). This behavior renders **2b** a simple and efficient alternative to tripeptide **3b** for assays employing these KDACs.

In our initial screening, both HDAC10 and 11 were able to deacetylate substrate **4a** at the relatively low enzyme concentration applied, and HDAC11 also showed deacetylase activity against substrate **3a** in the same range as HDAC1, 2, and 6. The activity of HDAC11 against **3b**, which was recently reported,^[25] proved to be significantly lower (Figure 1b), thus

pointing to the use of substrate **3a** or **4a** for studies involving HDAC11 and substrate **4a** for studies of HDAC10. Enzyme loading experiments showed a final enzyme concentration of 5–10 nM (≥ 100 ng/reaction) should be sufficient for end-point assays with substrate **4a** (Figure S3 in the Supporting

Information). This concentration is similar to the amount used previously for HDAC1.^[30]

HDAC3–NCoR1 was able to process acetamides as well as trifluoroacetamides efficiently, and was the only recombinant enzyme showing a measurable effect against the crotonylated substrate **6**, which will be discussed below in further detail. Finally, none of the KDAC isoforms were able to desuccinylate substrate **7** under our conditions (Figure S1 in the Supporting Information).^[29]

As mentioned, a comparison of the catalytic efficiencies of the different enzyme isoforms on various substrates requires determination of kinetic parameters (i.e., Michaelis–Menten constant K_m , maximal velocity V_{max} , and catalytic rate k_{cat}). We thus performed Michaelis–Menten analyses with selected enzymes against substrates **3a** and **3b** since these were the substrates applied by Bradner et al.^[18] Because of the poor conversion of **3a** by HDAC7 and 8, we used **4a** as Kac substrate for these two isozymes (Table 1).

HDAC3 and HDAC8, which are both believed to be functional deacetylase enzymes in vivo,^[3,4] were able to process Ktfa substrate **3b**. HDAC3–NCoR1 proved to be

Table 1: Kinetic parameters for selected enzyme–substrate combinations.^[a]

Enzyme	Substrate	K_m [μ M]	k_{cat} [s^{-1}]	k_{cat}/K_m [$s^{-1} M^{-1}$]
Class I				
HDAC3 ^[b]	3a	10 ± 3 (6) ^[c]	7.1×10^{-2}	7.1×10^3
	3b	140 ± 33	0.35	2.6×10^3
HDAC8	3b	440 ± 120 (190) ^[c]	16.5	3.8×10^4
	4a	90 ± 60	1.7×10^{-2}	2.0×10^2
Class IIa				
HDAC4	3b	10 ± 5 (10.3) ^[c]	0.77	8.2×10^4
	3a	20 ± 12	2.2×10^{-4}	11
HDAC7	3b	(19.8) ^[c]	— ^[c]	— ^[c]
	4a	15 ± 4	3.4×10^{-4}	23
Class IV				
HDAC11	3a	11 ± 3	2.0×10^{-3}	1.9×10^2

[a] Values are based on at least two individual experiments performed in duplicate in standard HDAC assay buffer at ambient temperature. For calculation of the k_{cat} values from measured V_{max} values, we relied on the enzyme stock solution concentrations and purities given by the vendors. See Figure S2 in the Supporting Information for Michaelis–Menten plots. [b] In complex with NCoR1. [c] Values in parentheses are from the literature, k_{cat} values were not given in the cited publication.^[18]

just two to three times more efficient against Kac substrate **3a** than against Ktfa counterpart **3b**, albeit at a significantly higher K_m value for **3b**. These findings show that the trifluoroacetamide substrate **3b** has a lower affinity for the binding pocket of HDAC3 (higher K_m) while it is turned over more rapidly (higher k_{cat}) than the corresponding acetamide **3a**. Although HDAC8 was significantly more substrate-specific in its deacetylase activity, as it exclusively deacetylated substrate **4a** of the Kac substrates in our panel, a similar trend concerning the difference in turnover of acetamide (**4a**) and trifluoroacetamide (**3b**) substrates was observed when comparing the kinetic parameters of HDAC8 (Table 1). For this isoform, however, the difference in turnover was significantly greater (ca. 1000-fold), which resulted in a 190-fold

increase in the catalytic efficiency favoring the detrifluoroacetylase activity. It is perhaps a bit counter-intuitive that the chemically more labile trifluoroacetamides were processed to a lower extent than the corresponding acetamides by some KDAC isoforms. Now kinetic data provide evidence that the increased steric bulk of the trifluoromethyl group may play an important role by affecting substrate affinity.^[31] Kinetic investigations were not possible for HDAC1 and 2, since these enzymes did not show any detrifluoroacetylase activity in our assays; further experiments are therefore required to determine whether this may also be explained by steric reasons.

In light of the poor deacetylase activity generally reported for HDAC4, it was not surprising to find that its catalytic efficiency ($k_{cat} \times K_m^{-1}$) was more than 7000 times greater with Ktfa substrate **3b** than with **3a**. Interestingly however, the K_m values for HDAC4 with the two different substrates were in the same range. The other class-IIa enzyme tested (HDAC7) also exhibited a low catalytic efficiency with substrate **4a**, and similar K_m values for Ktfa and Kac substrates (Table 1). These findings are all in agreement with our initial screening results. Moreover, the low micromolar K_m values (relatively high affinity) combined with very low k_{cat} values (low turnover) obtained for the acetamide substrates support the recently proposed hypothesis that HDAC4 and 7 may fulfill a role as Kac recognition domains rather than (or in addition to) being substrate-specific functional hydrolases.^[18] Our results therefore complement the evidence from reported Ktfa–Kac competition experiments.^[19]

Interestingly, HDAC3–NCoR1 showed a measurable effect against Kcr substrate **6** at the applied enzyme concentration; thus this decrotonylase activity was also investigated in further detail (Figure 2). The catalytic efficiency shows that HDAC3–NCoR1 is able to process Kcr in vitro, albeit at a significantly lower rate than processing Kac (substrate **3a**, Table 1). On the other hand, the catalytic efficiency of decrotonylation by HDAC3–NCoR1 was in the same range as the efficiency of deacetylation by HDAC8 and HDAC11. For those reasons we find it likely that the decrotonylase activity of HDAC3–NCoR1 may be physiologically relevant. In the publication reporting on the initial discovery of Kcr as a PTM, an evaluation of the in vitro decrotonylase activity of the eleven KDACs was addressed briefly by using Boc-Kcr-

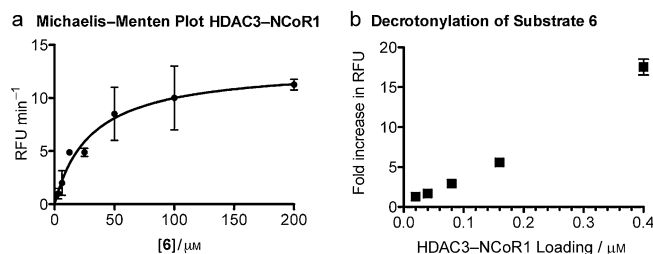


Figure 2. a) Michaelis–Menten plot for the decrotonylase activity of HDAC3–NCoR1 measured against substrate **6**. The derived kinetic constants are $K_m = (19 \pm 15) \mu$ M, $k_{cat} = (0.003 \pm 0.001) s^{-1}$, and $k_{cat} \times K_m^{-1} = 160 M^{-1} s^{-1}$. b) Titration of the amount of HDAC3–NCoR1 that is necessary to decrotonylate substrate **6** in an end-point assay (one hour incubation time).

AMC as the substrate.^[12] Though weak effects were observed for HDAC1–3, the authors drew the conclusion that KDACs were not likely to be decrotonylases, but our kinetic evaluation of HDAC3–NCoR1 decrotonylation now suggests that the issue requires further attention.

We furthermore observed that HeLa cell nuclear extract, which contains a variety of proteins including KDACs, gave rise to decrotonylation of **6** (Figure S1 in the Supporting Information). Thus, inhibition of the decrotonylase activities of recombinant HDAC3–NCoR1 as well as HeLa cell nuclear extract were tested using the approved drug vorinostat (SAHA) and the natural product apicidin, a cyclic tetrapeptide (Figure 3). The two inhibitors were chosen, because they have different selectivity profiles, that is, vorinostat has been

by well-known histone deacetylase inhibitors was demonstrated. Elucidation of the possible implications of this discovery in cellular environments will be an important future objective.

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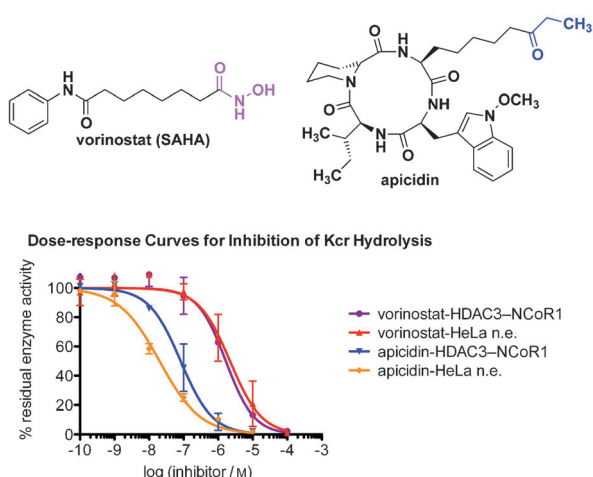


Figure 3. Dose-response curves for inhibition of the decrotonylase activity of HDAC3–NCoR1 and HeLa nuclear extract (n.e.) by vorinostat and apicidin.

shown to inhibit KDACs of class I, IIb, and IV, while apicidin is selective for class I.^[18] These dose-response experiments clearly showed that vorinostat and apicidin inhibit the decrotonylase activity of HDAC3–NCoR1; this inhibition followed trends analogous to inhibition of the deacetylation activity of HDAC3–NCoR1.^[18] The successful inhibition of the decrotonylase activity of HeLa nuclear extract by the two different histone deacetylase inhibitors indicates that KDAC enzymes may at least in part be accountable for decrotonylation. Hydrolases or multiprotein complexes with specific decrotonylase activity, however, may well exist, although they have not yet been discovered. Thus, further experiments aimed at the discovery of putative decrotonylases as well as the possible role of HDAC3–NCoR1 as a decrotonylase *in vivo* are warranted.^[32]

In conclusion, the present investigation, rooted in a systematic screening of the activities of the eleven human zinc-dependent KDAC enzymes against a series of fluorogenic substrates, has led to several discoveries as outlined above. Most importantly, we found that HDAC3–NCoR1 exhibited decrotonylase activity with a catalytic efficiency that is comparable to the deacetylase activity of other KDAC isoforms. Moreover, inhibition of the decrotonylase activity

- [1] T. Kouzarides, *Cell* **2007**, *128*, 693.
- [2] C. Choudhary, C. Kumar, F. Gnäd, M. L. Nielsen, M. Rehman, T. C. Walther, J. V. Olsen, M. Mann, *Science* **2009**, *325*, 834.
- [3] M. Biel, V. Waschowski, A. Giannis, *Angew. Chem.* **2005**, *117*, 3248; *Angew. Chem. Int. Ed.* **2005**, *44*, 3186.
- [4] M. Haberland, R. L. Montgomery, E. N. Olson, *Nat. Rev. Genet.* **2009**, *10*, 32.
- [5] a) M. Paris, M. Porcelloni, M. Binaschi, D. Fattori, *J. Med. Chem.* **2008**, *51*, 1505; b) J. E. Bolden, M. J. Peart, R. W. Johnstone, *Nat. Rev. Drug Discovery* **2006**, *5*, 769.
- [6] P. A. Marks, R. Breslow, *Nat. Biotechnol.* **2007**, *25*, 84.
- [7] R. Furumai, A. Matsuyama, N. Kobashi, K. H. Lee, M. Nishiyama, H. Nakajima, A. Tanaka, Y. Komatsu, N. Nishino, M. Yoshida, S. Horinouchi, *Cancer Res.* **2002**, *62*, 4916.
- [8] A. G. Kazantsev, L. M. Thompson, *Nat. Rev. Drug Discovery* **2008**, *7*, 854.
- [9] Z. Zhang, Y. Q. Cai, F. Zou, B. Bie, Z. Z. Pan, *Nat. Med.* **2011**, *17*, 1448.
- [10] a) D. M. Hutt, D. Herman, A. P. Rodrigues, S. Noel, J. M. Pilewski, J. Matteson, B. Hoch, W. Kellner, J. W. Kelly, A. Schmidt, P. J. Thomas, Y. Matsumura, W. R. Skach, M. Gentzsch, J. R. Riordan, E. J. Sorscher, T. Okiyoda, J. R. Yates 3rd, G. L. Lukacs, R. A. Frizzell, G. Manning, J. M. Gottesfeld, W. E. Balch, *Nat. Chem. Biol.* **2010**, *6*, 25; b) D. M. Hutt, C. A. Olsen, C. J. Vickers, D. Herman, M. Chalfant, A. Montero, L. J. Leman, R. Burkle, B. E. Maryanoff, W. E. Balch, M. R. Ghadiri, *ACS Med. Chem. Lett.* **2011**, *2*, 703.
- [11] J. Gräff, D. Rei, J. S. Guan, W. Y. Wang, J. Seo, K. M. Hennig, T. J. Nieland, D. M. Fass, P. F. Kao, M. Kahn, S. C. Su, A. Samiei, N. Joseph, S. J. Haggarty, I. Delalle, L. H. Tsai, *Nature* **2012**, *483*, 222.
- [12] M. Tan, H. Luo, S. Lee, F. Jin, J. S. Yang, E. Montellier, T. Buchou, Z. Cheng, S. Rousseaux, N. Rajagopal, Z. Lu, Z. Ye, Q. Zhu, J. Wysocka, Y. Ye, S. Khochbin, B. Ren, Y. Zhao, *Cell* **2011**, *146*, 1016.
- [13] a) Z. Zhang, M. Tan, Z. Xie, L. Dai, Y. Chen, Y. Zhao, *Nat. Chem. Biol.* **2011**, *7*, 58; b) J. Du, Y. Zhou, X. Su, J. J. Yu, S. Khan, H. Jiang, J. Kim, J. Woo, J. H. Kim, B. H. Choi, B. He, W. Chen, S. Zhang, R. A. Cerione, J. Auwerx, Q. Hao, H. Lin, *Science* **2011**, *334*, 806; c) C. Peng, Z. Lu, Z. Xie, Z. Chang, Y. Chen, M. Tan, H. Luo, Y. Zhang, W. He, K. Yang, B. M. M. Zwaans, D. Tishkoff, L. Ho, D. Lombard, T.-J. He, J. Dai, E. Verdin, Y. Ye, Y. Zhao, *Mol. Cell. Proteomics* **2012**, DOI: 10.1074/mcp.M111.012658.
- [14] J. Taunton, C. A. Hassig, S. L. Schreiber, *Science* **1996**, *272*, 408.
- [15] I. V. Gregoret, Y. M. Lee, H. V. Goodson, *J. Mol. Biol.* **2004**, *338*, 17.
- [16] a) W. Fischle, F. Dequiedt, M. Fillion, M. J. Hendzel, W. Voelter, E. Verdin, *J. Biol. Chem.* **2001**, *276*, 35826; b) W. Fischle, F. Dequiedt, M. J. Hendzel, M. G. Guenther, M. A. Lazar, W. Voelter, E. Verdin, *Mol. Cell* **2002**, *9*, 45.
- [17] A. Lahm, C. Paolini, M. Pallaoro, M. C. Nardi, P. Jones, P. Neddermann, S. Sambucini, M. J. Bottomley, P. Lo Surdo, A.

- Carfi, U. Koch, R. De Francesco, C. Steinkuhler, P. Gallinari, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 17335.
- [18] J. E. Bradner, N. West, M. L. Grachan, E. F. Greenberg, S. J. Haggarty, T. Warnow, R. Mazitschek, *Nat. Chem. Biol.* **2010**, *6*, 238.
- [19] a) H. Y. Kao, C. H. Lee, A. Komarov, C. C. Han, R. M. Evans, *J. Biol. Chem.* **2002**, *277*, 187; b) D. D. Fischer, R. Cai, U. Bhatia, F. A. Asselbergs, C. Song, R. Terry, N. Trogani, R. Widmer, P. Atadja, D. Cohen, *J. Biol. Chem.* **2002**, *277*, 6656.
- [20] L. Gao, M. A. Cueto, F. Asselbergs, P. Atadja, *J. Biol. Chem.* **2002**, *277*, 25748.
- [21] D. M. Fass, R. Shah, B. Ghosh, K. Hennig, S. Norton, W. N. Zhao, S. A. Reis, P. S. Klein, R. Mazitschek, R. L. Maglathlin, T. A. Lewis, S. J. Haggarty, *ACS Med. Chem. Lett.* **2011**, *2*, 39.
- [22] M. Bantscheff, C. Hopf, M. M. Savitski, A. Dittmann, P. Grandi, A. M. Michon, J. Schlegel, Y. Abraham, I. Becher, G. Bergamini, M. Boesche, M. Delling, B. Dumpelfeld, D. Eberhard, C. Huthmacher, T. Mathieson, D. Poeckel, V. Reader, K. Strunk, G. Sweetman, U. Kruse, G. Neubauer, N. G. Ramsden, G. Drewes, *Nat. Biotechnol.* **2011**, *29*, 255.
- [23] a) C. J. Chou, D. Herman, J. M. Gottesfeld, *J. Biol. Chem.* **2008**, *283*, 35402; b) C. P. Xu, E. Soragni, C. J. Chou, D. Herman, H. L. Plasterer, J. R. Rusche, J. M. Gottesfeld, *Chem. Biol.* **2009**, *16*, 980.
- [24] a) A. Montero, J. M. Beierle, C. A. Olsen, M. R. Ghadiri, *J. Am. Chem. Soc.* **2009**, *131*, 3033; b) S. Hanessian, L. Auzzas, A. Larsson, J. B. Zhang, G. Giannini, G. Gallo, A. Ciacci, W. Cabri, *ACS Med. Chem. Lett.* **2010**, *1*, 70; c) L. Auzzas, A. Larsson, R. Matera, A. Baraldi, B. Deschenes-Simard, G. Giannini, W. Cabri, G. Battistuzzi, G. Gallo, A. Ciacci, L. Vesci, C. Pisano, S. Hanessian, *J. Med. Chem.* **2010**, *53*, 8387; d) K. V. Butler, J. Kalin, C. Brochier, G. Vistoli, B. Langley, A. P. Kozikowski, *J. Am. Chem. Soc.* **2010**, *132*, 10842.
- [25] E. S. Inks, B. J. Josey, S. R. Jesinkey, C. J. Chou, *ACS Chem. Biol.* **2012**, *7*, 331.
- [26] a) C. A. Olsen, *Angew. Chem.* **2012**, *124*, 3817; *Angew. Chem. Int. Ed.* **2012**, *51*, 3755; b) M. D. Hirschey, *Cell Metab.* **2011**, *14*, 718.
- [27] For examples, see: a) D. Wegener, C. Hildmann, D. Riester, A. Schwienhorst, *Anal. Biochem.* **2003**, *321*, 202; b) D. Riester, D. Wegener, C. Hildmann, A. Schwienhorst, *Biochem. Biophys. Res. Commun.* **2004**, *324*, 1116; c) B. Heltweg, F. Dequiedt, B. L. Marshall, C. Brauch, M. Yoshida, N. Nishino, E. Verdin, M. Jung, *J. Med. Chem.* **2004**, *47*, 5235; d) Z. A. Gurard-Levin, M. Mrksich, *Biochemistry* **2008**, *47*, 6242; e) Z. A. Gurard-Levin, K. A. Kilian, J. Kim, K. Bahr, M. Mrksich, *ACS Chem. Biol.* **2010**, *5*, 863; f) A. L. Garske, S. S. Oliver, E. K. Wagner, C. A. Musselman, G. LeRoy, B. A. Garcia, T. G. Kutateladze, J. M. Denu, *Nat. Chem. Biol.* **2010**, *6*, 283; g) B. C. Smith, B. Settles, W. C. Hallows, M. W. Craven, J. M. Denu, *ACS Chem. Biol.* **2011**, *6*, 146.
- [28] E. Michishita, R. A. McCord, E. Berber, M. Kioi, H. Padilla-Nash, M. Damian, P. Cheung, R. Kusumoto, T. L. A. Kawahara, J. C. Barrett, H. Y. Chang, V. A. Bohr, T. Ried, O. Gozani, K. F. Chua, *Nature* **2008**, *452*, 492.
- [29] A. S. Madsen, C. A. Olsen, *J. Med. Chem.* **2012**, *55*, 5582.
- [30] a) W. S. Horne, C. A. Olsen, J. M. Beierle, A. Montero, M. R. Ghadiri, *Angew. Chem.* **2009**, *121*, 4812; *Angew. Chem. Int. Ed.* **2009**, *48*, 4718; b) C. A. Olsen, M. R. Ghadiri, *J. Med. Chem.* **2009**, *52*, 7836; c) C. J. Vickers, C. A. Olsen, L. J. Leman, M. R. Ghadiri, *ACS Med. Chem. Lett.* **2012**, *3*, 505.
- [31] The inherent chemical lability of the trifluoroacetamide functional group also resulted in shorter shelf lives of these substrates when diluted in KDAC assay buffer. This issue, however, could be easily circumvented by preparing fresh substrate dilutions (from dimethyl sulfoxide (DMSO) stock solutions kept at -20°C) immediately prior to use.
- [32] C. H. Kim, M. Kang, H. J. Kim, A. Chatterjee, P. G. Schultz, *Angew. Chem.* **2012**, *124*, 7358; *Angew. Chem. Int. Ed.* **2012**, *51*, 7246.