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6. AUTHORS
Terry Watt

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
Xavier University of Louisiana
1 Drexel Drive

New Orleans, LA 70125 -1098

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14. ABSTRACT
Lysine deacetylases (KDACs) mediate control of numerous cellular processes through the regulation of post-translational modification of lysine residues in substrate proteins. Our objective was to determine the molecular characteristics of the substrate binding surface of lysine deacetylases and the interactions of that surface with substrates and small molecule regulators. Key outcomes of our work include development of a novel, general-purpose assay for KDACs; determination that "activator" molecules of KDAC8 are not biologically relevant and

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Final Report: Molecular Characteristics of Lysine Deacetylase Interactions

ABSTRACT

Lysine deacetylases (KDACs) mediate control of numerous cellular processes through the regulation of post-translational modification of lysine residues in substrate proteins. Our objective was to determine the molecular characteristics of the substrate binding surface of lysine deacetylases and the interactions of that surface with substrates and small molecule regulators. Key outcomes of our work include development of a novel, general-purpose assay for KDACs; determination that "activator" molecules of KDAC8 are not biologically relevant and only increase activity for enzyme preparations that are inherently low activity; characterization of the activity effects of fluorescent labels used in common KDAC assays; development of a reliable assay for T4 lysozyme; and development of robust expression, purification, and reaction conditions for KDACs. We also characterized the impact of several mutations to KDACs to gain insight into the biological roles of these enzymes.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received		Paper
01/09/2016	15	Subramanya Pingali, Tasha B. Toro, Thao P. Nguyen, Destane S. Garrett, Kyra A. Dodson, Kyara A. Nichols, Rashad A. Haynes, Florastina Payton-Stewart, Terry J. Watt, Jinsong Zhang. KDAC8 with High Basal Velocity Is Not Activated by N-Acetylthioureas, PLoS ONE, (01 2016): 0. doi: 10.1371/journal.pone.0146900 380,670.00
03/30/2017	20	Tasha Toro, Jenae Bryant, Terry Watt. Lysine deacetylases exhibit distinct changes in activity profiles due to fluorophore-conjugation of substrates, Biochemistry, ( ): . doi: 1,036,667.00
06/02/2015	5	Tasha B. Toro, Thao P. Nguyen, Terry J. Watt. An improved 96-well turbidity assay for T4 lysozyme activity, MethodsX, (05 2015): 256. doi: 10.1016/j.mex.2015.05.004 358,779.00
11/09/2015	11	Tasha Toro, Thao Nguyen, Terry Watt. An improved 96-well turbidity assay for T4 lysozyme activity [corrected], MethodX, (05 2015): 256. doi: 376,030.00
11/09/2015	12	Tasha Toro, Terry Watt. KDAC8 substrate specificity quantifiedby a biologically relevant, label-freedeacetylation assay, Protein Science, (10 2015): 0. doi: 376,031.00

TOTAL: **5**

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Imbragulgio SA, Hylton BJ, Toro TB, Watt TJ. Optimizing the Buffer System for Lysine Deacetylase Activity. Experimental Biology Meeting 2017, Chicago, IL, April 2017.

White SE, Toro TB, Watt TJ. Probing the mechanism of acetate release in lysine deacetylases. Annual Biomedical Research Conference for Minority Students, Tampa, FL, November 2016.

Number of Presentations: 2.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

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Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received		Paper
08/06/2015	6.00	Tasha B. Toro, Terry J. Watt. KDAC8 substrate specificity quantified by a biologically-relevant, label-free deacetylation assay, Protein Science (05 2015)
11/09/2015	13.00	Tasha Toro, Subramanya Pingali, Thao Nguyen, Destane Garrett, Kyra Dodson, Kyara Nichols, Rashad Haynes, Florastina Payton-Stewart, Terry Watt. N-acetylthioureas do not activate KDAC8 under most conditions, PLoS ONE (10 2015)

TOTAL: **2**

Number of Manuscripts:

Books

Received	Book

TOTAL:

Received	Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Tasha Toro	0.20
FTE Equivalent:	**0.20**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	DISCIPLINE
Imani Bijou	11	Chemistry
Brandon Hylton	21	Biosciences
Samantha Imbraguglio	48	Chemistry
Kyara Nichols	7	Chemistry
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Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	3.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:......	3.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:......	3.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):......	2.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:......	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:......	0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED

FTE Equivalent:

Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See attachment

Technology Transfer

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Statement of the problem studied

Lysine deacetylases (KDACs) mediate control of numerous cellular processes through the regulation of post-translational modification of lysine residues in substrate proteins. Acetylation and deacetylation of proteins has been been directly associated with biological processes as diverse as the formation of memories, metabolic regulation, and the normal development and repair of organisms. In addition, the mis-regulation of KDAC activity is associated with many diseases and physiological stresses, including cancers, asthma, infectious diseases, and neurological, vascular, and muscular disorders. However, few specific substrates of the eleven known metal-dependent human KDACs have been identified, and therefore the biochemical basis for the association between a particular KDAC and the macroscopic effect on a person is often unclear. Lack of knowledge regarding the molecular interactions between KDACs and substrates is a critical barrier to the development of novel, targeted drugs with the potential to restore proper regulation of KDAC-controlled cellular pathways and to potentially enhance human performance in areas regulated by KDACs.

Our objective was to determine the molecular characteristics of the substrate binding surface of lysine deacetylases and the interactions of that surface with substrates and small molecule regulators. Our central hypothesis is that each KDAC has molecular features that contribute to specific binding of substrates and small molecular regulators and that these features allow for differential targeting of KDACs. The identification of the molecular factors controlling substrate, inhibitor, and activator binding is expected to enable both targeted development of novel drugs and a greater understanding of the pathways regulated by each KDAC. Expected outcomes include identification of molecular factors influencing human performance, possible routes to novel therapies for diseases and enhancement of human performance under stress, and enhanced model systems for studying KDAC-substrate interactions. The application of these methods will enable improved development of small molecule regulators of KDAC activity for drug therapy and contribute to an understanding of the appropriate KDAC to target for regulating particular cellular pathways.

Summary of the most important results

Aim 1. Construct a structural model of interactions between substrates and KDACs.

We proposed to utilize T4 lysozyme as a framework protein, in which we would incorporate desired peptide sequences corresponding to known acetylated proteins in regions of defined secondary structure. To ensure that we would be able to quickly determine whether the resulting

proteins retained the expected overall structure, we used a combination of activity and structural (circular dichroism spectroscopy) measurements. No previously reported quantitative and reliable assay for T4 lysozyme had previously been reported in the literature. Therefore, we developed a turbidity assay for use with our constructs, as well as an optimized expression and purification protocol. This work was reported in MethodsX.[1] Figure 1 demonstrates use of the assay with the wild-type protein.

We created a range of T4 lysozyme-based substrates for the KDACs. These structures presented the desired substrate peptides as an α-helix or within a loop structure. Although the α-helical-based proteins were usually expressed and purified with a reasonable yield, the loop-based proteins rarely did so, suggesting that our insertion was too disruptive of the overall protein structure. The α-helix-based proteins were tested as KDAC substrates, but low or no activity was measured. This proved to be consistent with concurrent computational calculations based on docking of different 3-D structures into the KDAC activity, in which we observed that α-helix-based structures had the lowest affinity, and β-sheet would be a more promising 3-D structure to pursue. T4 lysozyme does not contain any β-sheets in a convenient location for use as a model substrate, so we turned to ubiquitin. Unfortunately, the mutated ubiquitin constructs did not express to sufficient levels for testing. We are presently pursuing a more complicated strategy involving the use of several known substrates of particular KDACs, which can then specifically modify and/or selectively acetylate to generate model substrates to compare to activity on peptide substrates. Simultaneous with our efforts, other research groups have published extensive modeling data of small substrate binding to KDACs that we can draw on for our design of model substrates,[2–4] without duplicating the calculation efforts ourselves.

We extend some of this work into an advanced undergraduate lab for CHEM 4150L, Genomics & Proteomics Laboratory. Students are provided with a cell pellet containing one of the T4 lysozyme variants that expresses to a reasonable level, and then must purify the protein and characterize its activity. As there are a variety of proteins used in the class, the students then pool their data. The students then must use known literature data, the changes in activity, and 3-D modeling to draw conclusions about the role of specific residues in the function of the protein, based on the increase or decrease in activity observed due to the mutations present. This work is expected to be submitted for publication during summer 2017.

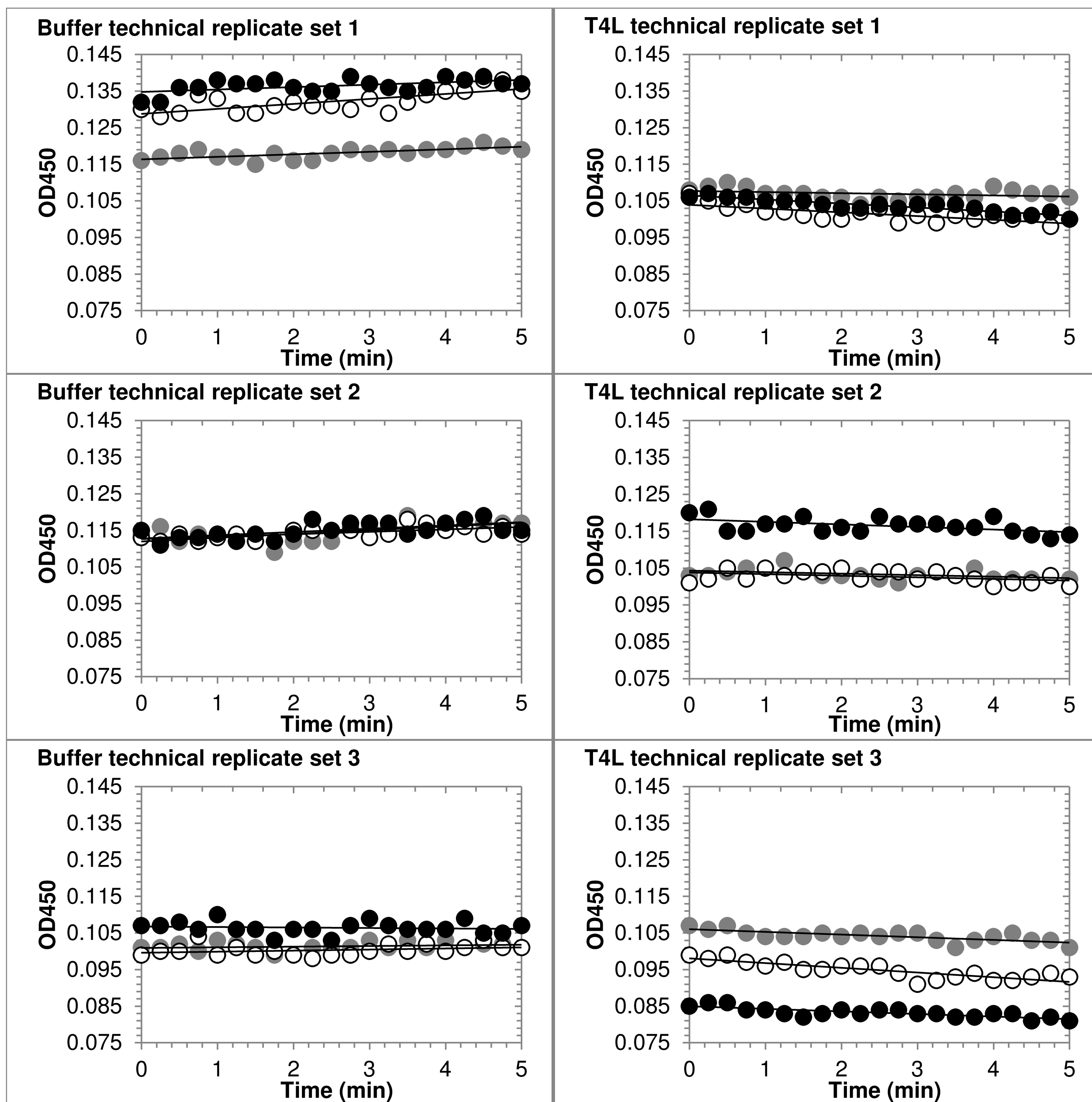

Figure 1. Activity of T4 lysozyme in low ionic strength buffer. *M. luteus* substrate (0.3 mg/ml) in 30 mM potassium phosphate pH 7.2 was added to a 96-well plate in triplicate containing T4 lysozyme (right) or buffer (left). OD_{450} was measured at 15 second intervals. Data shown represents technical triplicates for three independent experiments (each replicate series as white, grey, or black). Lines represent the best fit to each data set, to illustrate overall slope of the data within each replicate. (Figure from Toro et. al. 2015.[1])

Aim 2. Determine the roles of specific residues in the KDAC binding sites in substrate discrimination and binding of small molecule regulators.

Although several methods have been previously developed to monitor protein deacetylation, none are particularly suited for identifying enzyme-substrate pairs of label-free substrates across the entire family of lysine deacetylases. We developed a fluorescamine-based assay which is more biologically relevant than existing methods and amenable to probing substrate specificity, and which was published in Protein Science.[5] Using this assay, we evaluated the activity of KDAC8 and other lysine deacetylases, including a sirtuin, for several peptides derived from known acetylated proteins. (This assay was also utilized for the T4 lysozyme results described above.) KDAC8 showed clear preferences for some peptides over others, indicating that the residues immediately surrounding the acetylated lysine play an important role in substrate specificity. Steady-state kinetics suggest that the sequence surrounding the acetylated lysine affects binding affinity and catalytic rate independently. Our results provide direct evidence that potential KDAC8 substrates previously identified through cell based experiments can be directly deacetylated by KDAC8. Conversely, the data from this assay did not correlate well with predictions from previous screens for KDAC8 substrates using less biologically relevant substrates and assay conditions (Figure 2).

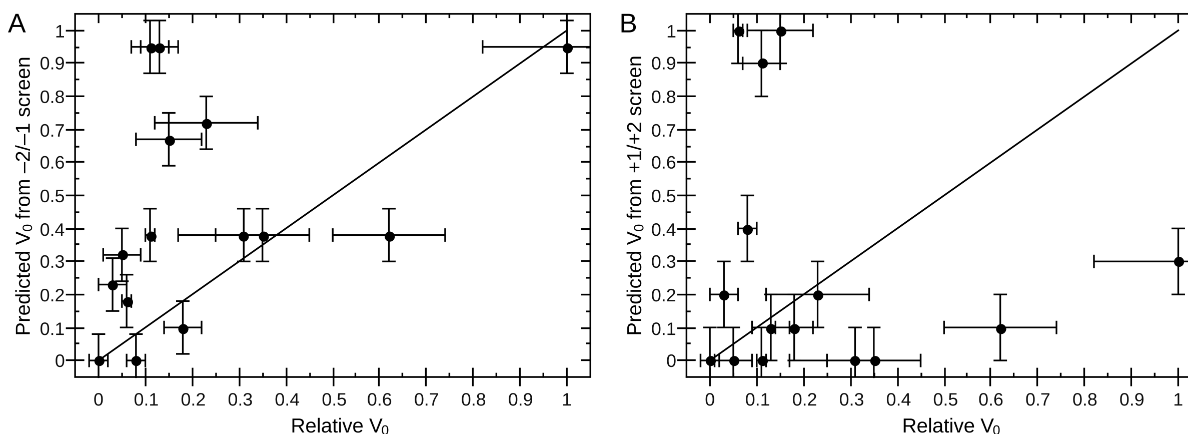

Figure 2. Screens with labeled substrates do not reflect activity in fluorescamine assay. Normalized activity of endpoint assays does not correlate with (A) a screen of all possible -2 & -1 sequences using fluorophore-labeled peptides ($r^2 = 0.18$)[6] or (B) a screen of all possible +1 & +2 sequences using surface-attached peptides ($r^2 = 0.02$).[7] Lines represent a hypothetical perfect correlation; deviation from the line indicates disagreement between observed and predicted results. Error bars come from experimental results (x-axis) or are extrapolated based on the reported precision of values used for predictions (y-axis). (Figure from Toro & Watt 2015.[5])

Recently, a series of N-acetylthioureas were synthesized and reported to enhance the activity of KDAC8 with a fluorogenic substrate.[8] To determine if the activation was general, we synthesized three of the most potent N-acetylthioureas and measured their effect with peptide substrates and the fluorogenic substrate under multiple reaction conditions and utilizing two enzyme purification approaches. No activation was observed for any of the three N-

acetylthioureas under any assayed conditions (Tables 1 and 2). Further characterization of KDAC8 kinetics with the fluorogenic substrate yielded a k_{cat}/K_M of 164 ± 17 in the absence of any N-acetylthioureas. This catalytic efficiency is comparable to or higher than that previously reported when KDAC8 was activated by the N-acetylthioureas, suggesting that the previously reported activation effect may be due to use of an enzyme preparation that contains a large fraction of inactive enzyme. Further characterization with a less active preparation and additional substrates leads us to conclude that N-acetylthioureas are not true activators of KDAC8 and only increase activity if the enzyme preparation is below the maximal basal activity. This work was published in PLOS One.[9] Based on these outcomes, we did not pursue further work with "activating" molecules of the KDACs.

Table 1. Effect of N-acetylthioureas on KDAC8 activity with peptide substrates. (Table from Toro et. al. 2016.[9])

	Activity (pmol min^{-1} μg^{-1})			
Substrate	No N-acetylthiourea	10 μmol L^{-1} TM-2-51	10 μmol L^{-1} TM-2-88	10 μmol L^{-1} TM-2-104
ac-FR-{K-ac}-RW-am	21.0 ± 1.3	20.3 ± 2.1	19.6 ± 1.9	19.7 ± 1.4
ac-IS-{K-ac}-FD-am	10.0 ± 2.6	9.9 ± 2.7	9.8 ± 2.9	9.9 ± 2.7

Table 2. Effect of N-acetylthioureas on KDAC8 activity with AMC substrate. (Table from Toro et. al. 2016.[9])

		Activity (pmol min^{-1} μg^{-1})			
Buffer	Enzyme preparation	No N-acetylthiourea	10 μmol L^{-1} TM-2-51	10 μmol L^{-1} TM-2-88	10 μmol L^{-1} TM-2-104
Assay buffer 1	Co^{2+} resin, cleaved His_6	3.7 ± 0.4	4.5 ± 0.4	4.4 ± 0.9	4.4 ± 0.3
Assay buffer 2	Co^{2+} resin, cleaved His_6	18.2 ± 3.4	21.9 ± 4.3	21.0 ± 2.4	17.8 ± 2.7
Assay buffer 2	Ni^{2+} resin, His_6 tagged	5.3 ± 1.4	4.9 ± 1.0	4.6 ± 1.0	4.5 ± 1.3
Assay buffer 1	Commercial, His_6 tagged	0.35 ± 0.04	0.68 ± 0.14	0.81 ± 0.26	0.54 ± 0.11

Commercially available peptide substrates that are conjugated to fluorescent dye molecules, such as 7-amino-4-methylcoumarin (AMC), are commonly used to monitor deacetylation by KDACs in studies addressing both substrate specificity and small molecule modulators of activity. We compared the activity of several KDACs, representing all major classes of KDACs, with substrates in the presence and absence of AMC as well as peptides for which tryptophan has been substituted for AMC. Our results unequivocally demonstrate that AMC has a significant effect on activity for all KDACs tested (Figure 3 and Table 3). Furthermore, the effect is not consistent across KDACs, neither in nature of the effect nor magnitude, making it impossible to predict the

effect of AMC on a particular enzyme-substrate pair. AMC did not affect acetyllysine preference in a multiply acetylated substrate. In contrast, AMC significantly enhanced KDAC6 substrate affinity, greatly reduced Sirt1 activity, eliminated substrate sequence specificity of KDAC4, and had no consistent effect with KDAC8 substrates. These results indicate that profiling of KDAC activity with labeled peptides is unlikely to produce biologically relevant data. These results have been submitted for publication in Biochemistry and are presently under review.

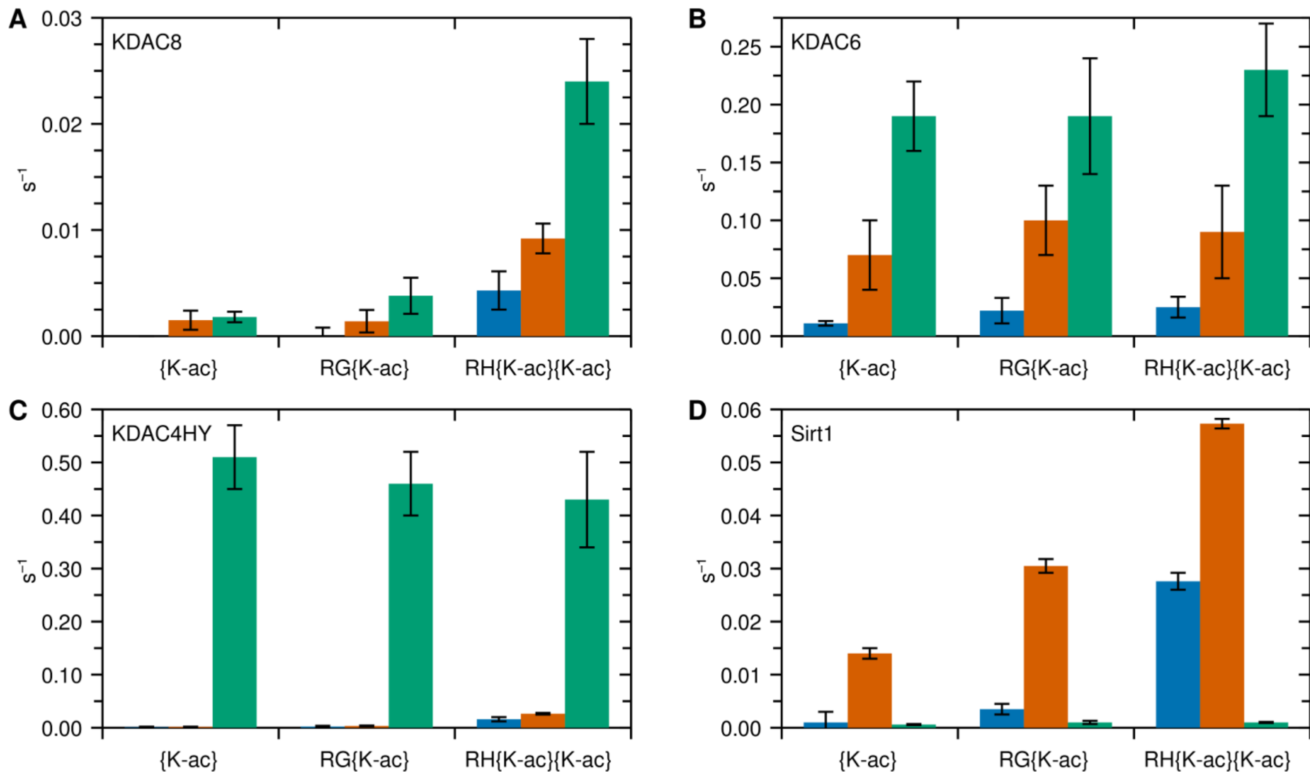

Figure 3. Endpoint activity for KDACs with peptide substrates. Three sets of peptide substrates where the C-terminus ends in an acetylated lysine (blue), tryptophan (orange), or AMC (green). Each peptide reacted with KDAC8 (A), KDAC6 (B), KDAC4HY (C), or Sirt1 (D). The average specific activity and standard deviation for at least three replicates is shown for each enzyme-substrate pair.

Table 3. Steady state kinetic parameters for peptides with KDAC6 and KDAC8.

	KDAC6			KDAC8		
Substrate	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})
{K-ac}	0.123 ± 0.018	800 ± 300	150 ± 30	0.011 ± 0.003	800 ± 400	1.4 ± 0.3
{K-ac}W	0.19 ± 0.02	70 ± 30	2500 ± 1000	> 0.02	> 5000	3.5 ± 0.6
{K-ac}-AMC	0.27 ± 0.04	15 ± 7	19000 ± 7000	0.07 ± 0.02	4200 ± 1700	16.3 ± 1.5
RG{K-ac}	0.32 ± 0.06	600 ± 200	530 ± 130	0.09 ± 0.05	> 5000	4.4 ± 0.4
RG{K-ac}W	0.25 ± 0.04	60 ± 30	4300 ± 1800	0.041 ± 0.014	2700 ± 1700	15 ± 4
RG{K-ac}-AMC	0.40 ± 0.02	11 ± 3	36000 ± 7000	0.011 ± 0.003	500 ± 200	23 ± 6
RH{K-ac}{K-ac}	0.23 ± 0.03	370 ± 150	620 ± 180	0.10 ± 0.03	3100 ± 1100	34 ± 4
RH{K-ac}{K-ac}W	0.24 ± 0.02	90 ± 40	2600 ± 900	0.131 ± 0.014	1200 ± 300	106 ± 13
RH{K-ac}{K-ac}-AMC	0.148 ± 0.016	9 ± 3	17000 ± 5000	0.34 ± 0.06	1300 ± 300	259 ± 19

In parallel with the experimental procedures described above, we developed a simplified expression and purification system for metal-dependent KDACs. Our method was used to purify a range of KDACs for use in all the experiments described here. This method is expected to be submitted for publication during summer 2017. We also studied the optimal reaction conditions to ensure high, consistent activity. This study was prompted by the observation that reported KDAC activity, in particular for KDAC8, is widely varying across the literature. We determined that trace levels of zinc present present in many common lab reagents is likely a major contributor to this variation, and have determined conditions to minimize variation between buffer reagents and enzyme preparations. We are presently preparing a manuscript for publication on this topic, with submission expected during summer 2017.

We created a series of KDAC8 variants containing mutations at a position previously reported to be important for release of acetate, one of the reaction products.[10] Our objective was to evaluate theoretical predictions made by previous computational studies. However, we found that all mutations at the critical site resulted in an unstable, poorly expressed protein. Therefore, we are unable to determine whether the arginine residue naturally found at that site is important for substrate release, as it appears to be (also or instead) critical for protein folding and structure.

Finally, we initiated a study of the role of the two deacetylase domains present in KDAC6, as a follow-up to the work described above. KDAC6 is unusual in having two catalytic domains; only KDAC10 also has two domains, all other deacetylases have a single domain. This study will continue, and we anticipate a future publication focusing on these results.

Aim 3. Probe the effects of natural variation in an active site residue on substrate and small molecule regulator interactions with KDACs.

We created variants of KDAC4, KDAC7, and KDAC8 that contain mutations in a catalytic residue. This residue exhibits natural variation, and our goal was to determine the effect of that residue on activity of model substrates, and eventually to measure the effect of the mutations on the effectiveness of small molecule regulators. Our hypothesis is that this residue has significant impact on the binding of small molecules but much less on biologically relevant substrates. We expect that this data will lead to new approaches to designing regulators specific for particular KDACs. We have made substantial progress on this project, identifying potential substrates for both KDAC4 and KDAC7 (neither of which has any previously reported biologically-derived substrates). Approximately a dozen mutated proteins, containing different residues to isolate particular chemical features, have been prepared and partially characterized. Final experiments are still in progress, and we anticipate submission of a manuscript describing all of this work early in 2018.

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