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RPPR Final Report
as of 12-Jan-2023

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Proposal Number: 72555BBREP **Agreement Number: W911NF-18-1-0450**
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Final Report for Period Beginning 29-Sep-2018 and Ending 28-Sep-2022
Title: Characterizing the Specificity of Class IIa Lysine Deacetylases
Begin Performance Period: 29-Sep-2018 **End Performance Period:** 28-Sep-2022
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Major Goals: Our overarching hypothesis for this project is that class IIa KDACs are catalytically active in vivo, based in part on our recent discovery of several biologically relevant peptides that are active with a class IIa KDAC. Our objectives are to identify potential substrates of these enzymes and evaluate contributing factors to the catalytic activity.
Specific aim 1: characterize the molecular determinants of interactions between class IIa KDACs and substrates, by:
(1a) determining substrate preferences using peptide libraries;
(1b) investigating predictive ability of peptide screens; and
(1c) determining the contribution of the non-catalytic domain to substrate specificity.
Specific aim 2: identify class IIa KDAC substrates in vivo, by:
(2a) characterizing changes in the acetylome due to modified KDAC activity;
(2b) directly validating potential substrates; and
(2c) evaluating inhibitor specificity and identify target regulatory features.

Key novel results will include demonstration of direct deacetylation with biologically relevant substrates, linking of model substrates to in vivo substrates, and determination of intracellular acetylation changes that are directly linked to catalytic activity by these enzymes. As a whole, our results will provide new approaches for enhancing Soldier performance through regulation of critical biological pathways. Finally, the project will directly contribute to the research training of at least five undergraduate students per year. Students from predominantly underrepresented groups will make key contributions to all aspects of this project.

Accomplishments: See attached PDF

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Training Opportunities: 4 undergraduate students have contributed to the project during the current reporting period. The students have received training in a variety of relevant experimental techniques. Specific experimental methods include enzyme activity assays, expression and purification of proteins, cell culture, immunoblotting, and mass spectrometry of proteins. Two students presented at the national American Society for Biochemistry and Molecular Biology (Experimental Biology) meeting in April 2022. Both students took advantage of a variety of undergraduate-oriented professional development sessions and training at the national meeting, in addition to attending research talks.

Results Dissemination: Two posters were presented by students at a national meeting.

Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: Nothing to Report

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Person Months Worked: 3.00 **Funding Support:**
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National Academy Member: N

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Project Contribution:
National Academy Member: N

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Project Contribution:
National Academy Member: N

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Project Contribution:
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Article Title: Critical review of non?histone human substrates of metal?dependent lysine deacetylases.
Authors: Tasha Toro, Terry Watt
Keywords: histone deacetylases, HDAC, KDAC, substrate specificity
Abstract: Lysine acetylation is a posttranslational modification that occurs on thousands of human proteins, most of which are cytoplasmic. Acetylated proteins are involved in numerous cellular processes and human diseases. Therefore, how the acetylation/deacetylation cycle is regulated is an important question. Eleven metal?dependent lysine deacetylases (KDACs) have been identified in human cells. These enzymes, along with the sirtuins, are collectively responsible for reversing lysine acetylation. Despite several large?scale studies which have characterized the acetylome, relatively few of the specific acetylated residues have been matched to a proposed KDAC for deacetylation. To understand the function of lysine acetylation, and its association with diseases, specific KDAC?substrate pairs must be identified. Identifying specific substrates of a KDAC is complicated both by the complexity of assaying relevant activity and by the non?catalytic interactions of KDACs with cellular proteins. Here,
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I certify that the information in the report is complete and accurate:
Signature: Terry Watt
Signature Date: 1/8/23 11:47PM

Abstract
Metal-dependent lysine deacetylases (KDACs) are multi-functional proteins that mediate control of numerous cellular processes. Acetylation and deacetylation of proteins have been directly associated with a wide range of biological processes, including metabolic regulation and organismal development as well as numerous diseases. Few specific substrates or indirect targets of KDACs have been identified despite widespread acetylation of proteins, which is a critical limitation in the ability to effectively regulate KDACs and their associated pathways. Class IIa KDACs have been reported to have regulatory roles in pain pathways and injury repair mechanisms, and so identifying their specific intracellular targets will provide new approaches for enhancing Soldier performance. Prior to this work, there have been no reports of catalytic activity for class IIa KDACs with biologically relevant substrates, so direct evidence of a catalytic role for these enzymes is lacking. When compared to other KDACs, class IIa KDACs also contain an additional protein domain of unknown function. Our overarching hypothesis for this project was that class IIa KDACs are catalytically active in vivo. Our objectives were to identify potential substrates of these enzymes and evaluate contributing factors to the catalytic activity. Major findings from this project include (1) determining that many putative class IIa KDAC substrates are not deacetylated by class IIa KDACs; (2) identification of a small number of more directly established putative substrates; (3) evidence that the class IIa KDACs are catalytically active in vivo, using a novel assay method; (4) identification of both overlapping and unique specificity determinants for each class IIa KDAC; (5) development of novel engineered cell lines for evaluating KDAC activity and identification of substrates; (6) identification and characterization of specific cellular changes in response to highly targeted inactivation or enhancement of class IIa KDACs while retaining biologically-relevant expression levels of each protein; and (8) extensive training of undergraduate research students leading to several students pursuing research-oriented careers.

Accomplishments.
Aim 1. Characterize the molecular determinants of interactions between class IIa KDACs and substrates, by:

(1a) determining substrate preferences using peptide libraries;
(1b) investigating predictive ability of peptide screens; and
(1c) determining the contribution of the non-catalytic domain to substrate.

We established that many putative class IIa KDAC substrates are not active with KDACs 4, 5, or 7, at least in peptide form. However, all 3 enzymes are active with several putative substrates of other KDACs, suggesting that the class IIa enzymes are being mis-attributed to target proteins due to methodological limitations of prior work. In addition, we have confirmed that the wild-type class IIa enzymes are measurably active as deacetylases, which has been a point of contention in the literature (Table 1).

We have determined that the class IIa KDACs have highly similar substrate preferences, while also identifying a few subtle differences in their interactions and activity that can be exploited for targeted inhibitor design and prediction of unique substrates for each enzyme (Table 2). It is noteworthy that all the regions identified as differentiating between the three enzymes (for example, the DTD sequence in KDAC7 with the -1 substrate position) are highly conserved in sequence, but subtle differences in structure result in significant

Table 1. Sample of peptide sequences active with at least one wild-type class IIa KDAC.

Peptide sequence	Corresponding protein
RSFSK^{ac}AFGQ	Nucleolar RNA helicase 2
GRK^{ac}YK^{ac}RR	Estrogen receptor α
RRK^{ac}EK^{ac}SR	Hypoxia-inducible factor 1α
SRHK^{ac}K^{ac}LM	p53

Table 2. Observed key enzyme residues driving selectivity for substrate sequences at positions relative to the acetyllysine residue for catalytically-enhanced class IIa KDACs.

Substrate position		KDAC4	KDAC5	KDAC7
-2	Preference	not D, not E	not D, not E	not D, not E
	Key residues	G868, N869, F870, F871, P872, G873	G898, N899, F900, F901, P902, G903	G774, N775, F776, F777, P778, G779
-1	Preference	none	none	R, not D, not E
	Key residues	-	-	D663, T664, D665
+1	Preference	W, not R	not R	W, not R, not F
	Key residues	F870, F871, P940, P942, L943	L970, P972, P973	R579, H580, P581, T664, D665, F718
+2	Preference	P, R, W	F, P, R	D, P, R
	Key residues	N869, P942, L943	N899, L970, P972, L973	R579, P846, A847, P848, L849, G850

variation in the contributions. The wild-type KDACs exhibit similar preferences in simulations to the catalytically-enhanced variants.

Completion status of sub-aims:

Aim 1a. Complete for 2 of the 3 target proteins (KDAC4 and KDAC7), and > 50% complete for KDAC5.

Aim 1b. Partially completed. Predictions made using peptide libraries have been evaluated on additional peptides. However, predictions for full-length proteins have not been completed.

Aim 1c. Partially completed. Expression and purification of full-length class IIa KDACs has been unsuccessful in producing adequate quantities of active enzyme for in vitro assays, despite attempting several different expression systems. However, we have successfully developed a method of evaluating enzyme activity directly in cell lysate, using the low amount of KDAC expressed by human cells, including the engineered cell lines developed in aim 2. A limited subset of peptides have been assayed using both wild-type and engineered cell lines, and the difference (excess activity of wild-type compared to cell line with inactivated KDAC) attributed to activity by the target KDAC.

Aim 2. Identify class IIa KDAC substrates in vivo, by:

(2a) characterizing changes in the acetylome due to modified KDAC activity;
(2b) directly validating potential substrates; and
(2c) evaluating inhibitor specificity and identify target regulatory features.

Selectively inactivating or enhancing the catalytic activity of KDAC4, KDAC7, or KDAC8, while retaining endogenous expression levels, results in a distinctive mRNA profile from either inhibitor-treated cells or cells with a knockout/knockdown of the corresponding KDAC (Figure 1). Our developed approach is providing insight into the cellular behaviors most directly controlled by catalysis of a single

KDAC, without the secondary effects caused by off-target inhibition (a major problem with KDACs, especially the class IIa group) or the loss of physical interactions when the expression is reduced. Table 3 lists representative cellular functions that can be attributed directly to the deacetylation activity of KDAC4 and KDAC7.

We have established a novel set of cell lines for probing KDAC interactions, as well as novel methods for assaying KDAC activity under biologically relevant conditions that allow for determination of the active KDAC without greatly manipulating expression levels. Figure 2 shows representative protein-based method data that rules out a direct deacetylation for a putative substrate, while Table 4 lists proteins that are likely direct substrates based on analysis of expression-level data, and will be followed up on in future experiments.

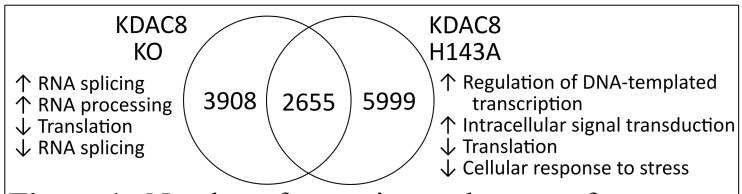

Figure 1. Number of genes in overlap sets of our KDAC8 cell line and a KDAC8 knockout from the literature (Hess L, et. al. (2022) *PLoS Genet*. 18, e1010376.) Representative most significant gene ontology terms, associated with either up-regulated or down-regulated genes (arrows), are shown outside the circles.

Table 3. Representative gene ontology (GO) terms that are highly associated with the effects of enhancing or inactivating the catalytic activity of class IIa KDACs.

KDAC4	KDAC7
• Regulation of transcription by RNA polymerase II	• Regulation of transcription by RNA polymerase II
• Regulation of RNA metabolic process	• Regulation of RNA metabolic process
• Aerobic electron transport chain	• Aerobic electron transport chain
• Regulation of signal transduction	• Chromatin remodeling
• Regulation of cell differentiation	• Proton motive force-driven mitrochondrial ATP synthesis
• Regulation of cell migration	• Cartilage development
• Regulation of nervous system development	

Completion status of sub-aims:

Aim 2a. Mostly complete. Target cell lines for KDAC4, KDAC7, and KDAC8 were completed. Cell lines for KDAC5 have not been completed. For all engineered cell lines, mRNA expression changes have been analyzed as one metric of changes in acetylation. Direct measurement of the complete acetylome was derailed by technical problems and delays related to Hurricane Ida, but remains work in progress. Specific measurements of selected proteins and their acetylation has been completed.

Aim 2b. Complete. Several target proteins have been identified and evaluated for KDAC activity from aim 2a, utilizing immunoblots and immunopreciptiation.

Table 4. Representative acetylated proteins likely regulated by class IIa KDACs (Uniprot ID).

KDAC4	KDAC7
GAPDH (P04406)	CTNNB1 (P35222)
LARP1 (Q6PKG0)	DNAJA1 (P31689)
UPF1 (Q92900)	TP53 (P04637)
ARNTL (O00327)	
ARPC2 (O15144)	
STK4 (Q13043)	

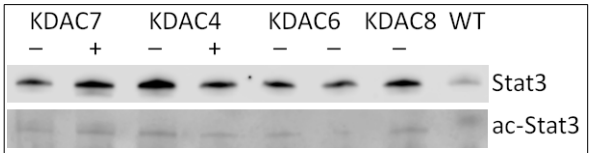

Figure 2. KDACs 4, 6, 7, and 8 do not change the acetylation status of Stat3 whether inactive (–, KDAC6 has 2 inactive forms) or enhanced activity (+) compared to wild-type cells, indicating that none of these KDACs are the Stat3 deacetylase.

Aim 2c. Partially complete. Selected mutations have been introduced into an active class I KDAC to make the active site resemble that of a class IIa KDAC, and the effects of those mutations on activity were probed to understand the different behaviors of class I and IIa KDACs. Direct measurement of inhibitor specificity remains an active work in progress, but we have already made comparisons to relevant existing data reported by other groups.

Changes in direction during project

Near the start of the final reporting period, the center of Hurricane Ida passed near our campus. The campus sustained significant damage and was completely closed for two weeks, then closed to students for a few additional weeks. During much of this period, the students, staff, and PI involved in this project were also displaced. The PI's research lab was partially flooded, and significant losses of material and equipment occurred. As a direct result of this natural disaster, progress on the project was largely stalled for 6 months, during which repairs were made and destroyed materials were replaced (many materials were not discovered to be destroyed until used in protocols, significantly extending the duration of storm impact). Fortunately all engineered cell lines and other irreplaceable materials survived. However, during the second half of the period we resumed measurable forward progress, as detailed below.

In addition, as noted in prior reports, we have replaced direct substrate binding measurements with molecular dynamics simulations, which enhanced our ability to isolate particular interactions as driving selectivity.

Key outcomes.

11 undergraduate students (all African American) participated in this research project over the entire project period. Of those students, five changed their career goals and decided to pursue a PhD directly as a consequence of their research experience. One of those students is currently in a PhD program, two are currently applying/interviewing for PhD programs while working as post-graduate research technicians, and two are currently applying for PhD programs while completing their senior year. A sixth student accepted a research position at a major company upon graduation, and has since also enrolled in a MS program. The seventh student has committed to military service upon graduation in 2023, with the intent to eventually pursue a career involving scientific research. The remaining four students have enrolled in/been accepted to medical schools.

One manuscript related to this work has been published, and several meeting presentations given. A second manuscript is being written with a target submission date of March 2023 (describing some cell line gene expression changes and related behaviors), followed closely by a similar manuscript (for additional cell lines) and a manuscript focused on characterizing the substrate preferences of the class IIa KDACs.