Critical Review of Non-Histone Human Substrates of Metal-Dependent Lysine Deacetylases

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Running Title: Non-Histone KDAC substrates

Nonstandard abbreviations: coA, coenzyme A; KAT, lysine acetyltransferase; KDAC, lysine deacetylase; PTM, post-translational modification

Acknowledgments

The authors acknowledge support from the National Science Foundation (MCB 1817358), the National Institutes of Health (5G1200MD7595, UL1GM118967, and R15GM129682), the U. S. Army Research Laboratory and the U. S. Army Research Office (W911NF1810450), and the Louisiana Cancer Research Consortium. The contents are solely the responsibility of the authors and do not necessarily represent the official views of these agencies.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Author Contributions

TBT and TJW reviewed the literature and wrote the paper.

Abstract

Lysine acetylation is a post-translational 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, we discuss *in vitro* and cell-based experimental strategies used to identify KDAC-substrate pairs and evaluate each for the purpose of directly identifying non-histone substrates of metal-dependent KDACs. We propose criteria for a combination of reproducible experimental approaches that are necessary to establish a direct enzymatic relationship. This critical analysis of the literature identifies 108 proposed non-histone substrate-KDAC pairs for which direct experimental evidence has been reported. Of these, five pairs can be considered well-established, while another thirteen pairs have both cell-based and *in vitro* evidence but lack independent replication and/or sufficient cell-based evidence. We present a path forward for evaluating the remaining substrate leads and reliably identifying novel KDAC substrates.

Keywords: histone deacetylases, substrate specificity, HDAC, KDAC

Introduction

Post-translational modifications (PTMs) have emerged as important regulatory tools for virtually all cellular processes. Acetylation was initially characterized for its role in gene expression mediated by histone proteins, but is now understood to be one of the most prevalent PTMs and has been observed on thousands of unique protein sequences *in vivo,* including many cytoplasmic and mitochondrial proteins.[1–4] A group of enzymes known as lysine acetyltransfereases (KATs) catalyze the transfer of an acetyl group from acetyl-CoA to the primary amine of specific lysine residues.[5] This activity is regulated by a variety of signaling mechanisms, and results in neutralization of the positive charge of the lysine side chain. The functional consequence of this change has been determined for some acetylated proteins; the outcomes include changes in DNA binding (transcriptional regulation), localization, protein-protein interactions, and protein stability.[5] Perhaps the most widely studied example of non-histone protein acetylation is α-tubulin. First reported over 30 years ago, it is widely accepted that acetylation of α-tubulin at K40 stabilizes microtubules.[6,7] Another well-established acetylated protein is p53 (TP53), which has at least 10 known acetylation sites. Acetylation at these sites ultimately affects gene transcription, although which genes are affected is dependent on which residues are acetylated. Furthermore, several of the acetylation sites in p53 are also sites of other PTMs, which suggests significant crosstalk between regulatory pathways.[8]

Like many other PTMs, acetylation is a reversible modification (Figure 1), allowing this feature to serve as a regulatory mechanism. Lysine deacetylases (KDACs, also known as histone deacetylases, EC 3.5.1.98) are metal-dependent enzymes that reverse this post-translational modification, by catalyzing the hydrolysis of ε-N-acetyllysine residues in proteins via a conserved mechanism.[9,10] Removal of the acetate group is essential for the use of acetylation as a regulatory mechanism, as the reversible nature of PTMs allow for their use to control cellular processes in a temporal manner or in response to various stimuli. The acetylation/deacetylation cycle has been implicated in many cellular processes, including DNA damage repair, RNA splicing, cell cycle control, metabolism, and cytoskeletal remodeling.[5] Thus, it is not surprising that aberrant KDAC activity has also been linked to numerous diseases, such as asthma, cancers, muscular disorders, and diabetes.[11] Because of these links to disease states, there is a large body of research aimed at identification of KDAC inhibitors that could potentially serve as therapeutics for a variety of diseases. Despite the large number of inhibitors that have been identified through these screening efforts and the initial excitement about the potential of these inhibitors as therapeutic agents, relatively few inhibitors have proven to be useful for treating patients. The few approved clinical uses of these compounds involve pan-KDAC inhibitors such as vorinostat, a chemotherapeutic agent for the treatment of lymphomas and myelomas.[12,13]

While there has been much effort dedicated to screening for inhibitors, there has been considerably less published work identifying substrates of particular KDACs. Identifying direct substrates of each particular KDAC is a critical step for understanding the biological functions of these enzymes, as well as understanding their role in disease. As lysine acetylation is a reversible modification, and parallels can be drawn with other PTMs such as phosphorylation, it is assumed that the vast majority of the thousands of identified acetylation sites can also be deacetylated. Understanding KDAC function, the consequences of aberrant KDAC function, and the effects of KDAC inhibitors can only be truly understood when the targets of each enzyme have been elucidated. This review discusses the progress that has been made and challenges that remain for determining biological human non-histone substrates of the metal-dependent KDACs.

Lysine deacetylases

Lysine deacetylases are grouped into two large families, determined by mechanism of action. One family, the sirtuins (also known as class III KDACs), use NAD^+ as a cofactor, and are outside of the scope of this review. Here, we focus on the second family, which consists of 11 metal-dependent KDACs in human cells. These enzymes all share a highly conserved catalytic domain (Figure 2) and utilize a divalent transition metal cation in the active site to promote hydrolysis, resulting in the removal of the acetate from lysine side chains.[9,14,15] The identity of the transition metal is usually Zn^{2+} *in vitro*, but evidence exists that some KDACs may utilize Fe^{2+} *in vivo*.[14,16] A reaction mechanism highlighting the key steps is shown in Figure 3. Because of this similarity, it is not surprising that KDACs can compensate for each other in some ways. For example, it has been shown that KDAC2 and KDAC1 have partially overlapping functional roles, and upregulation of one can sometimes compensate for elimination of the other.[17,18] Also, tubulin has been shown to be a substrate of both KDAC6 and Sirt2, indicating at least some overlap between the metal-dependent KDACs and sirtuins.[19] The extent to which various KDACs can compensate for one another and how much overlap exists between substrates is not widely known for the other family members.

The metal-dependent deacetylases are further divided into classes by phylogenetic analysis. Class I deacetylases (KDAC1, 2, 3, and 8) each contain only one domain, while class II deacetylases (KDAC4, 5, 6, 7, 9, and 10) contain at least two domains, one of which is not known to be associated with catalytic activity. The class II deacetylases are further divided into class IIa (KDAC 4, 5, 7, and 9) and class IIb (KDAC 6 and 10) enzymes. It is worth noting that recombinant class IIa enzymes frequently demonstrate little to no deacetylation activity *in vitro* with biologically relevant substrates and it is unclear to what extent they are active deacetylases in cells, although they are associated with a wide range of functions.[20] This observation could at least partially be explained by the fact that class IIa enzymes have a histidine residue in place of the tyrosine residue in the active

site (Figure 3b). Crystallographic evidence suggests that histidine is unable to stabilize the tetrahedral intermediate.[21,22] Mutating the histidine residue of a class IIa KDAC to tyrosine enhances activity of these enzymes *in vitro*.[22] Class IV currently only contains a single KDAC (11), which is most similar to the class I enzymes, but also shares some similarity with the class II enzymes.[23] Recent reports suggest that this enzyme has only weak activity as a deacetylase and may primarily function as a deacylase *in vivo*.[24,25]

In addition to the differences above, class I and II KDACs differ in their intracellular distribution. KDACs 1-3 (all Class I KDACs) are mainly found in the nucleus and are well characterized histone deacetylases. As such, they are important for regulating gene expression.[26] In contrast, the other metal-dependent KDACs (4-11) are frequently found in the cytoplasm and thus likely deacetylate non-histone targets.[27] For example, it has been proposed that the Class IIa KDACs shuttle between the cytoplasm and nucleus based on phosphorylation status.[28] KDAC8 was initially characterized as primarily nuclear, but it appeared to be excluded from the nucleolus (therefore it is probably not involved in transcription).[29] Later, it was found to be primarily cytoplasmic, at least in certain circumstances.[30] KDACs 1-3 are well-established in the literature as deacetylating histones, and there have been a large number of reports of potential histone activity of the other KDACs. However, much less emphasis has been placed on determining possible non-histone substrates, despite localization patterns that suggest that such substrates are probably common.

Approaches for KDAC substrate identification

Over the past several years, dozens of KDAC substrate proteins have been proposed in the literature through various experimental methods. Any discussion of these substrates must include a discussion of the experimental methods by which these potential substrates have been identified. Each of the experimental approaches has both strengths and weaknesses in terms of substrate identification, which provide essential context for evaluating the confidence of proposed enzyme-substrate pairs (Figure 4). The experiments can be broadly characterized as either cell-based, *in vitro*, or physical interaction studies. We propose, based on our discussion below, that a combination of experimental approaches is needed to identify putative KDAC-substrate pairs (Table 1). Substrates for which there is more limited evidence are classified as KDAC-substrate leads (Table 2).

Cell-based experiments have been widely utilized to identify functional relationships between KDACs and potential substrate proteins. Most of the cell studies were performed to understand the function of a particular protein of interest. Excluding studies that link functional changes to KDAC activity on histones (and therefore exert a functional effect via gene regulation rather than a change in target acetylation status), these studies rely on one or more tools to manipulate either the level of KDAC expression or activity in the cell and then measure

the change in acetylation of the target protein, usually by immunoblotting using an acetylation specific antibody.[31–82] (Note that we have only included studies that utilized human cells. Relatively few studies have reported KDAC interactions in non-human mammals, and all such studies focused on proteins with significant differences from the corresponding human protein.) The major advantage of these experiments is that effects on acetylation levels as a result of changing KDAC expression levels of the cell reveal a biologically relevant functional link between KDACs and potential substrates. Conversely, there are several caveats to this type of experiment that makes it virtually impossible to identify direct enzyme-substrate pairs using these methods alone.

First, many of these experiments have utilized KDAC inhibitors to reduce the amount of deacetylation, and then looked for changes in acetylation levels of putative substrate proteins.[31–39,83–85] While experimentally convenient, essentially all available inhibitors are capable of inhibiting multiple KDACs, due to the high degree of similarity between the KDAC active sites. Most identified inhibitors are pan-KDAC inhibitors or inhibit all members of a class.[13,86] There have been a few isoform-specific inhibitors reported, but even these have been shown to inhibit other KDACs in cells when treated with high enough doses.[13,86,87] Thus, using inhibitors has not been a particularly useful approach for definitively determining enzyme-substrate pairs or roles of specific KDACs in cells.

Other methods of manipulating protein levels, either increasing through overexpression or decreasing through siRNA-mediated knockdown or gene knockout, overcome the specificity limitation as they can target a single KDAC. However, there is an inherent caveat to any experiment that relies on changes of protein level in the cell. That is, overexpressed KDACs may deacetylate proteins that they would not interact with under endogenous conditions. Conversely, inhibition or knockdown of a particular KDAC may result in compensation by another KDAC, resulting in the inability to detect a change in acetylation level. Changes in KAT expression or activity levels may also be able to partially compensate for KDAC manipulation. Results of these approaches are often presented together to strengthen the evidence that a particular KDAC catalyzes removal of a particular acetylated lysine, but even together are unable to definitively establish direct enzymatic activity of KDAC on a putative substrate.[32–34,37–44]

There are also issues with the detection of acetylation changes, which can be visualized by immunoblot, mass spectrometry, or autoradiogram. For inclusion here, a clear change in acetylation must be visible in the raw data for whichever method has been utilized. Antibodies for specific acetylation sites are costly and time-consuming to produce, and commercially available antibodies are often are not rated to detect endogenous levels of protein

expression, especially if the acetylation frequency is relatively low, further complicating these experiments. To overcome these challenges, antibodies raised against acetylated lysine in a non-sequence-specific manner have often been employed. However, such antibodies are only useful if an immunoprecipitation step is incorporated in the experiment first, and the unknown affinity of such antibodies for any particular acetylated lysine makes for poor reliability. Also, non-specific antibodies are not effective for determining specific sites of acetylation/deacetylation on a target protein. Mass spectrometry similarly is not always able to detect endogenous acetylation in the absence of specialized workup, and reliable identification of a specific site still requires sophisticated experimentation and analysis. Together, these experimental challenges demonstrate the difficulty of interpreting the results of cell-based studies: these approaches are powerful tools for demonstrating a functional link but lack the resolving power to definitely establish a substrate. Thus, putative substrates of particular KDACs identified in this manner must be validated using a more direct *in vitro* method to determine whether a *bona fide* enzyme-substrate pair has been identified or whether, instead, changes in KDAC activity has indirectly affected acetylation status though some unknown regulatory pathway.

Unlike the cell-based studies*, in vitro* studies have consisted largely of limited screens conducted for the purpose of identifying new KDAC substrates. These experiments most often use purified, recombinant KDAC enzymes to assess deacetylation of putative substrates. *In vitro* experiments provide the most direct quantitative test of whether a particular, specific acetylated lysine can be deacetylated by a particular KDAC, and allow kinetic parameters to be carefully calculated. In this way, the catalytic efficiency of a particular KDAC for different acetylation sites can be compared.[83,88] Most *in vitro* studies screen several acetylated peptides, which correspond to partial sequences of acetylated proteins, as substrates.[83,88–91] In other reports, individual peptides corresponding to a particular protein of interest or known substrate were shown to be deacetylated.[74,92–94] However, it is not known whether these peptides are a reliable model for actual protein substrates. Recent research suggests that long range interactions, which are obviously not present with peptide substrates, at least sometimes do play a significant role in KDAC reactivity.[95] Note that we are excluding from consideration studies that utilize acetylated lysine in an unnatural context, such as conjugated to a fluorophore, as such substrates have not proven useful for understanding biologically relevant substrates.[88,96] In addition, we have only considered peptide studies in which the substrate peptide contains at least 4 residues other than acetylated lysine, as shorter peptides can generally not be assigned to a specific putative substrate. Finally, we excluded *in vitro* peptide sequences for which we were unable to locate evidence of *in vivo* acetylation in the corresponding protein at that site, such as from matching cell-based experiments or proteomics analysis of lysine acetylation in human cells.[1–4,97]

Other *in vitro* studies have utilized full-length proteins that have been purified from cells.[51,72,73,98–100] This experimental approach is fairly convincing; however, immunoprecipitation from cells often results in the purification of not only the target protein, but also other interacting proteins. This possibility renders it difficult to determine with certainty whether there is a direct enzyme-substrate relationship between the recombinant KDAC and the purified protein being investigated. In a related approach, deacetylation of an acetylated substrate is monitored by immunoblotting lysate from bacteria which are simultaneously expressing recombinant human deacetylase and a putative acetylated substrate.[101] Although this experiment is carried out in a bacterial cell, we have classified it as an *in vitro* experiment because the proteins are being assayed outside of their natural context. Ideally, a direct enzyme-substrate relationship would be determined by presenting purified enzyme with a purified, recombinant substrate; however, the logistical challenges involved in obtaining an adequate quantity of recombinant substrate protein that is site-specifically acetylated render this approach not broadly feasible. Additionally, experimental conditions have been shown to have significant effects on KDAC activity *in vitro*;[14,102,103] thus, it is difficult to determine how well the *in vitro* conditions mimic the cellular environment and how the behavior of the enzyme *in vitro* relates to its cellular behavior. Thus, experiments of both types (cell-based and i*n* vitro) identifying the same enzyme-substrate pair are necessary to show direct, biologically relevant deacetylation of a target protein.

In addition to these experimental approaches, several groups have reported physical interactions between particular KDACs and other cellular proteins. These experiments range from co-immunoprecipitation to using a KDAC variant that can be cross-linked to proteins that bind near the active site in cells.[89] While these experiments do capture physical interactions, they do not, on their own, provide evidence of an enzyme-substrate relationship. Furthermore, such experiments are biased toward abundant cellular proteins. Indeed, one report with KDAC8 indicated that the authors were able to validate only approximately 30% of the crosslinked proteins as substrates by *in vitro* methods. Additionally, their approach did not identify a known substrate of KDAC8, SMC3.[89] Thus, this type of experiment can be useful for identifying potential substrate leads for further study and, perhaps, strengthening evidence for suspected enzyme-substrate pairs that have previously been identified by other means. For this reason, we have not included putative substrates that have only been linked to particular KDACs through physical interaction methods.

Putative KDAC-substrate pairs

The experimental challenges with establishing that a particular protein is a substrate for a particular KDAC mean that a combination of *in vitro* and cell-based experiments must be interpreted together. To be highly confident about a direct enzyme-substrate relationship, we propose that the following criteria must be met: (1)

at least two different cell-based experimental approaches must yield consistent data, (2) an *in vitro* experiment must show direct deacetylation of a specific, identified lysine residue, and (3) independent replication or confirmation of the enzyme-substrate pair. Figure 4 illustrates these criteria. Here, we consider the 15 putative KDAC non-histone substrate proteins (two of which are putative substrates for multiple KDACs) for which we were able to find both cell-based and *in vitro* evidence supporting the claim (Table 1). At present, only KDAC6-tubulin, KDAC8-SMC3, KDAC6-p53, KDAC3-RELA, and KDAC6-CTTN meet our criteria to confidently claim a direct enzyme-substrate relationship; KDAC1-p53 also meets these criteria but counterevidence suggests that their relationship may be more complex.

TUBA1A (α-tubulin). KDAC6-tubulin is the best established non-histone KDAC-substrate pair.[104] KDAC6 has been shown by multiple groups in many different ways to deacetylate tubulin at lysine 40 (K40) both *in vitro* and *in vivo*.[31–33,55,84,88,105] It has been proposed that the acetylation/deacetylation of tubulin at this site serves to regulate the stability of microtubules and cilia.[6,7,106] KDAC8 has been reported to be capable of deacetylating tubulin at K40 as well, but the importance and prevalence of this event *in vivo* has not been well established or extensively replicated as it has with KDAC6.[56,89] The putative KDAC8-α-tubulin (K40) pair has been cited by multiple reports; however, the data in support of this are not strong. This claim is mostly based on a physical interaction between these two proteins in cells, as well as an inhibitor study showing increased acetylation specifically in HeLa cells but with a more ambiguous result in HEK293T cells.[56] In contrast, a previous study had reported no effect on tubulin acetylation in response to siRNA knockdown of KDAC8.[30] Similarly, a study reporting the isoform-specific KDAC8 inhibitor clearly demonstrated that treating cells with this compound did not increase α-tubulin acetylation, and an *in vitro* assay failed to detect any deacetylation of a tubulin K40-derived peptide by KDAC8.[88,107] The only published *in vitro* evidence in support of KDAC8 activity involves HPLC-based detection of K40 deacetylation of an α-tubulin derived peptide in the presence of a huge excess of KDAC8.[56] Additional research is necessary to resolve these evenly balanced positive and negative results, and so for now we retain KDAC8-tubulin in the list of putative substrates. A third deacetylase, KDAC5, has been linked to tubulin K40 in cell-based studies, but this has not been assessed *in vitro* to establish a direct link.[57,58] Additionally, several other acetylation sites have been identified on tubulin.[108] Unlike K40, the KDAC responsible for their deacetylation and the functional importance of these sites has not been determined. For example, KDAC8 was shown to deacetylate a peptide derived from tubulin *in vitro*, containing an acetylated K394 residue, but, thus far, there is no evidence that this occurs in cells (Table 2).[89]

SMC3 (structural maintenance of chromosomes protein 3). The second most frequently found enzyme-substrate pair in the literature is SMC3-KDAC8, as multiple groups have reported this relationship both *in vitro* and in

cells (Table 1). SMC3 is a member of the cohesion complex, and its acetylation status affects its ability to bind an N-terminal fragment of Rad21.[37] Unlike for tubulin, the targeted acetylation site is not completely clear. The most frequently attributed site of deacetylation includes two adjacent lysine residues (K105 and K106 in the human protein) which have both been identified as conserved acetylation sites.[109,110] Reported *in vitro* deacetylation reactions were performed with a peptide substrate that was only acetylated at K106, whereas cell studies were carried out using an antibody that recognizes acetylation at both K105 and K106.[37,83,92] Thus, it is unclear whether one or both sites are being deacetylated *in vivo.* Additionally, two other lysine residues have been identified as acetylation sites on SMC3 in a large-scale screen (K140 and K1190) but have not yet been reported as KDAC targets.[2] Conversely, a peptide derived from SMC3 containing yet another acetylated lysine (K215) has been deacetylated *in vitro* by KDAC8 (Table 2).[88] Overall, the evidence strongly suggests that KDAC8 deacetylates SMC3 at K106, but additional work is necessary to determine whether one or more of the other sites are also targets.

TP53 (cellular tumor antigen p53; p53). At least seven distinct acetylation sites have been identified on p53, and different acetylation sites have been proposed to regulate different functions of the protein. K120 is found in the DNA binding domain, and acetylation at this residue is involved in regulating apoptosis through modulating transcription of target genes. K120 is sometimes mutated in cancers, underscoring its potential importance in the cell.[8] KDAC5 and KDAC6 have both been shown to deacetylate p53 at K120 (Table 1). The evidence for KDAC5 all comes from a single report showing that KDAC5 can deacetylate a K120-containing peptide and that knockdown of KDAC5 in cells leads to increased acetylation at K120.[59] KDAC6, in contrast, has been linked to p53 at K120 by demonstrating peptide deacetylation *in vitro*, as well as cell-based studies done by an independent group utilizing both knockdown and overexpression of KDAC6.[42,111]

In addition, the acetylation status of a cluster of lysine residues found at the C-terminus have been proposed to regulate binding to DNA at target gene loci, although the mechanism for this has not been determined.[8] KDAC1 can deacetylate a peptide containing these acetylated residues *in vitro.*[45] Additionally, several groups have reported data consistent with a role for KDAC1 in p53 deacetylation in cells. K382 is deacetylated when KDAC1 is overexpressed, although differing detection methods make it impossible to determine whether neighboring acetylation sites are also affected by changes in KDAC1 expression.[45–48] These data meet our criteria for KDAC1-p53 as a high confidence enzyme-substrate pair. However, a more recent report suggests that the effect of KDAC1 modulation of cells on p53 acetylation status may be indirect, as decreasing KDAC1 levels in cells leads to an increase in an acetylatransferase which could affect p53 acetylation levels.[112] In addition, there is some evidence that both KDAC6 and KDAC8 can deacetylate p53 in this region (Table

2).[35,60,90,93] As only K120 has been shown to be a substrate by both *in vitro* and in cell-based experiments, evidence for this site as a specific substrate is stronger than for individual lysine residues in the C-terminal region.

RELA (transcription factor p65; NFκB subunit). RELA is a subunit of the NFκB trasnsciption factor complex. Acetylation status of this subunit affects protein-protein interactions and nuclear import/export.[113] Deacetylation of RELA by KDAC3 has been demonstrated multiple times in cells, as well as using full-length protein that was immunoprecipitated from cells.[53,114,115] Therefore, there is high confidence in RELA being a substrate of KDAC3. Using acetylation-specific antibodies targeted against specific lysine residues in RELA, deacetylation of three neighboring lysines (K310, K314, and K315) was observed in cells where KDAC3 was overexpressed.[53]

CTTN (cortactin). CTTN contains repetitive sequences, each of which includes a lysine residue that has been reported to be acetylated.[97] Acetylation status has been correlated to the ability of this protein to bind F-actin, which in turn affects cell motility and has potential implications for metastatic cancers.[61,62]Three independent cell-based reports identify CTTN as a substrate of KDAC6, using a combination of overexpression and knockdown experiments.[61–63] While several acetylation sites were identified in CTTN under endogenous *in vivo* conditions, including K124, the site of deacetylation *in vivo* was unable to be definitively worked out, due to the repetitive nature of the sequence.[62] An *in vitro* peptide study demonstrated KDAC6 activity using K124 of CTTN as a substrate.[91] While the peptide used in this study is unique, the residues surrounding the acetylated lysine occur several times in the protein. So, it is unclear whether K124 is the true target of KDAC6 and whether KDAC6 may deacetylate multiple residues of CTTN. However, based on the independent number of experiments that have all identified CTTN as a KDAC6 substrate, we have characterized this as a high confidence pair.

G3BP1(RasGTPase-activating protein-binding protein 1; G3BP-1). Acetylation status of G3BP1 K376 has been shown to regulate binding to c-myc and tau mRNA.[64] This site was identified in the same study as CTTN as a KDAC6 substrate based on deacetylation of a peptide.[91] A few months later, following up on a study that identified a physical interaction between KDAC6 and G3BP1,[116] a separate research group identified acetylation of this same residue from human cells.[64] They further demonstrated that this residue was deacetylated by KDAC6 both *in vitro* and *in vivo*, utilizing an antibody that specifically recognized G3BP1acetylated at K376. Using this antibody, they conclusively show that acetylation at this residue increases when KDAC6 is knocked out.[64] Together, the evidence for this enzyme-substrate pair is relatively strong and

independent validation from a cell-based study is all that would be required for this pair to be classified here as a high confidence substrate.

MAPK3 (mitogen-activated protein kinase 3; ERK-1). Acetylation of ERK-1 leads to decreased kinase activity of ERK-1 against its previously identified substrate ELK1.[32] The mechanism for this change is activity is unclear, and it has not been determined whether this effect is ELK1-specific. There are multiple lines of evidence to support the KDAC6-MAPK3 enzyme-substrate relationship; yet, these were all reported in a single study.[32] The only *in vitro* evidence for direct deacetylation was carried out using acetylated, recombinant MAPK3 and FLAG-tagged KDAC6 that was overexpressed and purified from HEK293T cells. Thus, it is possible that KDAC6 was not the only protein isolated from the cells. Furthermore, each of the *in vivo* experiments was assayed by immunoblotting and, on its own, does not provide convincing evidence of a significant change in MAPK3 deacetylation levels. While the authors propose a novel acetylation site, they do not show deacetylation in a site-specific manner. Therefore, additional cell-based experiments are necessary for high confidence in this relationship.

HIF1A (hypoxia-inducible factor 1α; HIF1α). The evidence for the KDAC4-HIF1A pair is also reported in a single paper, which proposes that KDAC4 regulates the stability of HIF1α.[51] In this report, the only cell-based evidence presented for deacetylation of HIF1α is a KDAC4 knockdown experiment demonstrating that HIF1A acetylation levels increase under these conditions. For the *in vitro* experiment, both the enzyme and the substrate were overexpressed and isolated from HEK293T cells, leaving the possibility that other proteins may have also been isolated along with the proteins of interest. Because the full *HIF1A* protein was used in the assay, and deacetylation was detected by immunoblotting using an antibody that was not site-specific, this study did not identify a specific lysine targeted by KDAC4.[51] When all evidence is considered together, it suggests that a direct relationship exists; however, further investigation is warranted to determine with confidence whether this is the case. Despite the limited evidence, this report has been cited numerous times, mostly by groups interested in developing KDAC inhibitors or determining disease connections, and so illustrates the need to establish relationships with greater confidence.

Other putative substrates. Seven additional putative KDAC8 substrates were identified in a cell-based inhibitor screen which identified proteins with increased acetylation upon KDAC8 inhibition: ARID1A (AT-rich interactive domain-containing protein 1A; ARID domain-containing protein 1A), KAT14 (cysteine-rich protein 2-binding protein; CSRP2-binding protein), NCOA3 (nuclear receptor coactivator 3; NCoA-3), KMTD2 (histone-lysine-N-methyltransferase 2D; lysine-N-methyltransferase 2D), RAI1 (retinoic acid-induced protein

1), SRSF5 (serine/arginine-rich splicing factor 5), and ZRANB2 (zinc finger ran-binding domain-containing protein 2; zinc finger protein 265). Each of the seven proteins were further validated in the same study by an i*n vitro* peptide assay.[83] The same sites in ARID1A, KAT14, and NCOA3 were also identified in an independent *in vitro* screen to identify novel, biologically relevant peptide substrates of KDAC8 (Table 1).[88] While this evidence is of high quality and certainly suggests that these proteins are substrates of KDAC8 in cells, further cell studies are required to strengthen this evidence, as the only cell-based evidence is inhibitor treatment. We were not able to identify any proposed functional effects of acetylation for these proteins.

Additional substrate leads

By the criteria used here, KDAC1, KDAC3, KDAC4, and KDAC5 each have only one putative substrate while most other KDACs (KDAC 2, 7, 9, 10, and 11) have no identified non-histone putative substrates (Table 1). In addition to these, numerous additional non-histone substrate leads have been reported in the literature (Table 2). These are proteins which have been linked to a particular KDAC through either cell-based or *in vitro* experiments, but not both (see references in Table 2). In total, 90 KDAC-substrate lead pairs, involving 81 unique proteins (of which 5 proteins have higher confidence relationships in Table 1), have been identified throughout the literature. A few of these lead proteins have multiple proposed deacetylation sites, and several lead proteins have been proposed to be deacetylated by more than one KDAC, as detailed in Table 2.

With only a handful of exceptions (KDAC8-CAD, KDAC6-HSPA1, KDAC8-LARP1, KDAC6-HSP90, and KDAC5-TUBA1A), each of these substrate leads were identified in a single study (see references in Table 2). Therefore, KDACs 2, 7, 9, 10, and 11 collectively only have a few substrate leads demonstrated by single research groups. For most substrate leads, specific sites of acetylation have been determined; however, in a significant number of cases the deacetylation site was not determined, because the change in acetylation level was detected by immunoblotting the candidate substrate protein with a non-specific anti-acetyl-lysine antibody.[32,34,36,43,49–51,54,65–74] For a subset of these studies, an acetylated lysine residue in the target protein was identified, but the experiments showing deacetylation could not confirm activity at a particular site. Thus, the acetylation site has been denoted as not determined in Table 2. For HSP90 (heat shock protein 90 α/β), *in vitro* experiments show deacetylation at K292;[94] however, the cell-based experiments do not identify a specific lysine residue.[67–70] Although several groups have reported a connection between KDAC6 and HSP90 based on cell-based data, the experiments were largely redundant and consistently showed at most a small effect with regards to a direct enzyme substrate relationship. Without enough information to determine whether the same deacetylation site was being observed in these experiments, we have assigned KDAC6-HSP90 to Table 2.

It is likely that many of the KDAC-substrate pairs proposed in the literature are true *in vivo* targets; however, there is not yet sufficient evidence for most of them. In other cases, the evidence is not strong enough to claim with confidence that a particular KDAC is responsible for deacetylating a putative substrate in humans. The field must take care to carefully evaluate these data, as over interpretation of limited data to claim a direct enzyme-substrate relationship can be detrimental to progress in determining the function of KDACs, their role in disease, and ultimately in designing therapies based on modulating KDAC activity. To move this field forward, a deliberate effort to validate these putative substrates through additional lines of experimentation is required, as presented in Figure 4.

Moving Forward

To date, the deacetylase field has focused largely on developing KDAC inhibitors to understand KDAC function and provide potential therapeutics for several diseases. Unfortunately, in part because of the challenges with developing specific KDAC inhibitors, this approach has only had limited success. However, even if a single KDAC could be targeted by an inhibitor and without compensatory effects from KATs and other KDACs (an apparently unlikely combination of events), inhibitor treatment would still result in changing acetylation levels of an unknown number of largely unidentified downstream targets. This would undoubtedly lead to unwanted side effects should these inhibitors be used as drugs. It may, instead, be more prudent to investigate particular KDAC substrates as potential therapeutic targets. Thus, it is important to identify the targets of each KDAC to understand how and why each KDAC has been associated with particular diseases.

To make progress toward understanding the discrete biological functions of this regulatory mechanism, we need to assign substrates to specific KDACs. The majority of metal-dependent KDACs lack even a single replicated non-histone substrate lead. More deliberate *in vitro* or cell-based screening with all KDACs is required, and putative substrates found by these methods will need to be validated using complementary methods. Substrate leads, especially for those KDACs without a putative substrate, should be followed up on using independent approaches. Critically, reporting of negative results will also be important, as represented by our analysis of whether KDAC8 is a tubulin deacetylase and to rule out some of the substrate leads that may prove not to be true substrates.

There is no proposed enzyme responsible for deacetylation of the majority of proteins reported to be acetylated, and the specific consequences of these acetylation events are unknown. Furthermore, we do not understand how the different KDACs discriminate between substrates, nor the extent to which they deacetylate the same targets. The high degree of similarity in catalytic domain structure of the KDACs makes it difficult to use features of the

enzyme to predict substrate specificity. Determining specific enzyme-substrate pairs in the manner described in Figure 4 will be critical for understanding the roles of each KDAC on cellular processes, and the functional outcomes of their aberrant activity.

Acknowledgments

The authors acknowledge support from the National Science Foundation (MCB 1817358), the National Institutes of Health (5G1200MD7595, UL1GM118967, and R15GM129682), the U. S. Army Research Laboratory and the U. S. Army Research Office (W911NF1810450), and the Louisiana Cancer Research Consortium. The contents are solely the responsibility of the authors and do not necessarily represent the official views of these agencies.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Author Contributions

TBT and TJW reviewed the literature and wrote the paper.

Table 1. Non-histone KDAC substrates validated by both *in vitro* and cell-based studies.

Substrate (Uniprot ID)	Ac site	References					
		KDAC1	**KDAC3**	**KDAC4**	**KDAC5**	**KDAC6**	**KDAC8**
ARID1A (O14497)	K1808						[83,88]
CTTN (Q14247)	K124					[61–63,91]	
G3BP1 (Q13283)	K376					[64,91]	
HIF1A (Q16665)	n.d.†			[51]			
KAT14 (Q9H8E8)	K292						[83,88]
KMTD2 (O14686)	K3579						[83]
MAPK3 (P27361)	n.d.†					[32]	
NCOA3 (Q9Y6Q9)	K687						[83,88]
RAI1 (Q7Z5J4)	K1087						[83]
RELA (Q04206)	K310/ K314/ K315		[53,114,115]				
SMC3 (Q9UQE7)	K106						[37,83,92]
SRSF5 (Q13243)	K167						[83]
TP53 (P04637)	K120				[59]	[42,111]	
TP53 (P04637)	K382	[45–48]					
TUBA1A (Q71U36)	K40					[31–33,55,84,88,105]	[56,89]
ZRANB2 (O95218)	K54						[83]

† n.d. = not determined

Table 2. KDAC substrate leads.

Substrate (Uniprot ID)	Ac site	KDAC (approach†)		Substrate (Uniprot ID)	Ac site	KDAC (approach†)
ACLY (P53396)	K540	KDAC8 (i)[89]		MYOD1 (P15172)	n.d.‡	KDAC1 (i)[117]
ADAP1 (O75689)	K272	KDAC6 (i)[88], KDAC8 (i)[88]		MAML1 (Q92585)	K188	KDAC8 (c)[83]
AIF1 (P55008)	K11	KDAC8 (i)[90]		MEF2D (Q14814)	n.d.‡	KDAC3 (c)[43]
AKAP8L (Q9ULX6)	K257	KDAC6 (i)[91]		MAP3K2 (Q9Y2U5)	K385	KDAC4 (i)[99]
AKT1 (P31749)	K163	KDAC6 (c)[85]		MSH2 (P43246)	n.d.‡	KDAC6 (c)[34]
AKT1 (P31749)	K377	KDAC6 (c)[85]		MSH2 (P43246)	K73	KDAC10 (c)[44]
AKT1 (P31749)	n.d.‡	KDAC8 (c)[75]		MSH6 (P52701)	K504	KDAC8 (i)[90]
ALDH3A1 (P30838)	K269	KDAC8 (i)[90]		NCOR2 (Q9Y618)	K878	KDAC8 (i)[90]
ALDOA/ALDOC (P04075/P09972)	K147	KDAC8 (i)[88]		NFKB2 (Q00653)	n.d.‡	KDAC1 (c)[50] KDAC3 (c)[50]
ARID1A (O14497)	K1808	KDAC6 (i)[88]		PDLIM1 (O00151)	K22	KDAC8 (i)[89]
AMPD2 (Q01433)	K516	KDAC8 (i)[90]		PFKP (Q01813)	K747	KDAC8 (i)[89]
ARHGDIA (P52565)	K52	KDAC6 (i)[100]		PGD (P52209)	K76	KDAC4 (i)[98]
ARNTL (O00327)	K537	KDAC5 (c)[38]		PGD (P52209)	K294	KDAC4 (i)[98]
ARPC2 (O15144)	K295	KDAC8 (i)[88]		PLA2G3 (O95768)	K909	KDAC6 (c)[77]
ATOX1 (O00244)	K60	KDAC6 (i)[91]		PLAGL2 (Q9UPG8)	n.d.‡	KDAC7 (c)[54]
BIRC5 (O15392)	K129	KDAC6 (c)[76]		PPIL2 (Q13356)	K482	KDAC8 (c)[83]
CAD (P27708)	K747	KDAC8 (i)[89,92]		PTBP3 (O95758)	K510	KDAC6 (i)[91]
CBX4 (O00257)	K149	KDAC8 (i)[90]		RELA (Q04206)	K310	KDAC6 (i)[101], KDAC8 (i)[101]
CDC25A (P30304)	n.d.‡	KDAC11 (i)[72]		RPL7 (P18124)	K31	KDAC8 (i)[90]
CENPF (P49454)	K1341	KDAC8 (c)[83]		SDHA (P31040)	K179	KDAC8 (i)[88]
CIDEC (Q96AQ7)	K56	KDAC6 (c)[39]		SIK2 (Q9H0K1)	K53	KDAC6 (i)[74]
CPOX (P36551)	K404	KDAC6 (i)[91]		SIK2 (Q9H0K1)	n.d.‡	KDAC6 (c)[74]
CS (O75390)	K76	KDAC6 (i)[91]		SLC25A1 (P53007)	K160	KDAC6 (i)[91]
CTTN (Q14247)	n.d.	KDAC8 (c)[118]		SMC1A (Q14683)	K713	KDAC8 (i)[90]
CTNNB1 (P35222)	K49	KDAC6 (c)[33]		SMC3 (Q9UQE7)	K215	KDAC8 (i)[88]

DDX21 (Q9NR30)	K779	KDAC8 (i)[88]		SP1 (P08047)	n.d.‡	KDAC1 (c)[49]
DNAJA1 (P31689)	n.d.‡	KDAC6 (c)[65]		SRSF6 (Q13247)	K101	KDAC6 (i)[91]
E2F1 (Q01094)	n.d.‡	KDAC1 (i)[119]		STAT3 (P40763)	K685	KDAC1 (c)[52] KDAC2 (c)[52] KDAC3 (c)[40]
EEF1A1 (P68104)	K55	KDAC8 (i)[90]		STK4 (Q13043)	n.d. ‡	KDAC6 (c)[71]
EIF3D (O15371)	K53	KDAC6 (i)[91]		TBK1 (Q9UHD2)	K241	KDAC9 (c)[78]
EIF5A (P63241)	K47	KDAC6 (c)[79]		TFRC (P02786)	K394	KDAC8 (i)[88]
EGR2 (P11161)	K247	KDAC6 (c)[80], KDAC10 (c)[80]		TKT (P29401)	K144	KDAC6 (i)[91]
ESRRA (P11474)	n.d.‡	KDAC8 (i)[73]		THRAP3 (Q9Y2W1)	K387	KDAC8 (i)[83]
EXOSC2 (A3KFL2)	K165	KDAC6 (i)[91]		TP53 (P04637)	K292	KDAC8 (i)[90]
FNDC3A (Q9Y2H6)	K384	KDAC6 (i)[91]		TP53 (P04637)	K379	KDAC8 (c)[60]
GAPDH (P04406)	K219	KDAC6 (i)[91]		TP53 (P04637)	K381/ K382	KDAC6 (c)[35],
HDAC2 (Q92769)	n.d.‡	KDAC5 (c)[66]		TP53 (P04637)	K382	KDAC8 (i)[93]
HSP90AA1/HSP90AB1 (P07900/P08238)	n.d.‡	KDAC6 (c)[67–70]		TP73 (O15350)	K331	KDAC6 (i)[91]
HSP90AA1/HSP90AB1 (P07900/P08238)	K292/K284	KDAC6 (i)[94]		TRIM29 (Q14134)	K116	KDAC9 (c)[81]
HSPA1A (P0DMV8)	K77	KDAC4 (c)[41]		TRIM33 (Q9UPN9)	K252	KDAC8 (i)[90]
HSPA1A/HSPA1B (P0DMV8 / P0DMV9)	n.d.‡	KDAC6 (c)[36,67]		TRIM33 (Q9UPN9)	K769	KDAC8 (i)[90]
HSPA5 (P11021)	K353	KDAC6 (c)[82]		TRIM33 (Q9UPN9)	K953	KDAC8 (i)[90]
IRF2 (P14316)	K78	KDAC8 (i)[90]		TUBA1A (Q71U36)	K394	KDAC8 (i)[89]
JPT1 (Q9UK76)	K8	KDAC8 (i)[90]		TUBA1A (Q71U36)	K40	KDAC5 (c)[57,58]
KAT6A (Q92794)	K355	KDAC8 (i)[90]		TYY1 ((P25490)	n.d.‡	KDAC1 (i)[120] KDAC2 (i)[120]
KAT6A (Q92794)	K604	KDAC8 (i)[90]		UPF1 (Q92900)	K386	KDAC8 (i)[89]
KAT6A (Q92794)	K815	KDAC8 (i)[90]		ZNF318 (Q5VUA4)	K1275	KDAC8 (i)[90]

LARP1 (Q6PKG0)	K1017	KDAC8 (i)[90,92]				

† i - *in vitro*; c - cell-based

‡ n.d. = not determined

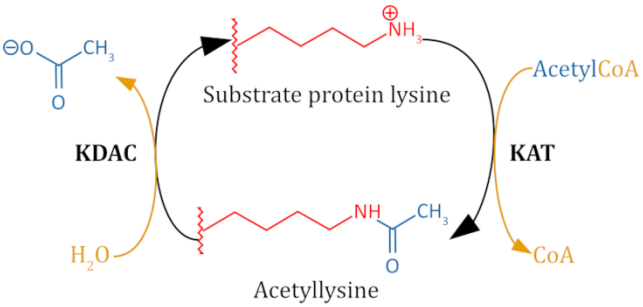


Figure 1. The post-translational acetylation cycle.

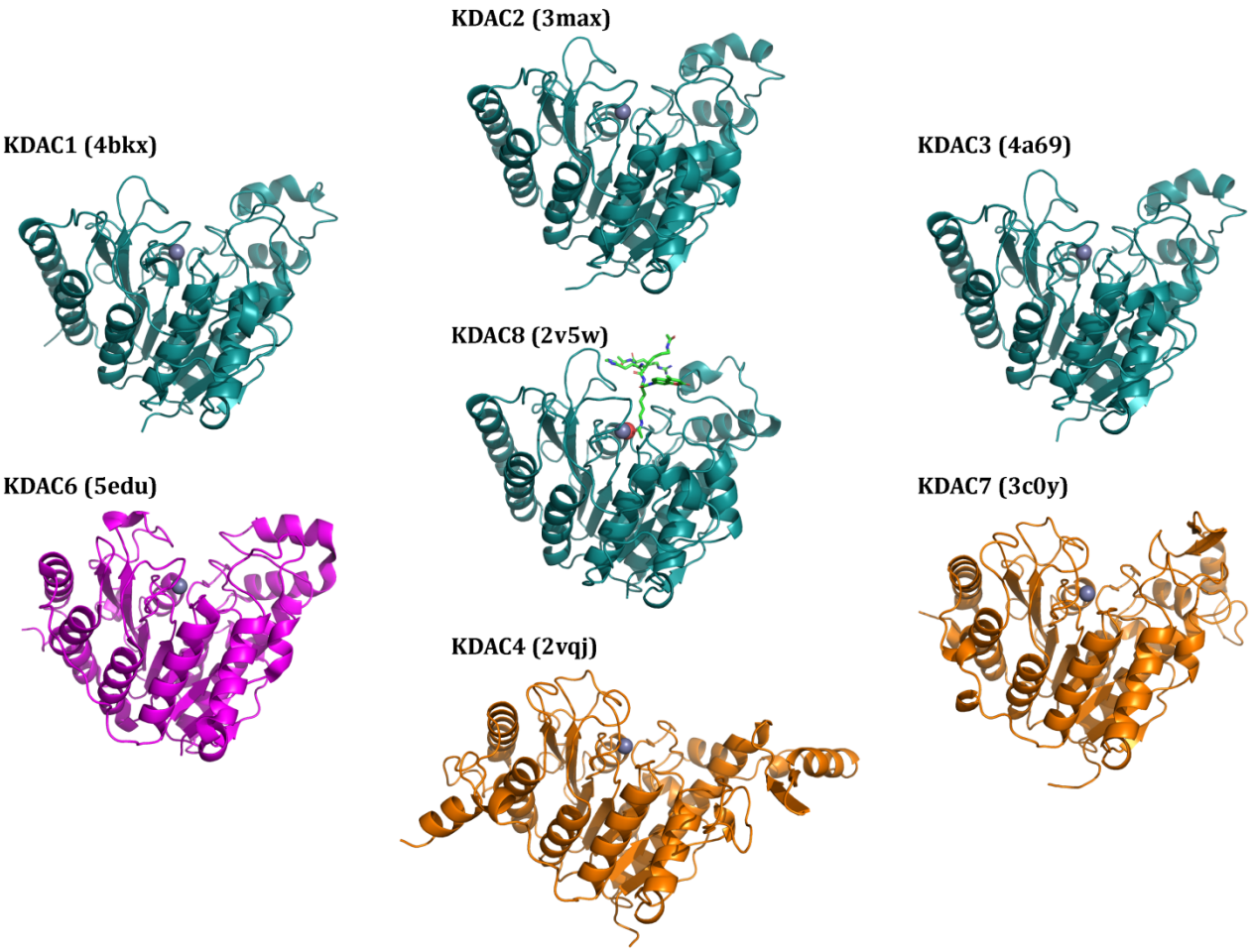
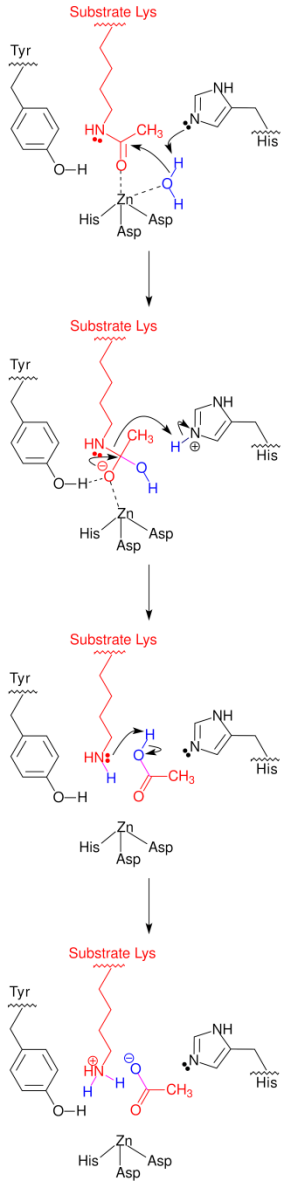


Figure 2.

Representative crystal structures of human KDAC catalytic domains. Crystal structures for all four class I members (KDACs 1, 2, 3, and 8; cyan), two class IIa members (KDACs 4 and 7; orange), and one class IIb member (KDAC6 second catalytic domain, magenta) have been solved. Structural alignment reveals the significant similarity in overall structure, especially within classes. All structures include the catalytic zinc atom (gray sphere). The KDAC8 structure also includes the active site water molecule (red sphere) and a synthetic substrate peptide bound to the active site (stick representation, colored by element); the peptide remains intact because this structure of KDAC8 has a Tyr306Phe mutation that renders the enzyme inactive without perturbing the structure. The Protein Databank ID of each structure is provided, and in all cases the first KDAC chain present in the structure was selected for this representation.

A



B

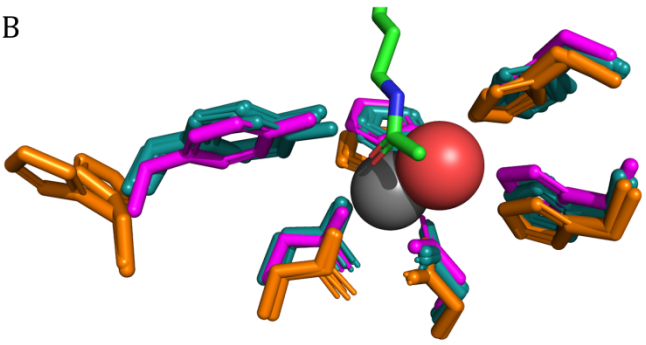


Figure 3. Minimal mechanism of metal-dependent KDACs. (A) The Zn^{2+} ion lowers the pK_a of the water molecule, allowing for deprotonation by a histidine residue and nucleophilic attack on the acetyllysine residue. A tyrosine residue contributes a hydrogen bond to stabilize the tetrahedral intermediate, which collapses to from the free lysine and acetic acid. After a proton rearrangement, the products are released. Further discussion of possible additional transition states and contributing residues can be found in Chen et. al.[10] (B) Alignment of the

active site of reported KDAC structures shows the similarity in residue positions (cyan, orange, and magenta for classes I, IIa, and IIb, respectively). Three residues coordinating the zinc atom (gray sphere from KDAC8 structure, center of image), as well as two histidine residues and one tyrosine residue that contribute to catalytic activity. The class IIa KDACs have a histidine in place of the tyrosine (left side of image), which does not occupy the same position as the tyrosine and causes the changes in activity described in the main text. Catalytic water (red sphere) and a crystallized substrate (colored by element) are from the KDAC8 structure. All PDB codes are the same as Figure 2.

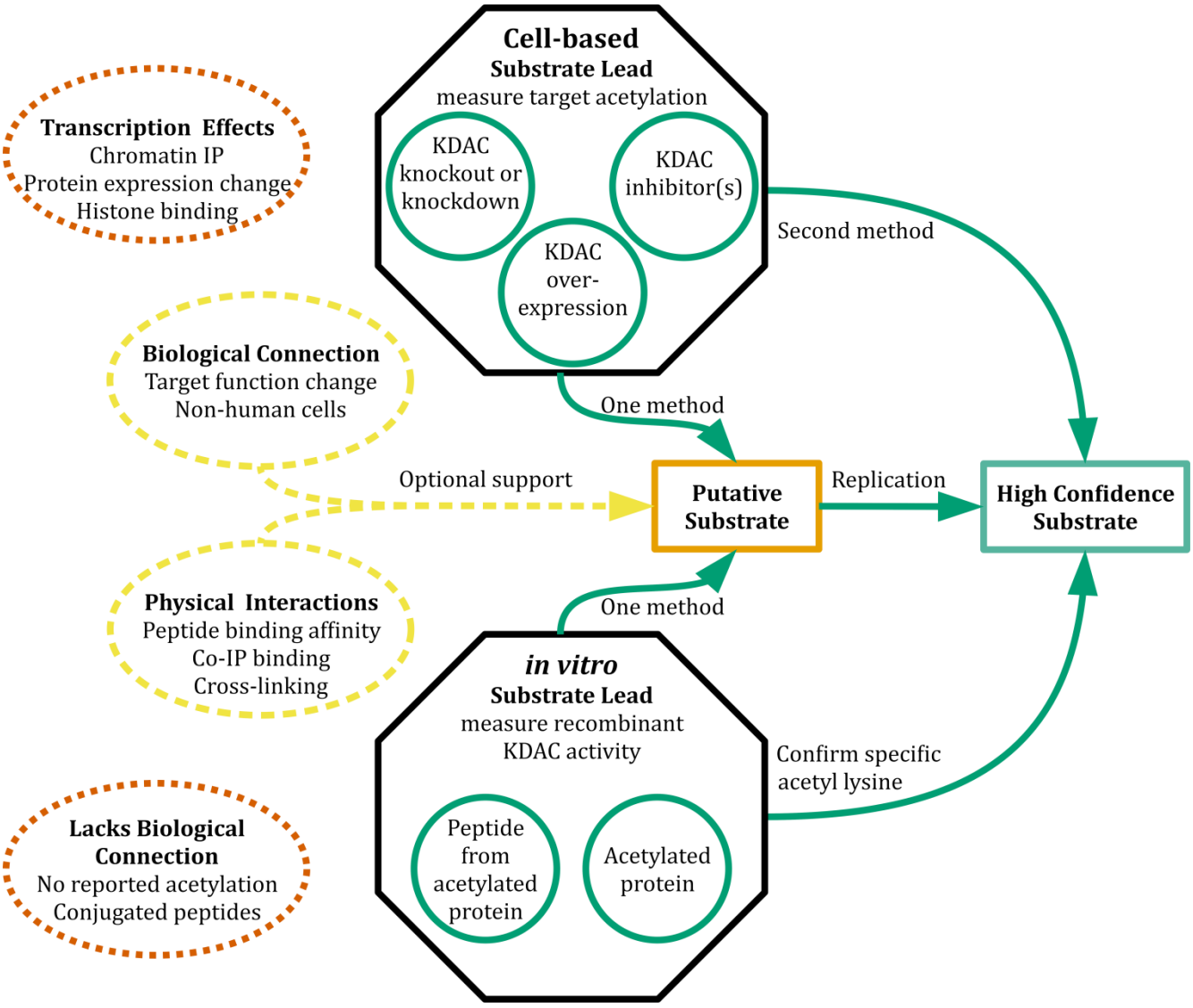


Figure 4. Relationships between experimental methods and the identification of high confidence substrates. Substrate leads can be found using either cell-based methods that determine acetylation status of a target protein with respect to some manipulation of a KDAC or by *in vitro* activity assays utilizing recombinant KDAC enzyme. Using any of these experimental methods generates substrate leads. A putative substrate has supporting evidence from at least one experiment of each type. Identification of a specific acetylated lysine residue that is the site of modification by the KDAC, additional cell-based verification by a second experimental method, and independent replication is necessary to identify the target protein as a substrate with high confidence. Additional methods (dashed ovals) can provide supporting evidence, but do not directly address the question of whether the target protein is a KDAC substrate. Other methods of probing KDAC activity and interactions (dotted ovals) are not relevant for determining whether a non-histone target protein is a KDAC substrate.

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