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Reconstitution of Mammalian Enzymatic Deacylation Reactions in Live Bacteria Using Native Acylated Substrates

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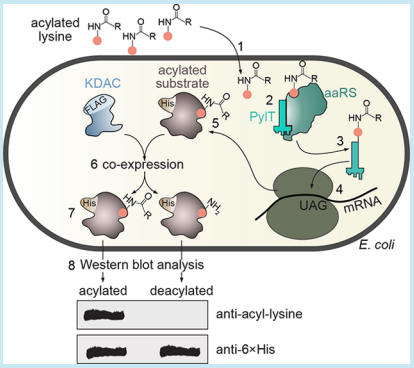
Supporting Information

ABSTRACT: Lysine deacetylases (KDACs) are enzymes that catalyze the hydrolysis of acyl groups from acyl-lysine residues. The recent identification of thousands of putative acylation sites, including specific acetylation sites, created an urgent need for biochemical methodologies aimed at better characterizing KDAC-substrate specificity and evaluating KDACs activity. To address this need, we utilized genetic code expansion technology to coexpress site-specifically acylated substrates with mammalian KDACs, and study substrate recognition and deacylase activity in live *Escherichia coli*. In this system the bacterial cell serves as a "biological test tube" in which the incubation of a single mammalian KDAC and a potential peptide or full-length acylated substrate transpires. We report novel deacetylation activities of Zn^{2+}-dependent deacetylases and sirtuins in bacteria. We also measure the deacylation of propionyl-, butyryl-, and crotonyl-lysine, as well as novel deacetylation of Lys310-acetylated RelA by SIRT3, SIRT5, SIRT6, and HDAC8. This study highlights the importance of native interactions to KDAC-substrate recognition and deacylase activity.



KEYWORDS: *histone deacetylase, sirtuin, KDAC, lysine acetylation, genetic code expansion*

Lysine acylation is a posttranslational modification (PTM) that involves the addition of an acyl group to the epsilon-nitrogen of lysine residues. Of currently known acylations, lysine acetylation—the posttranslational addition of an acetyl (Ac) group—is probably the most prominent and best-studied, primarily in the context of chromatin remodeling and regulation of gene expression.[1] That said, thousands of potential lysine acetylation (as well as acylation) sites have been identified in recent years, suggesting lysine acetylation to be a widespread PTM affecting proteins involved in diverse, highly regulated cellular processes (*e.g.*, cell cycle, splicing, nuclear transport, apoptosis, metabolism, *etc.*).[2−6] Lysine acetylation is carried out by a group of enzymes known as lysine acetyltransferases, which catalyze the transfer of an acetyl group from acetyl coenzyme A to a target lysine residue to form N^{ε}-acetylated lysine (AcK, Figure 1A, **1**). In humans, hydrolysis of the acetyl group is carried out by a group of 18 enzymes known as lysine deacetylases (KDACs). KDACs are generally divided into two major families, the Zn^{2+}-dependent histone deacetylases (HDACs) and the NAD^{+}-dependent sirtuins (SIRTs). The former can be inhibited by an array of inhibitors, such as suberanilohydroxamic acid (SAHA), while the latter can be inhibited by nicotinamide (NAM). The two enzyme families can be further divided into four classes, based on sequence homology. Class I consists of HDACs 1, 2, 3 and 8, class IIa includes HDACs 4, 5, 7, and 9, class IIb is made up of HDACs 6 and 10, class III comprises SIRT1 through 7, while class IV includes a sole member, HDAC11.[1,7] It was also found that some of these enzymes can act as deacylases, capable of catalyzing the hydrolysis of other acyl groups, such as propionyl (ProK, Figure 1A, **2**), butyryl (ButK, **3**) and crotonyl groups (CroK, **4**).[8] The increasing number of specific acylated positions revealed in numerous proteins begs the question of whether deacylation is part of a regulatory mechanism. If so, one can then ask what determines KDAC specificity toward different substrates, and to what degree different KDACs display cross-reactivity toward different substrates. In this work we aim to provide an experimental system that can be used to answer these questions.

At present, a variety of techniques and assays for detecting deacylation activity (mainly deacetylation) exist, such as fluorometric, charcoal-binding and NAM release assays.[9−12] In these assays, deacylation is quantified by monitoring changes in fluorescence upon deacylation of a fluorescent substrate, or by monitoring a fluorogenic/chromogenic reaction between a deacylation product and specific reagents. Although sufficient for detecting deacylation and measuring KDAC kinetics *in vitro*, such assays are not without their

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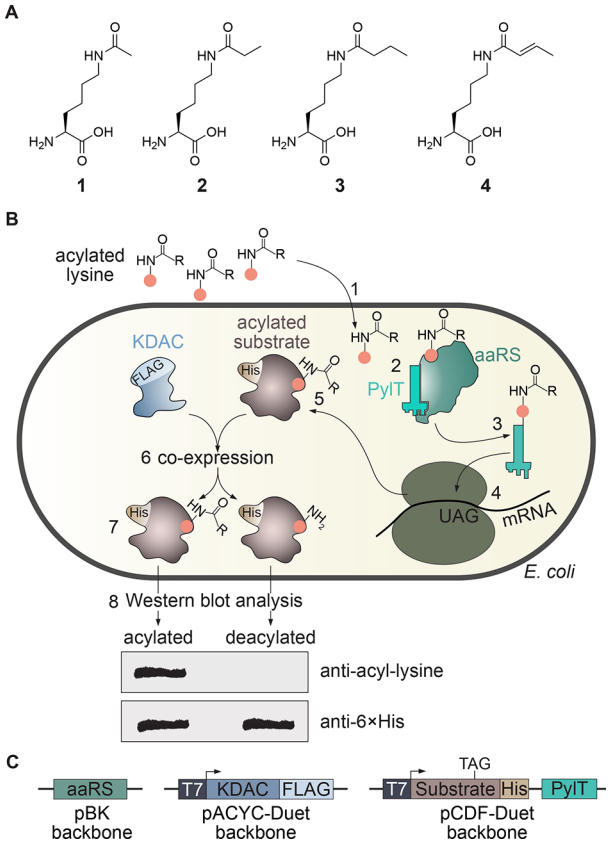

Figure 1. (A) Structure of acylated lysine residues. N^{ϵ}-acetyl-L-lysine (**1**), N^{ϵ}-propionyl-L-lysine (**2**), N^{ϵ}-butyryl-L-lysine (**3**), N^{ϵ}-crotonyl-L-lysine (**4**). (B) Overview of mammalian deacylase activity assay in transformed *E. coli*. Bacteria are incubated in the presence of acylated amino acid (1), which is recognized by an archeal aaRS capable of aminoacylating its cognate tRNA (PylT, 2). The aminoacylated tRNA (3) can suppress an in-frame TAG codon (4) to allow cotranslational incorporation of the acylated lysine residue. The C-terminal 6×His-tagged acylated substrate (5) is coexpressed with one of the KDACs (6). If the acylated substrate is recognized by the coexpressed KDAC, the acyl group will be hydrolyzed in-cell during incubation (7). The level of acylation is then quantified by Western blotting (8) as the ratio between anti-acyl-lysine and anti-His immunoblot intensities. (C) Schematic representation of the genetic components cloned in a modular three-plasmid-based system. aaRS and PylT are constitutively expressed/transcribed, while expression of a TAG mutant of the acylated substrate and the KDAC are induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) or lactose.

drawbacks; in particular, they require laborious purification of the KDAC of interest, and, in many cases, can only be used with short acylated peptide substrates (typically 5−15 amino acids long). Because only a peptide is used and not a folded, full-length protein or domain, such assays may, however, offer limited or even false insight into physiological KDAC-substrate interactions. For example, the frequently used Fluor de Lys assay (BIOMOL/Enzo) is based on monitoring the increase in fluorescence upon deacetylation of a fluorogenic peptide substrate by a purified enzyme, *in vitro*.[13] It has been argued that in this assay, the conjugation of a small molecule to a short peptide can affect the interaction between the studied KDAC and the peptide substrate, so as to yield false negative/positive results. This can have serious implications on drug screenings, for instance.[14,15] For more informative studies in cultured mammalian cells, the use of lines in which specific KDACs are deleted or knocked-down is usually required, with acylation levels being then detected and quantitated using different methods.[16−18] Although such strategies are more biologically relevant, they may still be affected by the catalytic activity of different KDACs toward the same substrate and might also be hindered by the acute phenotypic effects of deleting certain KDACs.[19−21]

RESULTS AND DISCUSSION

To overcome some of the obstacles researchers are currently faced with when studying KDACs, we utilized genetic code expansion technology and reconstituted mammalian KDAC-catalyzed reactions in *Escherichia coli* (*E. coli*, Figure 1B).[22] Genetic code expansion technology enables the expression of full-length acylated proteins by genetically encoding the site-specific incorporation of exogenously supplemented non-canonical amino acids (ncAAs) into ribosomally expressed proteins.[23−27] The ncAA is usually encoded by an in-frame stop codon (*e.g.*, the TAG 'amber' codon) and cotranslationally incorporated into a nascent poly peptide chain by an orthogonal pair of an aminoacyl-tRNA synthetase (aaRS) and its cognate suppressor tRNA.[23−25] We reasoned that catalytic deacylation activity can be evaluated by coexpressing a single KDAC with a putative acylated substrate. In this approach, the KDAC and its potential substrate are coincubated in the bacterial cell, with the level of substrate acylation being subsequently evaluated by Western blot analysis using anti-acyl-lysine antibodies (Figure 1B). In *E. coli*, deacetylation is predominantly catalyzed by CobB, a promiscuous, NAD^+-dependent enzyme homologous to the SIRT family.[28,29] Other potential deacetylases expressed in *E. coli* are LpxC and YcgC. However, the former catalyzes the deacetylation of UDP-3-*O*-(acyl)-*N*-acetylglucosamine, whereas the latter has disputable deacetylase activity.[30−32] Therefore, to avoid potential cross-reactivity with the principal endogenous deacetylase, a CobB deletion (Δ*cobB*) *E. coli* BL21(DE3) strain was first generated using the *Streptococcus pyogenes* type II CRISPR-Cas9 system (Figure S1).[33] In this *E. coli* mutant strain, NAM is not required for inhibiting CobB, making it suitable for studying the catalytic activity of introduced NAD^+-dependent sirtuins.[34]

For the coexpression of mammalian KDACs and their putative acylated substrates, we designed a modular expression system based on three cotransformed plasmids (Figure 1C). In our system, two plasmids carry all the required genetic components for expression of an acylated substrate, namely pyrrolysine amber suppressor tRNA (PylT), evolved or wild-type pyrrolysine aminoacyl synthetase (PylRS), and a potential substrate harboring a specific in-frame TAG mutation (encoding the ncAA). The KDAC under study is encoded on a third plasmid, thereby enabling a "mix-and-match" approach for studying different KDACs with different substrates. All mammalian KDACs were cloned into pACYC-Duet vector, bearing a C-terminal FLAG tag (Table S1). KDAC expression in Δ*cobB E. coli* BL21(DE3) cells was verified by Western blot using anti-FLAG antibodies (Figure S2). For those KDACs that were not expressed, truncated forms containing the catalytic domain were designed based on previous studies, and their expression was validated (hereafter, truncated KDACs are indicated by an asterisk).[35−38] Expression of full-length SIRT7 was achieved by fusion to an N-terminal maltose-binding protein (MBP) for improved solubility, as the truncated variant was not expressed as a

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soluble and/or active enzyme.[39] Protein expression was primarily carried out in defined lactose-based autoinduction (AI) media, which does not require active monitoring of culture density and induction, thus facilitating the study of several KDACs in parallel.[40] Additionally, density-based self-induction of protein expression, as in AI media, allows for reliable comparison of different samples and a high degree of reproducibility.

In evaluating our system, we first confirmed that the introduced KDACs were catalytically active in bacteria. To this end, we studied the deacetylation of ∼15 residue-long acetylated peptides, fused to the N-terminal of MBP bearing a C-terminal 6×His tag (Figure S3). Specifically, we considered Lys9-acetylated histone 3 (H3 AcK9)- and Lys310-acetylated RelA (RelA AcK310)-derived peptides, with the acetylated proteins being known substrates of several KDACs.[7,41−44] Indeed, acetylation plays an important role in the regulation of these proteins. Histone 3 K9-acetylation is recognized as a major epigenetic PTM that has been linked to various cellular processes and diseases, such as telomere maintenance, neural differentiation, and nonalcoholic fatty liver disease, among others.[45,46] RelA, the p65 subunit of nuclear factor kappa B (NF-κB), functions as a transcription factor that mediates important immune and inflammatory responses, and requires acetylation of Lys310 for full transcriptional activity.[47,48] Thus, the cloned KDACs were coexpressed with different acetylated peptide substrates. Substrate acetylation levels were estimated from Western blot performed with specific antibodies against the acetylated residue. Expression of full-length substrates was ncAA-dependent (Figure S4), therefore, the C-terminal 6×His tag was used for quantifying the expression levels of the acetylated substrates. As control, the same substrates were expressed without a KDAC (*i.e.*, with an empty pACYC vector) under identical conditions. The catalytic activity of the coexpressed KDACs was inferred from the ratio between the level of acetylation (*i.e.*, anti-AcK immunoblot intensity) and substrate expression levels (*i.e.*, the anti-6×His immunoblot intensity). Results were normalized to the control measurement.

In our evaluation, the mammalian NAD^+-dependent sirtuins displayed significant deacetylase activity in bacteria when coexpressed with H3 AcK9 peptide (Figure 2A), apart from SIRT4*, which is known to present ADP-ribosyltransferase activity and little to no deacetylase activity.[49] When the cloned Zn^{2+}-dependent HDACs were coexpressed with the same substrate, only HDAC6* and HDAC8 were capable of catalyzing the hydrolysis of the attached acetyl group. No significant catalytic activity was observed for the remaining HDACs, even when tested with known substrates (data not shown). We therefore reasoned that in comparison to mammalian Zn^{2+}-dependent HDACs, mammalian sirtuins are considerably more active in bacteria, due to their higher degree of homology to CobB.

The catalytic activity of mammalian KDACs is often dependent on PTMs, and may also depend on the formation of complexes with other factors.[7] For example, nuclear receptor corepressor 2 (NCOR2), a transcriptional coregulatory protein, is known to facilitate the catalytic activity of HDAC3.[50] Thus, NCOR2 (residues 395−489) was cloned into the same vector containing HDAC3, and the two proteins were coexpressed with an array of peptide substrates, including those derived from known substrates of HDAC3, such as Lys18-acetylated histone 3 (H3 AcK18).[51] However, HDAC3

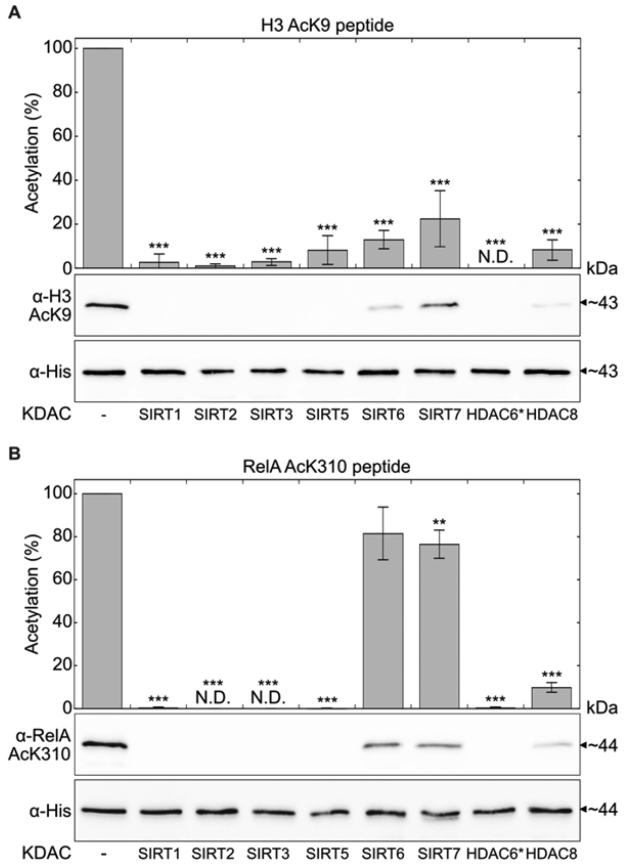

Figure 2. Catalytic activity of mammalian KDACs expressed in *E. coli*. The indicated KDACs were coexpressed with H3 AcK9 (A), or RelA AcK310 peptides (B). Levels of acetylation were estimated from the ratio between anti-AcK and anti-6×His immunoblot intensities, and are displayed relative to cells not expressing a KDAC (error bars indicate ±SD, $n = 3$). Statistical analyses were performed using a one-way ANOVA test together with a one-tailed Dunnett *post hoc* test to identify significantly lower acetylation levels, as compared to the control (-KDAC). *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$. N.D. = not detected.

showed no catalytic activity toward any substrate tested when coexpressed with NCOR2 in Δ*cobB* cells (Figure S5). Taken together, a total of eight mammalian KDACs expressed in Δ*cobB* cells (six sirtuins and two HDACs) demonstrated deacetylase activity. This experimental setup enables semi-quantitative assessment of relative deacetylation level of different substrates by a given KDAC, and qualitative comparison between the eight KDACs.

When these eight catalytically active KDACs were coexpressed with RelA AcK310 peptide, SIRT6 showed no significant catalytic deacetylase activity and SIRT7 displayed marginal activity (Figure 2B), demonstrating the selectivity of mammalian KDACs expressed in bacteria. In addition, the catalytic activity of the mammalian KDACs could be inhibited by known histone deacetylase inhibitors (HDACi) in a concentration-dependent manner (Figure 3). The catalytic activity of mammalian SIRT1 coexpressed with H3 AcK9 peptide was inhibited at NAM concentrations above 5 mM (Figure 3A), while inhibition of HDAC8 by SAHA was observed at concentrations above 10 nM (Figure 3B). This range of SAHA concentrations is higher than that used with cultured mammalian cells, possibly due to the limited uptake of hydroxamic acid-containing molecules by *E. coli*.[52,53] These

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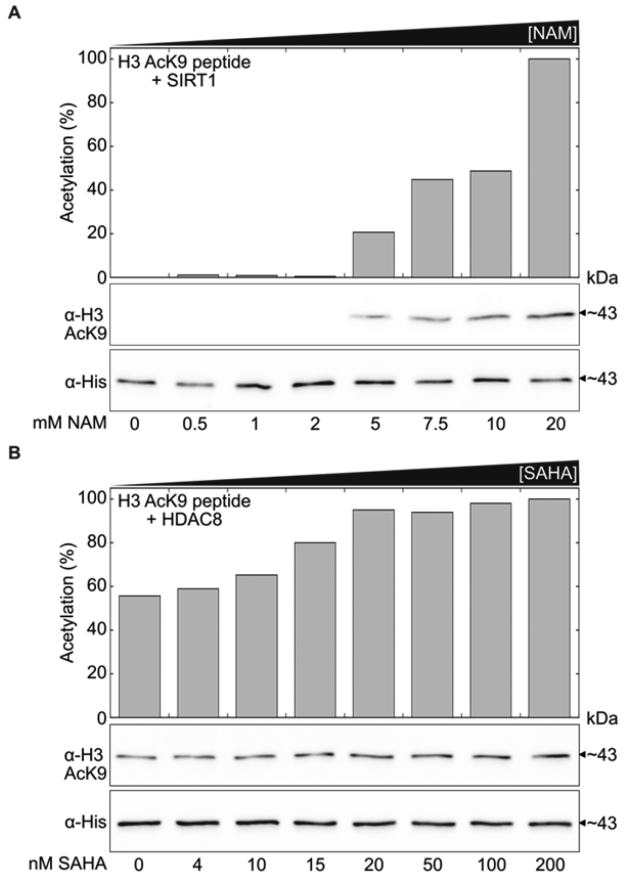

Figure 3. Inhibition of mammalian KDACs expressed in *E. coli*. Bacteria coexpressing H3 AcK9 peptide and SIRT1 (A) or HDAC8 (B) were incubated with increasing concentrations of the indicated HDACi (NAM for NAD^+-dependent SIRT1, or SAHA for Zn^{2+}-dependent HDAC8). Levels of H3K9-acetylation were quantified by Western blotting and are displayed relative to the acetylation level obtained at the highest concentration of the HDACi.

data show that our approach provides a method for evaluating the inhibitory effects of potential HDACi, which have emerged as a novel group of drugs with potent anti-cancer function.[54−56]

After verifying deacetylase activity of mammalian KDACs expressed in *E. coli*, we employed our experimental setup to measure the deacylase (rather than deacetylase) activity of certain mammalian sirtuins. It was previously shown that acylated lysine derivatives **2**−**4** can be incorporated into proteins by wild-type and evolved pyrrolysine synthetase.[27,57] Thus, as a proof of concept for the applicability and scope of our methodology, H3 peptides containing different acyl groups on Lys9 (H3 ProK9, H3 ButK9, and H3 CroK9) were coexpressed with mammalian KDACs in Δ*cobB* cells. H3 K9 butyrylation and crotonylation were shown to occur *in vivo*,[58−60] but all three modifications have been identified on histones, and their deacylation was previously studied *in vitro* using K9-acylated peptides.[57,61] Using anti-H3 AcK9 antibodies capable of recognizing other K9 acylations, we monitored the acylation levels of the modified substrates coexpressed with SIRT1−3 or SIRT6, which are known to hydrolyze acylated lysine residues.[8,62] The crotonyl group on Lys9 of the H3 peptide was hydrolyzed in the Δ*cobB* cells by SIRT2 and SIRT3, and to some extent by SIRT1, but not by SIRT6. In contrast, the propionyl and butyryl groups were hydrolyzed by the four coexpressed sirtuins (Figure 4). These results thus demonstrate that, similar to deacetylation, mammalian KDACs

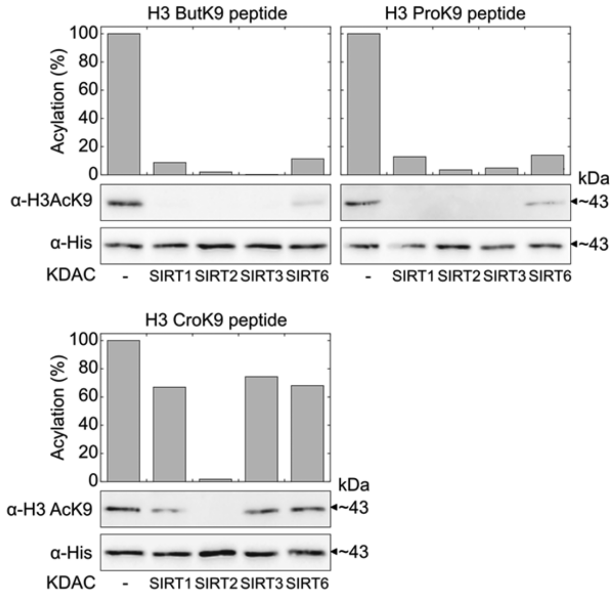

Figure 4. Deacylation activity of mammalian KDACs expressed in *E. coli* coexpressing the indicated acylated H3K9 peptide and mammalian KDAC. The peptide and KDACs were expressed in 2×TY media and level of H3K9-acylation was quantified by Western blotting using anti-H3 AcK9 antibodies and is displayed relative to the acylation level of the peptide when expressed without the KDAC. N.D. = not detected.

expressed in *E. coli* show deacylase activity, which can be monitored using our experimental system.

Following our analysis of short acylated peptide substrates, we next considered acylated proteins or domains. These considerably longer proteins are more biologically relevant substrates, and also more difficult to study by other methods. To allow comparison with the deacetylation of Lys310-acetylated RelA peptide, we coexpressed the eight active KDACs together with a truncated form of Lys310-acetylated RelA (trRelA AcK310, residues 1−323) harboring the functional Rel homology domain, which is essential for *in vivo* dimerization and DNA binding, and can be expressed in bacteria as a soluble and folded protein (Figure 5A).[63] The majority of KDACs coexpressed with trRelA AcK310 demonstrated the same deacetylation level as seen with the Lys310-acetylated RelA peptide (compare Figures 2B and 5A). However, SIRT6 was able to recognize and deacetylate trRelA AcK310, yet demonstrated no significant catalytic activity toward the short substrate. In addition, SIRT7 could not hydrolyze the acetyl group of trRelA AcK310 but displayed residual (yet significant) catalytic activity toward the acetylated RelA-derived peptide. These results highlight the advantage of performing deacetylation assays using fully folded and acylated substrates and stress the importance of protein-protein interactions that are not confined to the active site of the enzyme. Importantly, we ensured that catalytic activities, or lack of them, were independent of the ratio between the coexpressed enzyme and substrate, given that protein expression levels in AI medium may vary. To this end, we plotted the deacetylase activity of SIRT6 and SIRT7 as a function of the ratio between the deacetylases and coexpressed RelA AcK310 variants (Figure 5B). No apparent correlation

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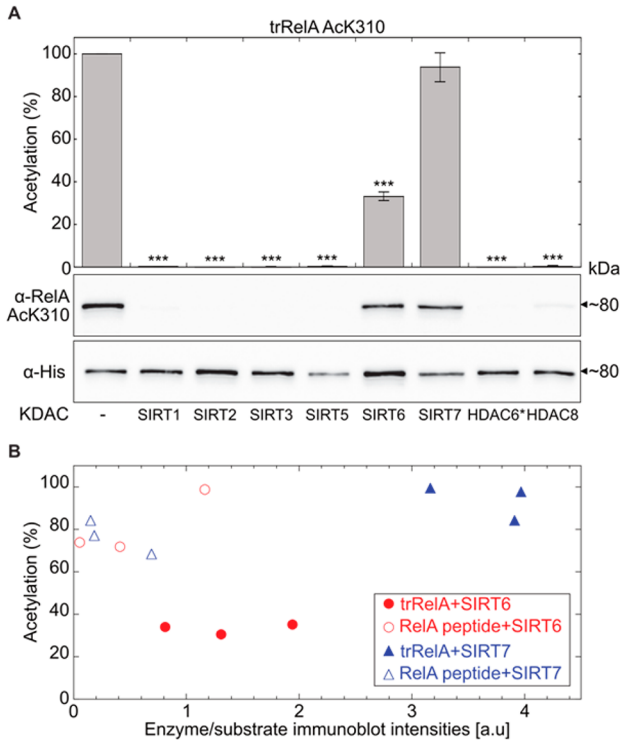

Figure 5. (A) Deacetylation of a folded substrate by mammalian deacetylases expressed in *E. coli*. The indicated KDACs were coexpressed with trRelA AcK310. Acetylation levels were estimated from the ratio between anti-AcK and anti-6×His immunoblot intensities, and are displayed relative to those seen in cells not expressing a KDAC (error bars indicate ±SD, $n = 3$). Statistical analyses were performed using a one-way ANOVA test together with a one-tailed Dunnett *post hoc* test to identify significantly lower acetylation levels, as compared to the control (-KDAC). *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$. (B) Deacetylation of trRelA AcK310 (filled marks) and RelA AcK310 peptide (hollow marks) by SIRT6 (red circles) and 7 (blue triangles), displayed as a function of the ratio between the coexpressed enzyme (anti-FLAG blot intensity) and its acetylated substrate (anti-6×His blot intensity). Each data point represents a single replicate.

between enzyme-to-substrate ratio (*x*-axis) and deacetylase activity (*y*-axis) was observed, demonstrating that measured deacetylation activities are reproducible despite natural variabilities in protein expression levels.

It was previously demonstrated that SIRT7 does not exhibit deacetylation activity toward RelA AcK310, a finding corroborated using our assay in live bacteria (Figure 5A).[64] Additionally, SIRT1 and SIRT2, as well as HDAC6, are known to deacetylate RelA Lys310 *in vivo*, with previous studies having suggested this protein to be a general substrate for class I HDACs.[21] That said, we could demonstrate that RelA AcK310 can also be deacetylated by SIRT3, SIRT5, SIRT6, and HDAC8. Our data clearly show that SIRT5 has the ability to recognize and directly deacetylate both the RelA AcK310-derived peptide and folded domain, in *E. coli*. It is of note that a recent study in mammalian cell culture found that Lys310 acetylation is positively correlated with SIRT5 expression *in vivo*.[65] That said, the results described here are to be expected, considering the differences between the native cellular environment and that within the bacterial cell. Much as an *in vitro* assay, our deacetylation assay conducted in bacteria shows that a given KDAC is *capable* of deacetylating a substrate. Whether this reaction indeed occurs in the native mammalian cell depends on numerous factors that either directly or indirectly associate with one another, or could be greatly affected by protein concentrations, subcellular compartmentalization, PTMs and other regulatory processes. It is therefore important to compare results of studies performed *in vitro* or in bacteria to those obtained in studies performed in mammalian cells.

To conclude, we have presented a novel system for monitoring the catalytic activity of mammalian KDACs with respect to native substrates in live bacteria, using genetic code expansion technology. We report the first examples of introduced SIRT6, SIRT7 and Zn^{2+}-dependent KDAC activity in bacteria, as well as mammalian enzyme-catalyzed hydrolysis of propionyl, butyryl and crotonyl groups in these cells. Furthermore, we showed that relative to other KDACs, SIRT6 has lower deacetylase activity toward all tested substrates. These results are in agreement with previous studies describing the poor *in vitro* deacetylase activity of SIRT6, and demonstrate that our method can reveal differences in substrate recognition by various KDACs.[8,62,66] Our methodology allows for the monitoring of deacylation reactions under more biologically relevant conditions, as compared to current peptide-based *in vitro* methods, since a site-specifically acylated full-length substrate is used. Also, lack of additional deacetylases in the bacterial cell makes it an ideal "biological test tube", given that different KDACs (which are normally present in a mammalian cell) may recognize and deacylate the same substrate, as demonstrated here. Furthermore, our assay does not require any protein purification or chemically modified peptides, is highly reproducible and allows simultaneous examination of multiple KDACs by following a simple protocol. We have also developed a modular acylated-peptide substrate that can be used to mimic the use of synthetic peptides and which allows for comparison with other experiments performed *in vitro*.

Devising strategies to express and characterize the ten remaining KDACs not studied in this work (*e.g.*, using truncated KDACS or upon coexpression with regulatory proteins) will significantly expand the scope of our assay. Moreover, with the increasing numbers of newly evolved aaRS-tRNA pairs,[23] as reflected here using ncAAs **2**–**4**, this strategy need not be limited to studying KDACs and deacylations, and can be further expanded to investigate additional important PTMs (*e.g.*, phosphorylation or methylation). We believe that the assay described here can yield more biologically relevant results than existing *in vitro* methods, when defining KDAC-substrate recognition. When used in parallel with existing *in vivo* methods, our assay can provide additional data, leading to more biologically accurate insight that could otherwise be overlooked.

■ ASSOCIATED CONTENT

Supporting Information

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Supplementary tables, supplementary figures, materials and methods, and supplementary references (PDF)

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Author Contributions

E.M.A., S.L., E.Z., and C.R. performed the experiments. O.B. provided technical assistance. E.M.A. analyzed the data. E.M.A. and E.A. wrote the manuscript with editorial assistance from all authors. E.A. conceived and supervised the study.

Notes

The authors declare no competing financial interest.

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