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## Targeted Protein Acetylation in Cells Using Heterobifunctional Molecules

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### Abstract

Protein acetylation is a central event in orchestrating diverse cellular processes. However, current strategies to investigate protein acetylation in cells are often nonspecific or lack temporal and magnitude control. Here, we developed an acetylation tagging system, AceTAG, to induce acetylation of targeted proteins. The AceTAG system utilizes bifunctional molecules to direct the lysine acetyltransferase p300/CBP to proteins fused with the small protein tag FKBP12<sup>F36V</sup>,

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Author Contributions

W.W.W. and L-Y.C. contributed equally to this work.

The authors declare the following competing financial interest(s): Scripps Research has filed provisional patent applications related to this work, listing W.W.W., L-Y.C., and C.G.P. as inventors.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.1c07850>.

Supplementary figures and tables; experimental details (PDF)

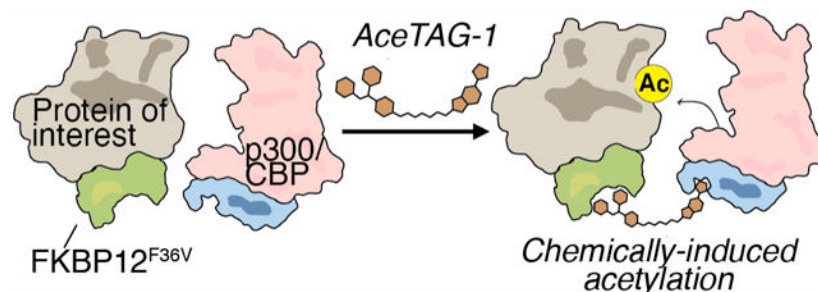
Compiled acetylproteomics data for AceTAG-1 and SAHA treated WT HeLa and H3.3-FKBP12 (F36V) cells (XLSX)

Complete contact information is available at: <https://pubs.acs.org/10.1021/jacs.1c07850>

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>65</sup> partner repository with the data set identifier PXD027617.

resulting in their induced acetylation. Using AceTAG, we induced targeted acetylation of a diverse array of proteins in cells, specifically histone H3.3, the NF- $\kappa$ B subunit p65/RelA, and the tumor suppressor p53. We demonstrate that targeted acetylation with the AceTAG system is rapid, selective, reversible and can be controlled in a dose-dependent fashion. AceTAG represents a useful strategy to modulate protein acetylation and should enable the exploration of targeted acetylation in basic biological and therapeutic contexts.

## Graphical Abstract



## INTRODUCTION

Lysine acetylation is one of the most frequent post-translational modifications (PTMs), occurring on >10000 sites on human proteins, and plays critical roles in human biology.<sup>1</sup> This covalent modification is reversible, and its dynamic equilibrium is mediated by a combined ~40 lysine deacetylases (KDACs) and acetyltransferases (KATs).<sup>2,3</sup> Acetylation not only imparts direct functional consequences on proteins but can also interplay with other PTMs, such as phosphorylation, ubiquitination, methylation, and SUMOylation, and its dysregulation has been implicated in diverse human diseases, including various cancers,<sup>4,5</sup> neurodegenerative disorders,<sup>6,7</sup> autoimmune conditions,<sup>8,9</sup> and metabolic diseases.<sup>5,10,11</sup> Despite its frequency and diverse roles, a substantial hurdle for accurate investigations of acetylation is the dearth of tools to selectively modulate protein acetylation in cells. Although conventional methods, such as genetic or chemical manipulation of KDACs or KATs, have yielded much insight into the myriad roles of acetylation,<sup>12,13</sup> they induce global alterations to their substrates, potentially complicating the interpretation of the biological effects relating to a single protein.<sup>14,15</sup> Alternatively, methods for site-specific modification of substrates, to either mimic or block acetylation,<sup>16,17</sup> could have undesirable effects on protein structure, obfuscate analysis of alternate PTMs on the same residues, and do not permit acute or graded investigations of acetylation in cells. To overcome these obstacles, we envisioned a potentially generalizable, chemical-based strategy to dynamically and selectively induce acetylation on a protein of interest (POI) directly in cells.

Encouraged by the transformative utility of chemical inducers of dimerization<sup>18,19</sup> and ubiquitin-inducing small molecules,<sup>20</sup> as well as recent examples of phosphorylation modifying bifunctional molecules,<sup>21,22</sup> we hypothesized that a similar heterobifunctional system could be employed to modulate the acetylation of any POI on-demand by proximity-induced acetylation. Acetylation can impart inhibitory effects on a protein as well as activating or gain-of-function outcomes; thus, a potential hurdle for the development

of such heterobifunctional small molecules is the identification of functionally “silent” ligands that selectively bind to target proteins without overt effects on protein function to prevent counterproductive or complicated outputs. Considering this, and inspired by various technologies that enable the selective targeting of genetically tagged proteins with small molecules,<sup>23–25</sup> we conceptualized a strategy to direct protein acetylation to targeted POIs, even in the absence of available small molecule ligands.

Here, we report the development of an “acetylation tagging” system, or AceTAG, which consists of a heterobifunctional molecule formed by covalently linking a KAT-binding ligand to an FKBP12<sup>F36V</sup>-binding ligand, resulting in chemically induced proximity between cellular acetylation machinery and a targeted protein. Using AceTAG, we demonstrate that the KAT p300/CBP can be recruited directly to histone H3.3, p65/RELA, and p53, resulting in their targeted acetylation at functionally relevant sites in cells. We demonstrate AceTAG-mediated acetylation is dose-controlled, reversible, rapid, and selective for tagged protein targets.

## RESULTS AND DISCUSSION

### Bifunctional AceTAG Molecules Mediate Binding between p300/CBP and FKBP12<sup>F36V</sup>.

Among KATs, the E1A-associated protein (p300) and its paralogue CREB-binding protein (CBP) are two of the most prominent members, playing key roles as transcriptional co-activators essential for myriad cellular processes, including growth and development, stress response, oncogenesis, and DNA damage.<sup>26</sup> p300/CBP collectively regulate >2/3 of the known acetylation sites in humans,<sup>1</sup> indicative of a broad substrate tolerability, and therefore represent high-priority candidates for the design of generalizable chemically induced acetylation strategies. Recently, inhibitors targeting the conserved p300/CBP bromodomain (BRD)<sup>27</sup> have been co-opted into bifunctional molecules to target chromatin machinery. This includes their conjugation to DNA-targeting polyamides<sup>28</sup> to coordinate histone acetylation *in vitro* and alter gene expression *in cellulo* as well as their use to recruit p300/CBP to gene loci for targeted gene transcription via catalytically inactive Cas9,<sup>29</sup> the latter presumably through proximity-induced histone acetylation. However, it remained an open question as to whether a potentially generalizable strategy might be developed to recruit p300/CBP directly to protein targets, resulting in their targeted acetylation in living cells.

Recently, in an approach called degradation TAG (dTAG), it has been shown that synthetic ligands that recognize the engineered FKBP12 variant FKBP12<sup>F36V</sup> can be conjugated to E3 ligands,<sup>30</sup> resulting in selective ubiquitination and proteasome-mediated degradation of FKBP12<sup>F36V</sup>-tagged proteins.<sup>25</sup> We hypothesized a similar strategy could be adopted for targeted protein acetylation (Figure 1a). To this end, we appended the previously reported FKBP12<sup>F36V</sup>-binding ligand<sup>25,31</sup> to the 5-isoxazoly-benzimidazole p300/CBP BRDinhibitor<sup>27</sup> to generate a series of compounds, AceTAG 1–3, with different linker compositions (Figure 1b and Figure S1a). We first assessed the ability of AceTAG 1–3 to mediate complex formation between soluble recombinant FKBP12<sup>F36V</sup> and the BRD domain of p300 (BRD-p300) *in vitro* using an AlphaScreen assay<sup>31</sup> (Figure S1b). We observed characteristic bell-shaped autoinhibitory curves for all AceTAG analogues, the

result of AceTAG molecules saturating both FKBP12<sup>F36V</sup> and BRD-p300, effectively outcompeting ternary complex formation.<sup>32</sup> We noted that AceTAG-1 has increased potency relative to other analogues, with maximum complex formation occurring at ~1  $\mu\text{M}$  (Figure 1c). In addition, the luminescence signal is considerably diminished upon co-treatment of either terminal binding molecules (FKBP-c and p300-c) in a concentration-dependent fashion (Figure 1d,e and Figure S1c), confirming that observed ternary complex formation is dependent on the simultaneous binding of both BRD-p300 and FKBP12<sup>F36V</sup>. To verify that AceTAG molecules can engage their protein binding partners in cells, we constructed “fully functionalized” photoaffinity probes<sup>33</sup> of both the FKBP12<sup>F36V</sup> (FKBP-p) and p300/CBP (p300-p) ligands and confirmed respective binding to recombinantly expressed p300 and FKBP12<sup>F36V</sup> can be efficiently competed when cells are co-treated with increasing concentrations of AceTAG-1 (Figure 1f,g). Together, these biochemical data indicate that AceTAG 1–3 effectively engage FKBP12<sup>F36V</sup> and p300/CBP and are capable of inducing complex formation.

### AceTAG Molecules Induce Targeted Protein Acetylation in Cells.

We next evaluated whether AceTAG molecules could induce acetylation of FKBP12<sup>F36V</sup>-tagged proteins in cells. We chose first to target histone H3.3 given that p300/CBP is a predominant histone acetyl transferase. Although the specific sites on H3 targeted by p300/CBP are not firmly established, recent proteomic studies have indicated that pharmacological inhibition and genetic ablation of p300/CBP result in decreased levels of H3K18ac, H3K27ac, and H3K36ac.<sup>15</sup> In addition, *in vitro* studies suggest H3K14, H3K18, H3K23, H3K64, and H3K122 are also major acetylation targets.<sup>34–36</sup> We first stably transfected HeLa cells with H3.3-FKBP12<sup>F36V</sup>-HA and confirmed that the H3.3FKBP12<sup>F36V</sup> chimera localizes primarily to chromatin along with endogenous H3.3 (Figure S2a). We next evaluated AceTAG compounds AceTAG 1–3 for their relative ability to induce acetylation at H3.3 K18 using acetyl histone H3 K18 selective antibodies. We observed AceTAG-mediated K18 acetylation for all analogues, with AceTAG-1 inducing modestly higher levels of acetylation relative to the other analogues (Figure 2a and Figure S2b).

We observed that AceTAG-mediated acetylation of H3.3FKBP12<sup>F36V</sup> is dose-dependent, with highest levels of acetylation achieved between 625 nM and 3  $\mu\text{M}$ , near the concentration needed for maximum ternary complex formation *in vitro* (Figure 1c). In addition, acetylation levels demonstrate a decreasing trend at the highest AceTAG-1 concentrations, consistent with acetylation being a consequence of ternary complex induction in cells (Figure 2a). We also confirmed increased H3.3-FKBP12<sup>F36V</sup> acetylation upon incubation of cells with the broad histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA), indicating the fusion protein is a substrate of endogenous HDACs (Figure 2b). We next examined levels at other known H3.3 sites using available antibodies, including K9, K14, K23, K27, and K79 (Figure 2a and Figure S2c), detecting induced acetylation only at K18, K23, and K27 (Figure 2a). Notably, we did not observe changes in acetylation of untagged, endogenous H3.3 after AceTAG-1 treatment in either WT HeLa or H3.3-FKBP12<sup>F36V</sup> HeLa cells, suggesting that AceTAG-1 does not modulate p300/CBP KAT activity (Figure S2d,e). In addition, acetylation can be

blocked by co-incubation with either A-485, a p300/CBP KAT inhibitor,<sup>37</sup> with excess FKBP12<sup>F36V</sup> ligand (FKBP-c, Figure 2b) or with excess p300/CBP ligand (p300-c, Figure S2f). Collectively, these data indicate that AceTAG-induced acetylation in cells is dependent on p300/CBP enzymatic activity as well as binding to both p300/CBP and the FKBP12<sup>F36V</sup>-tagged protein.

We next set out to assess the kinetics of AceTAG-mediated acetylation in cells by monitoring acetylation levels of H3.3 K18 over multiple time points. Surprisingly, we observed that induced acetylation occurs almost immediately, after only ~5 min exposure to AceTAG-1 (Figure 2c) as well as after continuous incubation (up to 24 h). In addition, when AceTAG-1 is removed from cells, H3.3 K18 acetylation steadily dissipates, reaching near basal levels within 2 h after compound removal (Figure 2d). This re-equilibration is likely the result of endogenous KDAC activity, together indicating that the targeted acetylation is reversible and dependent on the heterobifunctional compound.

### AceTAG Strategy Extendable to Multiple Proteins.

Satisfied with the successful compound-induced acetylation of H3.3, we next used our AceTAG system to assess chemically mediated acetylation of other targeted proteins. To avoid potential obfuscation by endogenous target proteins, we generated FKBP12<sup>F36V</sup>-RelA and FKBP12<sup>F36V</sup>-p53 constructs, two proteins wherein p300/CBP-mediated acetylation is known to impart consequences<sup>38,39</sup> on their transcriptional activity. Subsequently, FKBP12<sup>F36V</sup>-RelA was stably transfected in a HeLa *RelA*<sup>-/-</sup> cell line while FKBP12<sup>F36V</sup>-p53 was stably transfected in H1299 non-small cell carcinoma (NSCLC) cells, which have a homozygous partial deletion of *TP53* and lack p53 protein expression. Treatment of FKBP12<sup>F36V</sup>-RelA expressing cells with AceTAG-1 resulted in dose-dependent acetylation at K310, a site previously proposed to be a predominant target of p300/CBP (Figure 3a,b),<sup>40-42</sup> with minimal effects on neighboring K314/315 (Figure S3a). In FKBP12<sup>F36V</sup>-p53 H1299 cells, we monitored acetylation of the C-terminal domain of p53, specifically at K305, K373, and K382, sites previously suggested to be substrates of p300/CBP,<sup>43-46</sup> where strong AceTAG-1-dependent acetylation was also observed (Figure 3c,d). Consistent with our previous observations, we observe apparent acetylation autoinhibition for both targets at higher AceTAG-1 concentrations (Figure 3a, c) and blockade of induced acetylation upon co-treatment of cells with p300/CBP KAT inhibitor A-485 or competing FKBP ligand (Figure 3b,d). In addition, a significant level of p53 acetylation is detectable after ~10 min of AceTAG compound treatment (Figure S3b), similar to the kinetics observed for H3.3. Finally, we note that AceTAG-1-induced acetylation does not appear dependent upon the positioning of the FKBP12<sup>F36V</sup> tag, as similar acetylation effects were observed with C-terminally tagged p53 (Figure S3c).

### Selectivity of AceTAG-Induced Acetylation.

To more robustly characterize the site selectivity of induced acetylation of targeted proteins using AceTAG, we monitored AceTAG-1-induced acetylation in cells by quantitative mass spectrometry (MS). In these experiments, H3.3-FKBP12<sup>F36V</sup>-HA was enriched from stably transfected HeLa cells treated with (1) DMSO, (2) AceTAG-1, or (3) SAHA and trypsinized for protein identification and quantitation by using tandem mass tags (TMT) (Figure

S4a). In line with immunoblotting experiments (Figure 2a and Figure S2c), we measured substantial increases in acetylation at K18, K23, and K27 but detected no acetylation at other identified lysines on H3.3 (Figure 4a and Figure S4b). Together, these data suggest that when p300/CBP is recruited to H3.3 in cells by using our AceTAG system, the preferred acetylation sites are K18, K23, and K27. We next sought to evaluate the selectivity of AceTAG-induced acetylation across the human proteome. Treatment of HeLa cells with AceTAG-1 resulted in no observable acetylation changes in well-established p300/CBP substrates, including c-Myc<sup>47</sup> and STAT3<sup>48</sup> (Figure S5a) or broader acetylation perturbation via immunoblot assays (Figure 4b). To assess targeted acetylation selectivity more globally and quantitatively, we performed MS-based acetylproteomic analysis of AceTAG-treated H3.3-FKBP12<sup>F36V</sup> HeLa cells. Briefly, acetylated peptides from H3.3-FKBP12<sup>F36V</sup> HeLa cells treated either with (1) DMSO, (2) AceTAG-1, or (3) SAHA were digested, enriched, identified, and quantified by using TMT (Figure S5b). Relative to DMSO, we observed no substantial changes in acetylation of any detected protein in AceTAG-treated cells while SAHA treatment resulted in increases across ~30 proteins (Figure 4c,d, Figure S5c–e, and Table S1). We note that no substantial changes in endogenous histone lysine acetylation was observed upon AceTAG treatment, including H3.3, likely due to the substantially lower H3.3-FKBP12<sup>F36V</sup> acetylation and expression levels (~25-fold) relative to endogenous histones (Figure S5f). Collectively, these data suggest that AceTAG-1 does not broadly affect p300/CBP KAT activity on other substrates, and AceTAG-1 mediated acetylation is endowed with exquisite selectivity for FKBP12<sup>F36V</sup>-tagged proteins.

## CONCLUSION

Here, we describe a method for the selective acetylation of targeted proteins in live cells using heterobifunctional small molecules. We demonstrate that AceTAG molecules can bind to and recruit the lysine acetyltransferase p300/CBP to multiple protein targets genetically fused with FKBP12<sup>F36V</sup>, effectively inducing acetylation in live cells. We show AceTAG-mediated acetylation is rapid, occurring within minutes of molecule addition, selective for tagged proteins, reversible, and dependent upon KAT enzymatic activity. In addition, AceTAG molecules possess submicromolar efficacy in cells and, as expected, possess characteristics associated with heterobifunctional small molecules, including autoinhibition at increased concentrations both in vitro and in cellulo as well as reduced ternary complex formation with competing FKBP12<sup>F36V</sup> and p300/CBP-binding ligands.

Despite the consequential roles of protein acetylation, methods to study the effects of acetylation on specific protein targets in cells are limited. Common approaches include genetic or pharmacological ablation of acetylation machinery which affects global substrates, potentially complicating any downstream analyses, or substrate mutagenesis, which lacks dynamic control and can perturb substrate structure or potentially interfere with competing PTMs. Thus, the ability to selectively induce acetylation of targeted proteins overcomes many of these challenges. Toward this end, chemical-based approaches have yielded ligand-directed acetyl-donating reagents for stoichiometric, nonenzymatic transfer of acyl groups to lysine residues on specific proteins like androgen receptor,<sup>49</sup> histone H2B,<sup>50,51</sup> dihydrofolate reductase,<sup>52,53</sup> and phosphoglycerate mutase 1.<sup>54</sup> Though capable of targeting endogenous POIs, such reagents are “single use”, allowing for the transfer of

one acetyl group per molecule and, further, are limited to proteins with available ligands that bind within proximity to targeted recipient lysine residues. In addition, recent approaches have established that recruitment of p300/CBP to specific DNA sequences with bifunctional molecules can induce transcriptional modulation.<sup>28,29,55</sup> However, these approaches rely on DNA-targeting polyamides or sequence targeting using guide RNA's via engineered Cas9. Our AceTAG system enables the direct targeting of tagged proteins for acetylation, even in the absence of available targeting ligands, in principle enabling the study p300/CBP-mediated acetylation on a wide range of targets.

There are some important points when considering implementation of AceTAG to study protein acetylation. First, we recognize that our current studies have focused on three protein targets. Although current evidence deems p300/CBP as having the broadest substrate scope of all KATs,<sup>1</sup> it is unclear how generalizable this approach might be to induce acetylation of other substrates or even neo-substrates. Thus, future attention should be given to exploring a broader subset of targets from diverse functional classes. In addition, even though we conducted a brief exploration of linker length and composition, we do observe linker-dependent ternary complex formation and targeted acetylation (Figure 1c–e and Figure S2b). Like other bifunctional molecules,<sup>56–58</sup> we suspect that each protein target will likely have unique linker preferences, necessitating thorough linker optimization studies. In addition, we recognize that the functional effects of acetylation on protein targets are often site-specific, and it is not yet clear if site-selective acetylation can be achieved by using heterobifunctional molecule systems. In our studies, we note consistent acetylation of observable sites on p53 using both C- and N-terminal FKBP12<sup>F36V</sup> fusion constructs, suggesting that selectivity is largely driven by the substrate recognition of the recruited acetylation machinery. In this regard, future studies will focus on exploration of AceTAG selectivity on additional targets as well as its potential tunability for siteselective acetylation. Furthermore, AceTAG should be fully compatible with CRISPR-knockin technologies, which would benefit various investigations of acetylation in cells. We also note that although a strong “hook effect” is observed during our *in vitro* experiments (Figure 1c), we observe a somewhat blunted effect in cells while monitoring induced acetylation of H3.3, p65, and p53 (Figures 2a and 3a,c). Given that these targets are endogenous substrates of p300/CBP, we hypothesize there might be naturally occurring recognition that imparts positive cooperativity, effectively delaying autoinhibition, as observed in other heterobifunctional systems.<sup>32,59</sup> In addition, as we show in our washout studies (Figure 2d), there is likely competing HDAC activity of chemically induced acetylation, which could complicate equilibrium interpretation. Lastly, the utility of our AceTAG system is limited by the availability and reliability of methods to monitor acetylation. Currently, the majority of proteins with evidence of acetylation lack site-specific antibodies while acetylation detection by MS-based proteomics typically require pan anti-acetyl lysine antibodies for enrichment and detection, which themselves have limited coverage.<sup>60</sup> However, as we demonstrate in the case of H3.3, the use of the FKBP12<sup>F36V</sup>-HA fusion constructs enables the direct enrichment and monitoring of target proteins by quantitative MS, partially alleviating the dependence on antibodies to explore new target areas.

Finally, we believe that AceTAG-based methods can be applied to investigate functional consequences of acetylation in cells. Toward this end, we have shown that targeted

acetylation occurs at functional residues on proteins such as H3.3, p65/RelA, and p53, providing evidence that AceTAG is an attractive tool to study the various roles of acetylation in cells. Looking forward, we envision similar strategies can be extended to target endogenous proteins for acetylation, bypassing the requirement of genetic manipulation altogether. Given the manifold roles acetylation can have on protein function, we suspect that the identification of functionally “silent” ligands or binders<sup>61</sup> will prove necessary for the generation of such acetylation-targeting chimeric small molecules. In this regard, powerful ligand discovery technologies, including chemoproteomic-based methods<sup>62,63</sup> and DNA-encoded libraries (DELs),<sup>64</sup> should prove fruitful. In conclusion, we envision AceTAG will not only serve as useful tool to study basic biology but also could enable the exploration of chemically induced acetylation as a therapeutic strategy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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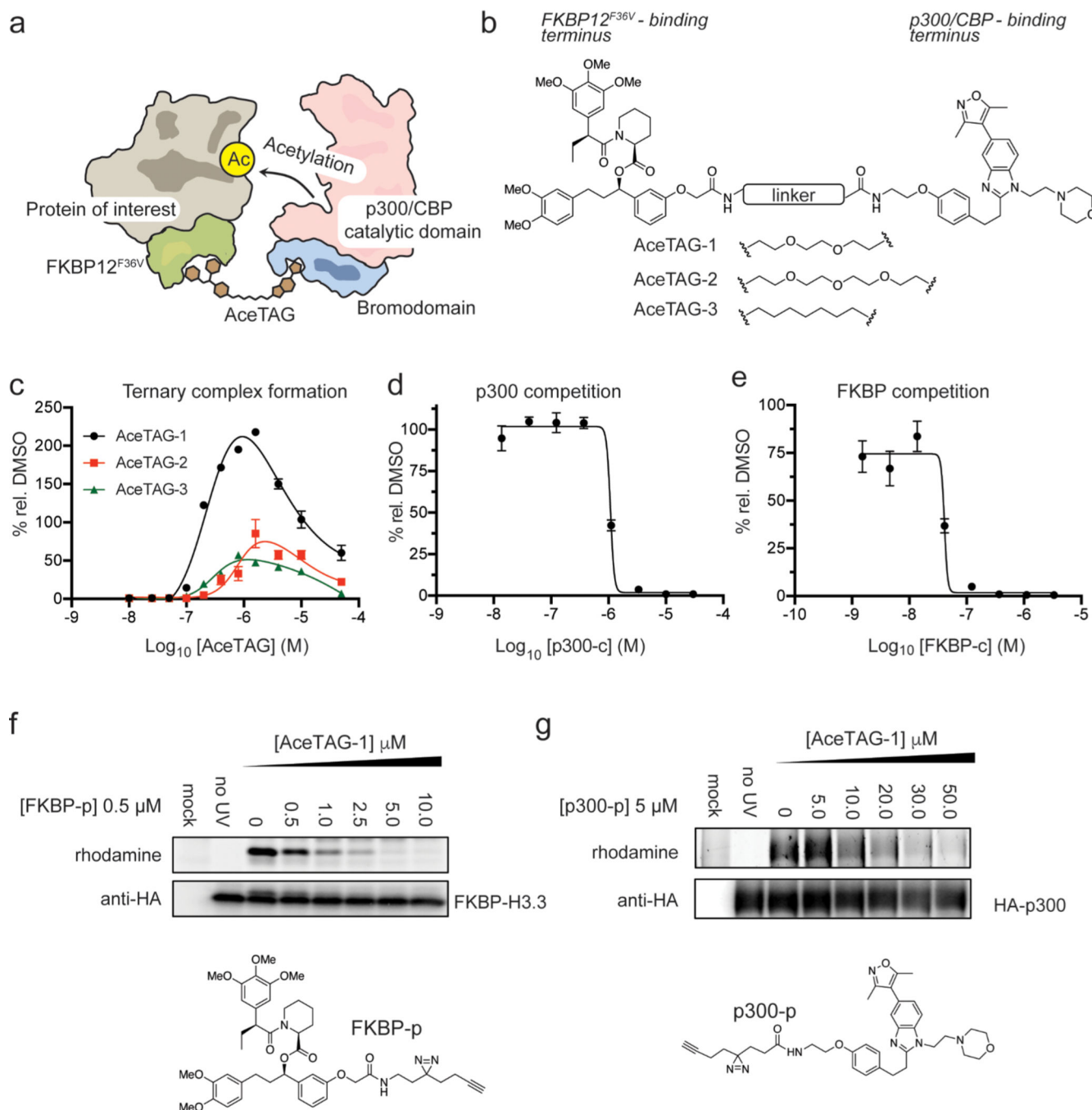
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**Figure 1.**

AceTAG molecules bind p300/CBP and FKBP12<sup>F36V</sup> and can mediate ternary complex formation. (a) Schematic overview of targeted acetylation tagging (AceTAG) strategy. Heterobifunctional AceTAG molecules induce acetylation of FKBP12<sup>F36V</sup>-POI fusions through recruitment of KAT p300/CBP. (b) Structures of AceTAG molecules composed of an FKBP12<sup>F36V</sup> binding ligand and a p300/CBP-binding ligand connected by a linker. (c) AceTAG molecules induce ternary complex between FKBP12<sup>F36V</sup> and the bromodomain of p300 (BRD-p300) as determined by AlphaScreen (Figure S1b). (d, e) AceTAG-1

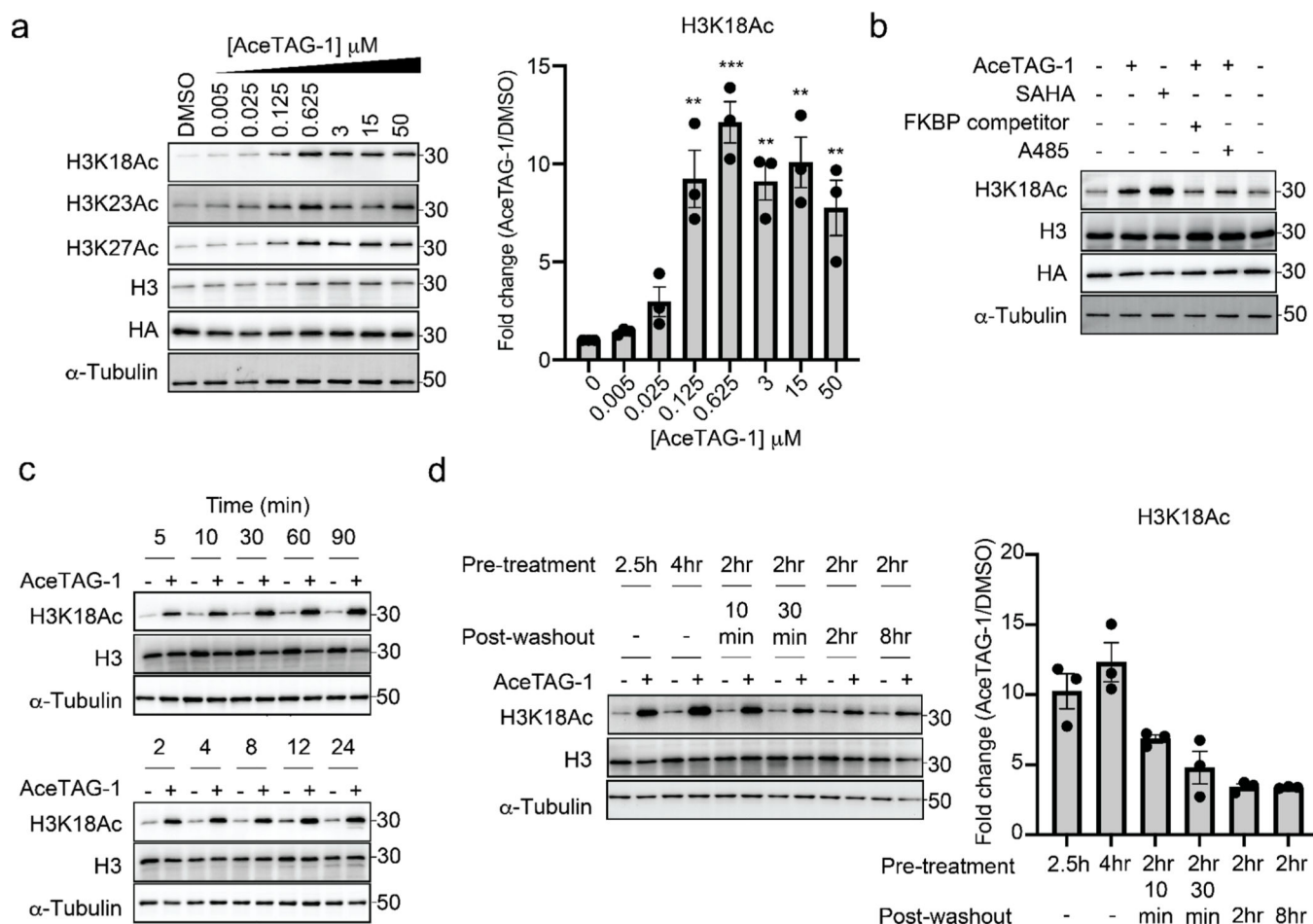
(300 nM) mediated ternary complex formation between FKBP12<sup>F36V</sup> and BRD-p300 can be blocked upon co-incubation of increasing concentrations of p300-binding (d) or FKBP12<sup>F36V</sup>-binding ligands (e, Figure S1c). Data in (c–e) represent as mean  $\pm$  s.d. of  $n = 3$  replicates. (f, g) Confirmation of AceTAG-1 target engagement in cells. FKBP12<sup>F36V</sup>-H3.3 (f) and full length p300 (g) were recombinantly expressed with HA epitope tags by transient transfection in HEK293T cells, which were then co-treated with a photoaffinity probe derived from the FKBP12<sup>F36V</sup> ligand (f, FKBP-p) or p300/CBP ligand (g, p300-p) and increasing concentrations of AceTAG-1, photo-cross-linked, and lysed, and proteomes were conjugated to an azide–rhodamine tag by CuAAC chemistry and analyzed by in-gel fluorescence staining. The results in (f, g) are representative of three independent biological replicates ( $n = 3$ ). Full images of gels are shown in Figure S6.

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**Figure 2.**

AceTAG molecules induce rapid, targeted acetylation of H3.3-FKBP12<sup>F36V</sup> in cells. (a) Concentration-dependent acetylation of H3.3-FKBP12<sup>F36V</sup> lysine residues by AceTAG-1. H3.3-FKBP12<sup>F36V</sup> HeLa cells were treated with increasing concentrations of AceTAG-1 for 2 h, and acetylation of K18, K27, and K23 was monitored by immunoblot. Shown in the right panel is quantitation of immunoblot signal of K18Ac relative to  $\alpha$ -tubulin as the mean  $\pm$  s.e.m. of  $n = 3$  biologically independent experiments. Statistical significance was calculated with unpaired two-tailed Student's  $t$  tests comparing DMSO- to AceTAG-1-treated samples. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ . (b) Immunoblot analysis of controls for AceTAG-mediated acetylation of H3.3. Cells were treated with the histone deacetylase inhibitor SAHA (5  $\mu$ M) or co-treated with AceTAG-1 (625 nM) and DMSO, an FKBP12<sup>F36V</sup> binding ligand (50  $\mu$ M, Figure S1c), or the p300/CBP KAT domain inhibitor A-485 (1  $\mu$ M) for 2 h which block AceTAG-1-induced acetylation. The results in (a, b) are representative of two independent biological replicates ( $n = 2$ ). (c) Immunoblot analysis of AceTAG-1 (625 nM) treated H3.3-FKBP12<sup>F36V</sup> HeLa cells over the indicated time course. (d) Washout experiments showing decreasing acetylation upon removal of AceTAG-1 from cells. Immunoblot of H3.3-FKBP12<sup>F36V</sup> HeLa cells pretreated with AceTAG-1 (625 nM) or vehicle for the indicated time, washed with DPBS, and resuspended in fresh media (without AceTAG-1) for the indicated time. Shown in the right

panel is quantitation of immunoblot signal of K18Ac relative to  $\alpha$ -tubulin as the mean  $\pm$  s.e.m. of  $n = 3$  biologically independent experiments. The results in (c, d) are representative of three independent biological replicates ( $n = 3$ ). Full images of blots are shown in Figures S7 and S8.

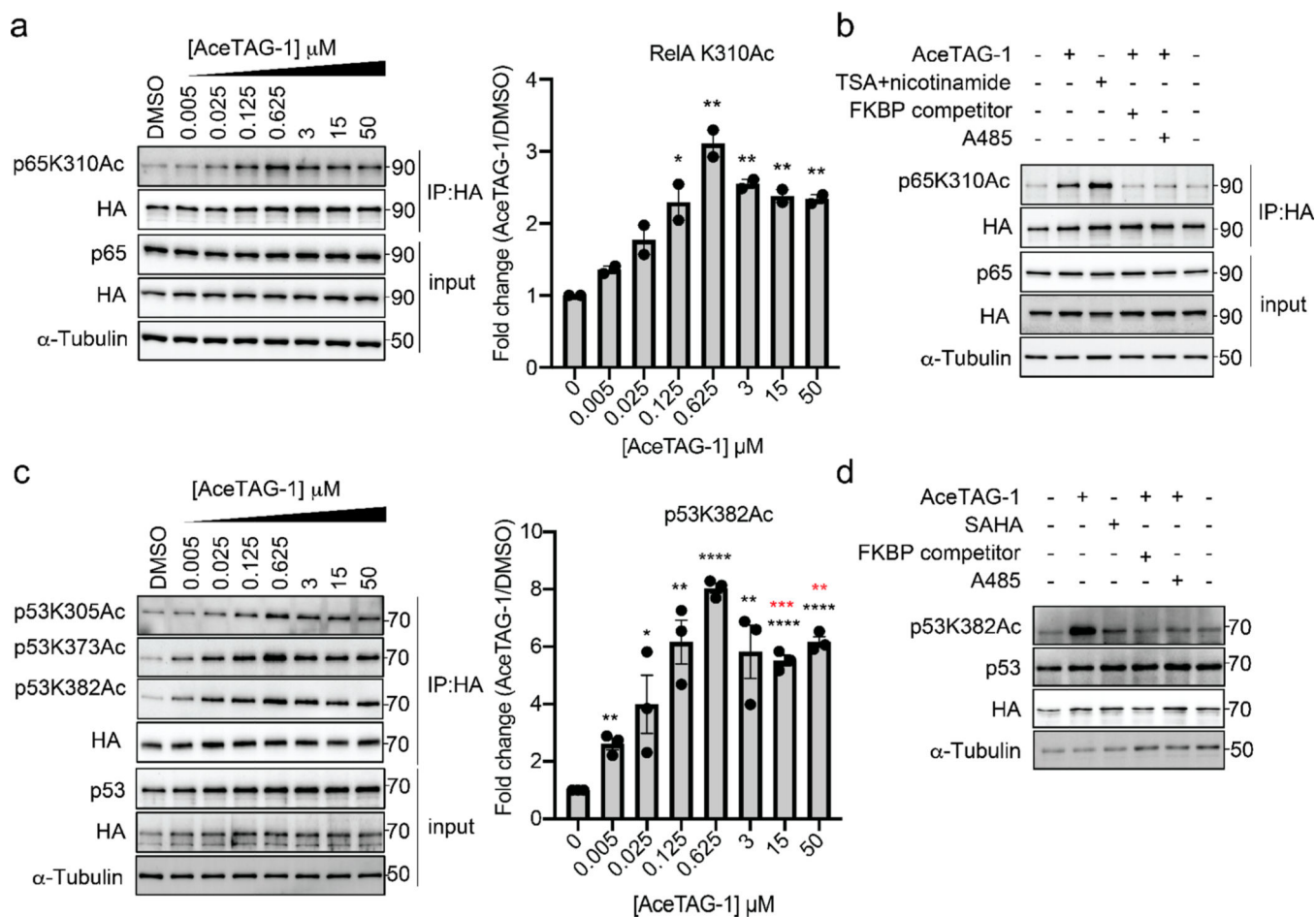
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**Figure 3.**

AceTAG targeted acetylation can be applied to multiple POIs. (a) AceTAG-1 induces acetylation of FKBP12<sup>F36V</sup>-p65/RelA fusion in HeLa cells. FKBP12<sup>F36V</sup>-RelA-HA RelA<sup>-/-</sup> HeLa cells were treated with increasing concentrations of AceTAG-1 (2 h) and lysed, and FKBP12<sup>F36V</sup>-RelA was enriched and blotted with noted antibodies. Shown in the right panel is quantitation of immunoblot signal for p65 K310Ac relative to FKBP12<sup>F36V</sup>-RelA-HA as the mean  $\pm$  s.e.m. of  $n = 2$  biologically independent experiments. (b) Immunoblot analysis of controls for AceTAG-mediated acetylation of RelA. Cells were treated with TSA (2  $\mu$ M) and nicotinamide (10 mM) as a positive control for p65 acetylation<sup>42</sup> or co-treated with AceTAG-1 (625 nM) and DMSO, an FKBP12<sup>F36V</sup> binding ligand (Figure S1c), or the p300/CBP KAT domain inhibitor A485 for 2 h which block AceTAG-1-induced acetylation. (c) AceTAG-1 induces acetylation of FKBP12<sup>F36V</sup>-p53 fusion in H1299 cells. FKBP12<sup>F36V</sup>-p53HA H1299 was treated with increasing concentrations of AceTAG-1 (2 h) and lysed, and FKBP12<sup>F36V</sup>-p53 was enriched and blotted with noted antibodies. Shown in the right panel is quantitation of immunoblot signal for p53 K382Ac relative to FKBP12<sup>F36V</sup>-p53-HA as the mean  $\pm$  s.e.m. of  $n = 3$  biologically independent experiments. (d) Immunoblot analysis of controls for AceTAG-mediated acetylation of p53. The results in (b, d) are representative of two independent biological replicates ( $n = 2$ ). Full images of blots are shown in Figures S9 and S10. Statistical significance (black asterisks) was calculated with unpaired two-tailed

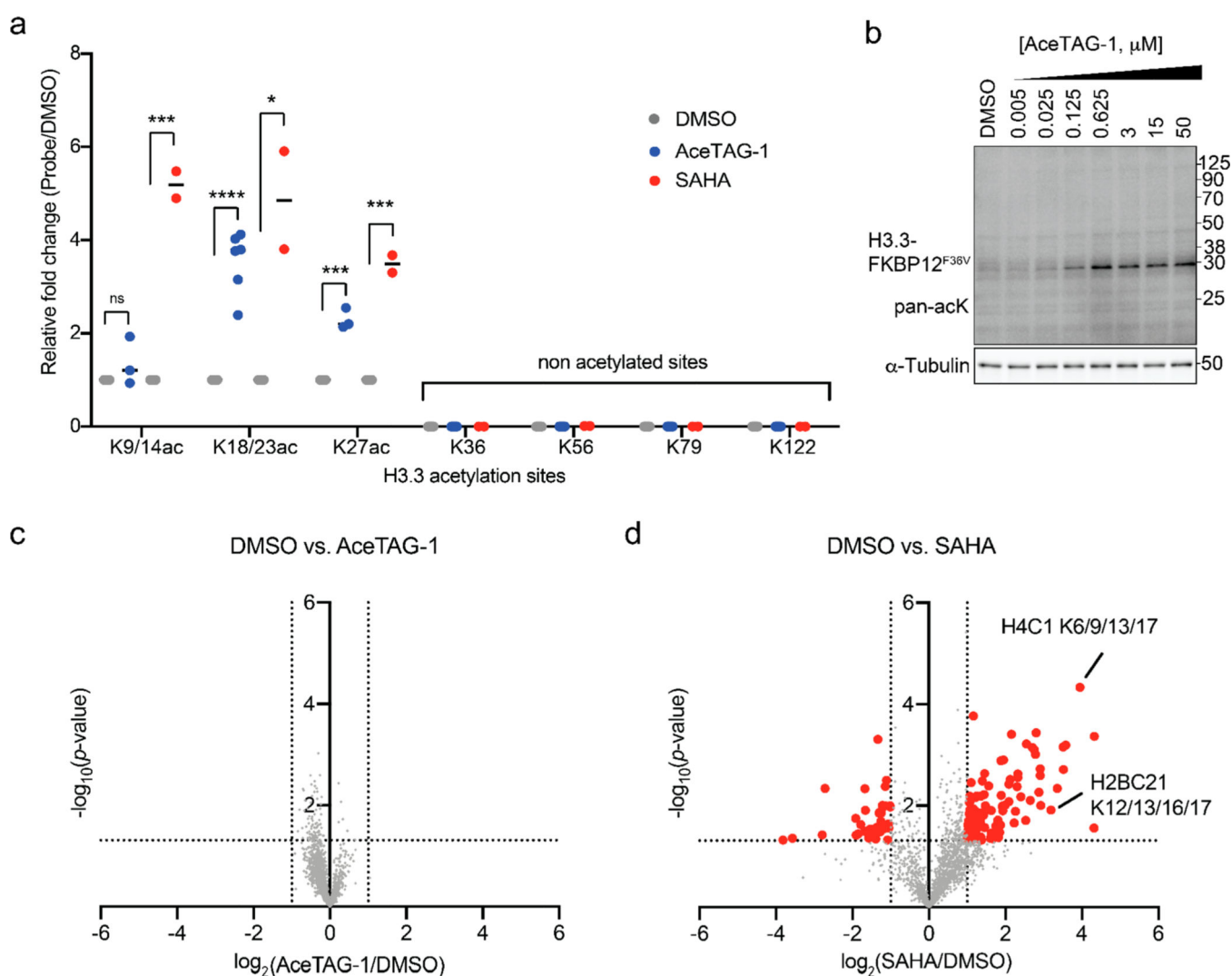
Student's *t* tests comparing DMSO- to AceTAG-1-treated samples. Statistical significance (red asterisks) of the autoinhibition effect was calculated with unpaired two-tailed Student's *t* tests comparing 625 nM AceTAG-1- and 15 or 50  $\mu$ M AceTAG-1-treated samples. \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001; \*\*\*\**p* < 0.0001.

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**Figure 4.**

Chemically induced acetylation selectivity assessment using quantitative proteomics. (a) Fold changes in H3.3 acetylated lysine abundances of H3.3-FKBP12<sup>F36V</sup> HeLa cells treated with AceTAG-1. Cells were treated with DMSO, AceTAG-1 (625 nM), or SAHA (5  $\mu\text{M}$ ) for 2 or 24 h and lysed; H3.3 was enriched, trypsinized, and labeled with isobaric TMT reagents and then subjected to quantitative MS (outlined in Figure S3a and Table S1). Data represent a median of  $n = 6$  biological replicates for AceTAG-1-treated cells and a median of  $n = 2$  biological replicates for SAHA-treated cells. Note that peptides may not have been detected in every replicate but are required to be detected and quantified in at least two biological replicates to be included in this analysis. No acetylation was detected for K36, K56, K79, and K122. Statistical significance was calculated with unpaired two-tailed Student's  $t$  tests comparing DMSO- to AceTAG-1- or SAHA-treated samples. \* $p < 0.05$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ . See Figure S4b for representative spectra. (b) AceTAG-1 does not induce broad changes in acetylation as indicated by immunoblot analysis of H3.3-FKBP12<sup>F36V</sup> HeLa cells treated with increasing concentrations of AceTAG-1 by using a pan acetyl-lysine antibody. The results are representative of three independent biological replicates ( $n = 3$ ).

Full images of blots are shown in Figure S11. (c, d) Volcano plots showing that AceTAG-1 does not induce broad changes in acetylation in the proteome as determined by quantitative MS. H3.3-FKBP12<sup>F36V</sup> HeLa cells treated with DMSO, AceTAG-1 (600 nM, c), or SAHA (2  $\mu$ M, d) for 2 or 16 h, lysed, trypsinized, and acetylated peptides enriched and labeled with TMT reagents, and then subjected to quantitative MS (Table S1). The vertical dashed lines correspond to 2-fold change in enrichment relative to DMSO, and the horizontal line corresponds to a P value of 0.05 for statistical significance. Red circles correspond to protein targets with >2-fold change ( $P < 0.05$ ) relative to DMSO. Each point represents an individual acetylated peptide plotted as a mean of  $n = 3$  biological replicates for DMSO and AceTAG-1 treatments and  $n = 2$  biological replicates for SAHA treatments combined in one TMT 10-plex experiment. An independent replicated TMT experiment is shown in Figure S5. Examples of histone-derived sites with increased acetylation upon SAHA treatment are noted in (d).