# Histone PTM Profiling Reveals Global and Specific Responses to Systematic Enzyme Ablations

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Post-translational modifications (PTM) regulate almost every aspect of protein function but their abundance is often unknown. To determine exact stoichiometries among PTM-modified isoforms we have recently developed a proteomic strategy that combines JPT's SpikeTides™_TQL synthetic peptides and a targeted liquid chromatography - mass spectrometry (LC-MS)-based method[1]. Application to histone acetylation and methylation in the fruit fly *D. melanogaster* suggests distinct abundance classes that may reflect needs for signalling (rare, combinatorial motifs) and chromatin structure (high-abundant acetylation sites). PTM profiling after systematic ablation of all known and suspected acetyltransferases and deacetylases revealed general properties of these enzyme classes and uncovered a systemic response that balances global histone acetylation levels upon individual perturbations[1].

## Introduction

Post-translational modifications (PTMs) alter the function of proteins and greatly contribute to proteome diversity. Technical progress in liquid chromatography-mass spectrometry (LC-MS) enabled the identification of over one hundred PTM types and revealed that in some proteins multiple PTMs co-occur. However, only little is known about the abundance of PTM-modified proteins relative to the corresponding unmodified form. Knowledge on the exact stoichiometries is desired to interpret biological functions and gauge significance.

Post-translational modifications on histone proteins serve as a model to elucidate PTM diversity and combinatorial cross-talk ('histone code hypothesis'[2]). Histones are among the most abundant cellular proteins and assemble into nucleosomes to organize nuclear DNA and regulate genome function. Although over 200 histone modifications have been described[3], their exact cellular abundance remains elusive. This is because the LC-MS workflow generates peptide and PTM-specific signal distortions that – if not corrected for – impair accurate quantification.

Recently, SpikeTides™ have been described as low cost peptide standards for development of MRM assays as well as for absolute and relative quantification of protein expression levels.[4] For absolute protein quantification the peptides are quantified (SpikeTides™_TQL) by application of a tagging strategy allowing accurate determination of synthetic peptide quantity[4] (**Figure 1**). Due to their ready availability as absolutely quantified reference peptides, SpikeTides™_TQL have been successfully used in a number of recent studies[5].

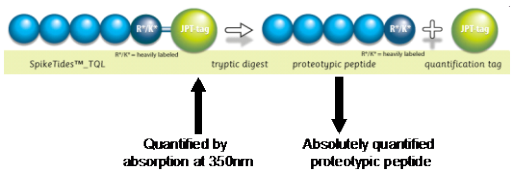


**Figure 1:** Quantification concept for SpikeTides™_TQL.

Others[6] and we[1] recently characterized a representative library of modified histone peptides (**SpikeTides™ Set Histone H3 – heavy – qunatified**) that was used to estimate the cellular abundance of over 40 acetylation and methylation isoforms to histones H3 and H4. Developing an optimized LC-MS workflow not only allowed us to systematically characterize the enzymes that set and erase histone modifications but also revealed unexpected insights into the cellular response upon perturbations[1].

## Results

SpikeTides™_TQL are quantified using a unique quantification tag (Figure 1). The tag is proteolytically labile and has UV-absorption properties that differ from those of the peptide, allowing quantification via HPLC in comparison to a standard. Unlike highly purified peptide standards quantified by AAA, JPT's TQL series contains a C-terminal quantification tag (Q-tag) that can be released by tryptic digestion in equimolar concentrations to the target peptide (Figure 1).

In the present study[1], we used the same tag to establish an alternative mass spectrometry-based quantification of modified histone peptides. Histone proteins were derivatized with d6-acetic anhydride to convert unmodified and mono-methylated lysines to d3-acetylated lysines (D3AA method[7]). The same procedure was applied in parallel to a library of SpikeTides™_TQL representing histone PTMs. In the subsequent trypsinisation step, cleavage occurs only after arginines to yield peptides with favorable LC-MS properties that are not accessible by regular tryptic digestion procedures. Importantly, LC-MS-based quantification of the target peptide released from the SpikeTides™_TQL allows correcting for different LC-MS response factors. Further, quantification of the released Q-tag enables normalization for deviations in the quantities of the SpikeTides™_TQL during the numerous steps of peptide delivery, resuspension and processing (Figure 2, reproduced with permission from[1], for a detailed discussion please refer to supplementary notes in[1]).

Using the described approach we characterized the LC-MS response factors for a library of histone H3 acetylation and methylation states. This analysis revealed strongly varying response factors with up to an order of magnitude differences (Figure 2). For example, a peptide with three methyl groups at lysine 9 of histone H3 (H3.K9me3) has a 34-fold lower LC-MS response factor than the mono-methylated isoform of the same peptide (H3.K9me1).

Accounting for the varying LC-MS response factors enabled us to estimate the cellular abundance of single and combinatorial histone modification motifs in *Drosophila* cells. For example, the HP1-type heterochromatin mark H3.K9me3 populates over 35% of

# SpikeTides™ Sets – heavy - quantified

histone H3 molecules, reflecting and quantifying the wide-spread occurrence of constitutive heterochromatin in flies.

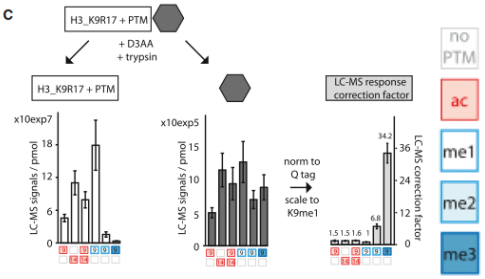


**Figure 2:** Correction for varying LC-MS response factors improves quantitative accuracy for motifs containing lysine methylation motifs (reproduced from[1] with permission from Elsevier ).

Our quantitative analysis allowed us to recognize three abundance classes for histone acetylation motifs. Most combinatorial acetylation motifs are of very low abundance (< 1%). For example, histone H4 with three acetylation sites on lysines K5, K8 and K16 (H4.K5acK8acK16ac) is present in only 0.13% of all H4 molecules, which corresponds to an estimated 8000 copies per diploid female cell. We speculate that this low cellular concentration enables specificity in epigenetic signaling pathways. In contrast, most histone motifs that contain a single acetylation site fall into a class of intermediate abundance (1% - 12%). Interestingly, acetylated lysine 23 on histone H3 (H3.K23ac) is by far the most abundant acetylation motif. It is present on 47% of all histone H3 molecules and may therefore almost saturate the genome if set in a hemi-modified manner with only one of the two nucleosomal histone H3 molecules bearing this mark. We speculate that high abundant acetylation motifs are used to configure global structural features of chromatin and thereby contribute to the stability of nuclei.

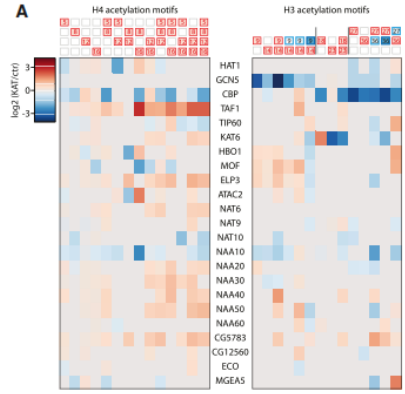


**Figure 3:** Changes in histone acetylation motifs after KAT RNAi depletion (reproduced from[1] with permission from Elsevier).

We next profiled the changes in histone acetylation and methylation marks after depleting all known and suspected lysine acetyltransferases (KATs) and deacetylases (KDACs) expressed in *Drosophila* KC cells (Figure 3). This allowed us to not only discover new and re-evaluate controversial enzyme-substrate relationships but also to recognize general properties for those enzyme classes. For example, we uncovered many examples where adjacent modifications modulate the target specificity of KATs whereas most KDACs seem to have relatively broad substrate specificity.
Our finding that most KATs show a narrow but not absolute substrate specificity contrasts common perceptions of rather promiscuous KAT activities and was made possible by the highly selective and quantitative LC-MS-based profiling strategy.
Most surprisingly, we observed not only reductions of putative bona fide primary KAT targets after individual enzyme depletions but also widespread increases in secondary sites. Summing up all gains and losses revealed that cells have balanced or even increased histone acetylation levels after individual KAT depletions. This finding was only possible because of the completeness of the data matrix (most acetylation motifs x most acetyltransferases) and the correction for different LC-MS response factors. We speculate that KAT ablation triggers a homeostatic response to maintain global charge neutralization of chromatin and the stability of nuclei[1].

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Christian Feller is a PostDoc at the Institute of Molecular Systems Biology of ETH in Zurich. After studying biology at the universities of Jena (Germany) and UNC Chapel Hill (USA) Christian performed his PhD studies with Peter Becker (LMU Munich) studying chromatin with a focus on histone acetylation and nuclear architecture. In 2015, Christian joined the group of Ruedi Aebersold at ETH Zurich to develop and apply methods to study changes in the composition of chromatin.

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