

INNOVATIVE MASS SPECTROMETRY TECHNOLOGY FOR THE STUDY OF CHROMATIN BIOLOGY

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This lecture will focus on posttranslational modification of histones and the mass spectrometry technologies developed to study them. By using electron transfer dissociation (ETD)-tandem mass spectrometry it is now possible to analyze intact proteins on a chromatographic times scale (1 protein/2-5 sec). Proteins are converted to gas-phase, positive ions by electrospray ionization and then allowed to react with fluoranthene radical anions. Electron transfer to the multiply charged protein promotes random fragmentation of amide bonds along the protein backbone. Multiply charged fragment ions are then de-protonated in a second ion/ion reaction with the carboxylate anion of benzoic acid. The m/z values for the resulting singly, doubly, and triply charged ions are used to read a sequence of 15-60 amino acids at both the N and C termini of the protein. This information, along with the measured mass of the intact protein, is used to identify unknown proteins, to confirm the amino acid sequence of a known protein, to detect post-translational modifications, and to determine the presence of possible splice variants. Applications of this technology to the study of histone posttranslational modifications associated with transcription, stem cell differentiation, and reprogramming of DNA in the egg will be discussed.

A proteomic screen to decipher the histone code interactome by SILAC-based proteomics

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Introduction

Post-translational modifications of core histones play a major role in regulating fundamental nuclear processes. Whereas a substantial amount of effort is invested to map and identify histone modifications using mass spectrometry, the functional consequences of these modifications are often still poorly understood. We have developed a peptide pull-down approach that allows for a rapid screening of histone modifications for potential interactors. We describe this procedure in detail and present biologically significant results.

Methods

We have developed a peptide pull-down approach making use of the SILAC technology, based on our previous phosphopeptide interaction screen (Schulze and Mann, 2004; Mann, 2006). Nuclear extracts derived from HeLaS3 cells grown in “light” and “heavy” medium are incubated with immobilized biotinylated histone peptides. Following in gel trypsin digestion and peptide extraction, the peptide mixtures are then analyzed on an Orbitrap mass spectrometer. Background proteins, binding equally well to the modified and non-modified peptide, show up in the mass spectrum with equal intensity between the “light” and “heavy” form, whereas proteins specifically interacting with the modification are present with a higher intensity in the “heavy” form.

Results

We have initially focussed on methylated lysines on histone H3. Eluates from control and methylated histone peptides showed no visible differences on 1D gels indicating that specific interactors are “buried” by a large number of background proteins. However, SILAC ratios different from 1:1 were found for a number of proteins in each pull-down. For methylated histone H3 lysine four (H3K4me3), we mainly identified PHD finger proteins as interactors, including the recently published BPTF and ING2 proteins. Furthermore, we found that the basal transcription factor TFIID binds to H3K4me3 via a PHD finger in its subunit TAF3 (Vermeulen et al., 2007). For H3K9me3 we identified the three HP1 isoforms, HP1 α , - β and - γ as well as two novel chromodomain proteins, CDYL1 and CDYL2 as specific interactors.

To investigate crosstalk between histone modifications on the same tail we established a triple pull-down approach making use of different heavy versions of lysine and arginine. Using this

approach we have been able to show agonistic binding effects between H3K4me3 and acetylated K9 and K14 of histone H3 as well as antagonistic effects between H3K9me3 and phospho serine 10 of histone H3.

Innovative aspects

- Applying SILAC to histone peptide pull-downs
- Novel interactors for all major lysine methylation sites on histone H3
- Novel insights in a combinatorial “histone code”

References

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- (2) Schulze W and Mann M. J Biol Chem. 2004 Mar 12;279(11):10756-64.
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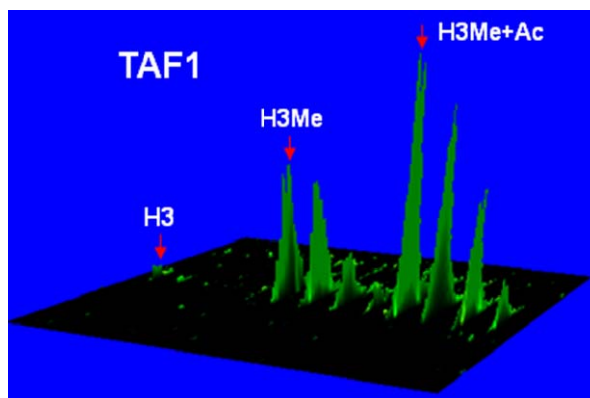


Figure legend. Shown is a TAF1 peptide (TFIID subunit) in a so called triple pull-down where the agonistic binding effects between H3K4me3 and H3K9 and H3K14 acetylation for TFIID binding can be seen (Vermeulen et al., 2007).

The maturation of histone modifications after chromatin assembly

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Abstract Every cell has to duplicate its entire genome during S-phase of the cell cycle. After replication the newly synthesized DNA is rapidly assembled into chromatin that is made up of parental and new histone molecules. After histone deposition, the newly assembled chromatin “matures” and adopts a variety of conformations. This differential packaging of DNA plays an important role for the maintenance of gene expression patterns and has to be faithfully copied in each cell division. Posttranslational histone modifications are prime candidates for the regulation of chromatin structure and it is therefore crucial to understand the replication of histone modification patterns.

In collaboration with the lab of G. Almouzni, we could demonstrate that histones carry a specific modification pattern before assembly, which is dependent on the H3 isoform [1]. In HeLa tissue culture cells we observe only very little lysine methylation on H3 or H4 before assembly but a much larger number of modifications on several lysines on chromatin associated histones. These findings prompted us to investigate the kinetics as well as the mechanisms of the maturation of histone modification patterns during assembly. We have initially focussed our investigations on the study of histone modifications during chromatin assembly *in vitro* using a well characterised assembly extract from *Drosophila* embryos that resembles most of the features of chromosome assembly *in vivo*. Using this assembly system we could show that H4 molecules are deposited in a diacetylated form, which gets progressively deacetylated during chromatin maturation. This deacetylation is facilitated by a monomethylation of K20 within the H4 tail, which occurs immediately after histone deposition and is almost complete after 3h of chromatin maturation. In contrast to H4, which is deacetylated and monomethylated during assembly *in vitro*, H3 is dynamically acetylated and deacetylated implying a role of H3 modifications in more dynamic processes like transcription or DNA repair.

In order to study the kinetics of histone modifications *in vivo* we use stable isotope labelling with amino acids in cell culture (SILAC) [2,3]. In order to establish the method in the lab, we have pulse labelled synchronised cells with an isotopically labelled arginine ($^{13}\text{C}_6$) that is 6 Da heavier than the naturally occurring $^{12}\text{C}_6$ -isoform. As the synthesis of histones is highly replication dependent, cells were arrested at the G1/S boundary using a double thymidine block, released into S-phase and simultaneously labelled with heavy arginine. A mass spectrometric analysis of the isolated histones showed a clear signal for the

labelled histone at 2hrs post-S-Phase entry. Allowing us a detailed analysis of the kinetics of various modifications in a quantitative manner. Experiments using various pulse/chase times show that particular modifications have considerably different kinetics until they have acquired a modification pattern that is indistinguishable from the parental histones.

In summary we observe a:

- Ordered addition and removal of particular histone modifications during assembly
- High degree of interdependency between various modifications arguing against the existence of a static histone code
- Major variation in the kinetics of histone modification establishment among different sites of modifications.

References

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Screen for differential transcription factor binding at Single Nucleotide Polymorphisms

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Introduction

Single nucleotide polymorphisms (SNPs) represent the smallest possible form of genetic variation occurring between individuals. They can nevertheless have a profound impact on phenotype. In some cases, SNPs in non-coding regions of a gene can alter the binding affinity for a sequence-specific transcription factor thereby influencing target gene expression. Novel sequencing technologies allow to map disease phenotype to SNPs in complete populations but systematic mapping of differential binding of putative transcription factors on large scale has not been performed to date.

Methods

We employed quantitative mass spectrometry to detect the differential binding of protein factors between a wild-type and a single nucleotide mutant sequence. Cells were labelled by stable isotope labelling of amino acids in cell culture (SILAC). Nuclear extracts of heavy and light cell populations were used in a DNA pull-down experiment with the biotinylated DNA oligonucleotide as a bait. A TECAN workstation has been adapted to allow a streamlined automated workflow for the pull-down experiments in a medium to high throughput manner. The tryptic peptides of the bound proteins were measured on a high accuracy LC-MS/MS system (LTQ-Orbitrap) and data was processed with the in-house developed software MaxQuant.

Results

Using a point mutated SELEX binding site for AP2 as a control against the wild-type AP2 SELEX sequence around 1000 proteins could be identified in the elution fraction. Nevertheless, only the tryptic peptides for AP2 family members resulted in a significant ratio different from 1:1. We identified peptides belonging to all five AP2 family members. Further we tested a known SNP (rs509813) situated in the human promoter region of the muscarinic acetylcholine receptor M1 gene (CHRM1). Whereas the C allele was predicted to be a binding site for SP1, the G allele abrupts the SP1 consensus sequence. Within the 1000 proteins identified and quantified, solely tryptic peptides of SP1, SP2, SP3 and SP4 as well as ZPF148 were quantified with a significant ratio distinct from the background. In compliance with

literature, binding of ZPF148 to SP1 sequences has already been reported.

We established proof of principle that quantitative mass spectrometry can be used to detect differential transcription factor binding to SNPs and are currently setting up a medium scale analysis to study DNA-protein interactions.

Innovative aspect

- Employing quantitative MS for DNA-protein interactions in a streamlined approach

References

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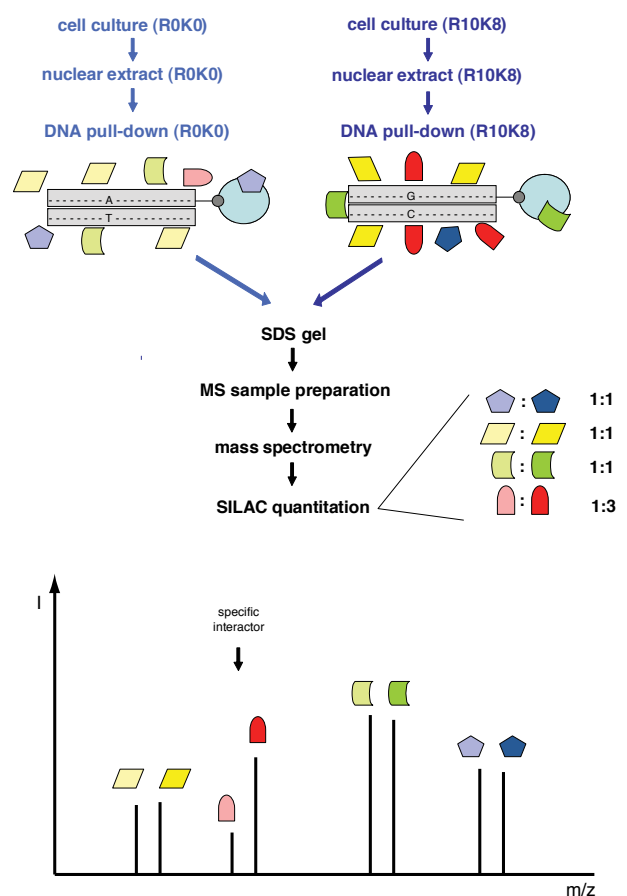


Figure 1: Experimental work-flow to study differential transcription factor binding to SNPs