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Novel genetic tools for probing individual H3 molecules in each nucleosome

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Abstract

In eukaryotes, genomic DNA is packaged into the nucleus together with histone proteins, forming chromatin. The fundamental repeating unit of chromatin is the nucleosome, a naturally symmetric structure that wraps DNA and is the substrate for numerous regulatory post-translational modifications. However, the biological significance of nucleosomal symmetry until recently had been unexplored. To investigate this issue, we developed an obligate pair of histone H3 heterodimers, a novel genetic tool that allowed us to modulate modification sites on individual H3 molecules within nucleosomes *in vivo*. We used these constructs for molecular genetic studies, for example demonstrating that H3K36 methylation on a single H3 molecule per nucleosome *in vivo* is sufficient to restrain cryptic transcription. We also used asymmetric nucleosomes for mass spectrometric analysis of dependency relationships among histone modifications. Furthermore, we extended this system to the centromeric H3 isoform (Cse4/CENP-A), gaining insights into centromeric nucleosomal symmetry and structure. In this review, we summarize our findings and discuss the utility of this novel approach.

Keywords

Nucleosome; Nucleosomal symmetry; Cryptic transcription; Histone modification crosstalk; Centromere; Hemisome

Introduction

The basic repeating unit of chromatin is the nucleosome, consisting of 147 bp of DNA wrapped around an octamer of core histone proteins (Luger *et al.* 1997). Core histones assemble in pairs, and each nucleosome contains two H2A-H2B dimers flanking an inner (H3-H4)₂ tetramer. Nucleosomes are intrinsically symmetrical, with each pair of core histone molecules located in a mirror image configuration relative to the dyad axis. This axis of symmetry is centered on the homodimeric interaction between the C-termini of the H3 molecules (Figure 1-left).

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In addition to their role in compaction of genomic DNA, core histones affect all aspects of chromosome function via various post-translational modifications. Although the backbones of the core histones are the same on each side of the dyad axis, until recently it was not known to what extent this symmetry might extend to post-translational modifications. Specifically, for each modifiable histone residue, there is the potential for three distinct stoichiometries of modification per nucleosome (0, 1 or 2). Thus, the presence of distinct modification patterns at each residue on the two halves of each nucleosome could provide an additional, complex layer of epigenetic information in chromatin, supplementing combinatorial modification of different histone residues. It became clear that this is more than a theoretical possibility when Reinberg and co-workers revealed the existence of nucleosomes with asymmetric histone H3K27me_{2/3} and H4K20me₁ modifications in mammalian cells (Voigt *et al.* 2012). They also detected ES cell nucleosomes carrying H3K4me₃/H3K36me₃ on opposite H3 tails from those marked with H3K27me₃. More recently, single molecule analyses have also shown that the majority of H3K4me₃/K27me₃-marked “bivalent” nucleosomes carry these modifications in an asymmetric configuration (Shema *et al.* 2016). Additionally, biochemical analyses with *in vitro*-reconstituted asymmetric nucleosomes have shown that the SAGA histone acetyltransferase complex binds cooperatively to histone marks, and has enhanced activity once one H3 tail is already acetylated (Li and Shogren-Knaak, 2008; Li and Shogren-Knaak, 2009). Furthermore, the PRC2 histone methyltransferase complex can be selectively inhibited depending on the symmetry of histone modifications (Voigt *et al.* 2012). Therefore, modifying enzymes can detect and respond to asymmetrically-modified nucleosomes, suggesting that asymmetric histone modifications could provide regulatory information in the chromatin of living cells.

However, because the inherent symmetry of the natural H3-H3 interface had made it impossible to manipulate nucleosomal symmetry *in vivo*, there had not been methods to test the biological significance of asymmetric modification states. To overcome this fundamental issue, we designed an obligate pair of H3 heterodimers which cannot homodimerize (Figure 1-right). These constructs allowed us to manipulate individual H3 molecules in each nucleosome *in vivo*. With this novel tool, we first reproduced asymmetrically modified nucleosome in yeast cells, and our molecular genetic studies revealed that H3K36 methylation on a single H3 molecule per nucleosome is sufficient to suppress cryptic transcription. Next, we used asymmetric nucleosomes to examine dependency relationships among histone modifications. Our mass spectrometric analysis clarified that crosstalk between H3S10 phosphorylation and H3K9 acetylation happens within the same tail. Furthermore, we extensionally applied this system to the centromere specific H3 variant (Cse4/CENP-A), gaining insights into centromeric nucleosomal symmetry and its significance. Our genetic and functional analysis demonstrated that an octomeric Cse4 nucleosome is essential for yeast viability, and a single binding site of important kinetochore proteins per centromeric nucleosome is sufficient for viability and efficient maintenance of chromosome segregation.

Breaking nucleosomal symmetry

In order to manipulate nucleosomal symmetry, we used protein design to generate “bumps and holes”-based heterodimeric interfaces, with energy minimization calculations to identify

candidate replacements at key hydrophobic residues at the H3 C-terminus (Ichikawa *et al.* 2017). These designs were subsequently optimized by *in vivo* selection, resulting in heterodimeric pairs that would not support growth in budding yeast unless both halves were present. After this genetic validation of our heterodimeric pair, termed H3X and H3Y, we validated our system biochemically. First, via intranucleosomal crosslinking and immunoprecipitation, we observed efficient coprecipitation of H3X with H3Y, but not homodimeric H3X-H3X or H3Y-H3Y pairs. We also demonstrated that recombinant human H3X and H3Y molecules preferentially form heterodimers during biochemical renaturation experiments. We had therefore established a novel tool for manipulation of nucleosomal symmetry *in vivo* and *in vitro*.

A caveat to these studies is that yeast strains expressing H3X and H3Y are viable, but grow slower than the parental strain in rich media (yeast extract/peptone/dextrose, YPD) at 30°C. In addition, they are temperature-sensitive, growing extremely poorly at 37°C. We note that another study identified an alternative heterodimeric histone H3 interface based on electrostatic interactions (Zhou *et al.* 2017). That interface also causes temperature-sensitive growth (Ichikawa *et al.* 2018), and provided similar conclusions about the roles of H3K36 stoichiometry (Zhou *et al.* 2017, see below). These data suggest that biological effects of asymmetric nucleosomes are generally independent of the specific residues used to create the asymmetry at the H3 C-terminus. It is not known whether the growth phenotypes caused by the asymmetric interfaces result from reduced kinetics of nucleosome assembly, reduced stability of nucleosomes, or other unintended interactions of the altered H3 proteins. In any case, all experimental observations were made by comparing experimental strains to a “pseudo wild-type” strain that expressed H3X + H3Y, to remove from consideration any changes caused by the altered histone backbones themselves.

Biological effects of asymmetric H3 mutations

Because of the inherent symmetry of the H3-H3 interface, it had previously been impossible to manipulate the modification status of individual residues within nucleosomes *in vivo*. For example, co-expression of both mutated and wild-type H3s would result in three co-existing configurations (Figure 2A). Our novel H3X + H3Y heterodimer made it possible to mutate a single H3 residue in each nucleosome, and we therefore generated nucleosomes harboring asymmetric mutations in H3 residues that undergo posttranslational modification (Figure 2B).

First, we focused on H3K36 methylation, which is catalyzed by the Set2 methyltransferase enzyme (Strahl *et al.* 2002). Set2 is recruited to active RNA polymerase II (pol II) molecules, and cotranscriptionally modifies H3 molecules along open reading frames (Li *et al.* 2002; Li *et al.* 2003; Krogan *et al.* 2003; Schaft *et al.* 2003; Xiao *et al.* 2003; Sims *et al.* 2004). The methylation of H3K36 in turn recruits the Rpd3S histone deacetylase complex, which removes histone acetylation marks that are incorporated into chromatin via histone exchange events that are also triggered by pol II movement (Smolle *et al.* 2013; Venkatesh and Workman 2015). Histone deacetylation by Rpd3S is important because it is required for silencing of cryptic internal promoters within genes. That is, in the absence of Set2/H3K36me3/Rpd3-dependent resetting of the acetylation status over genes, novel transcripts

appear (Carrozza *et al.* 2005; Pattenden *et al.* 2010). Therefore, we tested how this regulatory system would respond to changing the stoichiometry of H3K36 methylation within each nucleosome *in vivo*. To do this, we analyzed cryptic transcripts in single and double K36Q mutant strains (K36Q-X + Y, X + K36Q-Y and K36Q-X + K36Q-Y); this revealed that a single H3K36 methylation per nucleosome is sufficient to silence cryptic promoters. Additionally, we demonstrated that even dimethylation of a single H3K36 per nucleosome is sufficient. This was accomplished via use of the P38V mutation, which prevents proline isomerization in this region of the H3 N-terminus, and blocks trimethylation but not dimethylation of the nearby K36 residue (Youdell *et al.* 2008). Our system allowed us to construct nucleosomes with K36Q and P38V mutations in “trans” (that is, on opposite H3 molecules). These K36Q/P38V trans strains displayed no increase in cryptic transcription or stress sensitivity. Taken together, these data indicate that Rpd3S can repress cryptic transcription even if nucleosomes have only a single H3K36 methylation per nucleosome. Furthermore, the use of asymmetric nucleosomes allowed us to determine that either dimethylation or trimethylation on K36 is sufficient for this function.

In addition to repression of cryptic transcription, H3K36 methylation is also involved in checkpoint activation and double strand break repair (Jha and Strahl, 2014, Pai *et al.* 2017). Therefore, we used the asymmetric K36Q mutants to test DNA damage sensitivity, and found that the K36Q double mutant, but neither of the single K36Q mutants, displayed hypersensitivity to DNA damaging agents (methyl methanesulfonate and phleomycin). These data suggest that methylated H3K36 recognition factors are involved in this aspect of genome stability, although in this case this effect is independent of the Rpd3S complex (Jha and Strahl, 2014). As we observed for cryptic transcription, this mechanism also functions if nucleosomes have only a single H3K36 methylation per nucleosome. We hypothesize that this characteristic acts as a buffer system to ensure genome stability during transient reductions in H3K36 methylation density during DNA repair, histone turnover or replication (Figure 3A).

In addition to molecular genetic experiments, we showed that asymmetric nucleosomes are useful for biochemical investigations. For example, we applied this system to investigate crosstalk among histone modifications, in which one histone modification affects the level of another modification. Specifically, we tested whether the effects of H3S10 phosphorylation on Gcn5-dependent H3 acetylation (Lo *et al.* 2000; Liokatis *et al.* 2016) occur in *cis* (on the same H3 molecule) or *trans* (on the opposite H3 tail in the nucleosome). To do this, we constructed single and double H3S10A mutants (S10A-X + Y, X + S10A-Y and S10A-X + S10A-Y). In order to distinguish H3X from H3Y, we also added to the H3X N-terminus the 15 amino acid AviTag, which can be biotinylated by heterologously expressed BirA (*E. coli* biotin ligase) (Beckett *et al.* 1999). Thus, in the presence of conditions that dissociate nucleosomes (high salt and urea), streptavidin-agarose affinity chromatography captured only the H3X and not the H3Y molecules. Mass spectrometric and immunoblotting analyses of these samples showed that H3K9 acetylation levels were significantly decreased on the same histone tail carrying the S10A mutation (*cis*), but were not affected by the presence of S10A on the other tail (*trans*). Therefore, crosstalk between H3S10 phosphorylation and H3K9 acetylation happens within the same tail in yeast cells (Figure 3B). Our asymmetric system could be used for analysis of the many other modified residues within H3.

Asymmetric centromeric nucleosomes

Eukaryotic centromeres contain specialized nucleosomes in which the canonical H3 molecules are replaced by histone H3 variant CENP-A (termed Cse4 in budding yeast) (Palmer *et al.* 1987; Stoler *et al.* 1995; Meluh *et al.* 1998; Buchwitz *et al.* 1999; Henikoff *et al.* 2000; Takahashi *et al.* 2000; Oegema *et al.* 2001; Régnier 2005). CENP-A containing nucleosomes are required for centromere identity and function, because they are essential attachment points connecting centromeric DNA to the kinetochore proteins that are responsible for accurate segregation of chromosomes during mitosis (Biggins 2013; Foltz *et al.* 2006; Sullivan 2001).

Although it is clear that CENP-A/Cse4 is required for viability throughout eukaryotic organisms (Stoler *et al.* 1995; Howman *et al.* 2000), the oligomeric state of centromeric histones had been controversial, with evidence for two different structures presented (Dunleavy *et al.* 2013). Many experiments, including high-resolution structures obtained by crystallography (Tachiwana *et al.* 2011), mapping via chromatin immunoprecipitation (Furuyama and Biggins 2007), and molecular genetic experiments demonstrating the need for CENP-A dimerization (Zhang *et al.* 2012), supported the presence of an octomeric centromeric nucleosome (octasome). This resembles a canonical nucleosome, in which H3 is replaced by Cse4 (Figure 4A-left). In contrast, other experiments, including crosslinking, atomic force microscopy, chromatin digestion experiments and analysis of DNA supercoiling (Dalal *et al.* 2007; Furuyama and Henikoff 2009; Furuyama *et al.* 2013; Henikoff *et al.* 2014), had suggested a tetramer model (hemisome), containing one (H2A-H2B) dimer plus one (Cse4-H4) dimer (Figure 4A-right).

We realized that an asymmetric Cse4 dimerization interface could provide a test of whether both of these forms could support viability in budding yeast. We therefore integrated our asymmetric H3 interface into Cse4, resulting in the Cse4X and Cse4Y proteins. We reasoned that yeast cells expressing either Cse4X or Cse4Y should be able to assemble hemisomes but not octasomes, whereas yeast cells expressing both Cse4X and Cse4Y could in principle assemble both octasomes or hemisomes. We observed that yeast cells expressing Cse4X + Cse4Y were viable, but cells expressing either protein alone were not. These data indicate that an octomeric Cse4 nucleosome is essential for viability, although this experiment by itself cannot rule out whether hemisome forms also exist (Figure 4B). Additionally, we showed that the asymmetric Cse4 molecules could be used to test stoichiometries of essential protein interactions for centromere function. For example, mutated both the N-terminal and C-terminal tails of Cse4, which serve as binding sites for the Ctf19-Mcm21-Okp1 complex and CENP-C/Mif2, respectively (Chen *et al.* 2000; Kato *et al.* 2013; Xiao *et al.* 2017). We observed that a single binding site for each of these two sets of important kinetochore proteins per centromeric nucleosome is sufficient for viability and efficient maintenance of chromosome segregation. Thus, the asymmetric histone H3 interface we designed is a portable and versatile reagent for exploration of chromatin protein interactions.

Conclusions and perspectives

We engineered specific residues at the histone H3-H3 interface to generate the obligate pair of H3 heterodimers, termed H3X and H3Y, and this novel tool allowed us to manipulate nucleosomal symmetry in yeast cells. We had demonstrated that our asymmetric nucleosomes could be powerful tools for study of epigenetics, for example histone modification dependent transcriptional regulation, DNA damage response and histone modification cross talk. Additionally, we also showed that the asymmetric H3-H3 interface could be applied to histone H3 isoform CENP-A/Cse4, indicating that the universality of our approach.

These studies provide tools for a variety for future investigations. For example, asymmetric nucleosomes harboring mutations on modification sites could be used for exploring the binding mode of recognition enzymes/epigenetic readers. Combining such biochemical studies with gene expression profiling could provide detailed insights into transcriptional regulation via histone modifications and related factors. Additionally, structural studies of non-canonical chromatin structures including tetrasomes, or overlapping dinucleosomes (Rhee *et al.* 2014; Ramachandran *et al.* 2014; Kato *et al.* 2017) could be assisted by this system. Finally, because the H3-H3 interface is highly conserved throughout eukaryotes, we expect that our novel tool should be applicable to studies in many other organisms.

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Figure 1:

Design of an asymmetric H3-H3 interface. (left) Nucleosome structure with histone H3 in blue (PDB ID: 1KX5). The red circle indicates the histone H3-H3 interface. (right upper) Secondary structure and sequences of the C-terminal domains of wild type H3 (blue), H3X (green) and H3Y (yellow). Red residues indicate the asymmetric alterations and the substituted amino acids are shown below. (right lower) Schematic of the experimental strategy. The H3 N-terminal tail is indicated as a wavy line extending from the H3 globular histone fold domain. The H3X/H3Y pair makes an asymmetrical heterodimer, whereas H3X-H3X or H3Y-H3Y pairs cannot homodimerize, as indicated.

Figure 2:
Asymmetric mutations on H3 molecules in the nucleosome. (A) Schematic of co-expression of both mutated and wild-type H3s. Three possible configurations are indicated. (B) Manipulation of nucleosomes harboring asymmetric mutations on H3 residues. Our obligate pair of H3 heterodimers make it possible to generate various types of asymmetrically mutated nucleosomes as shown. Asterisks indicate mutated residues of H3, and each color (Black and Red) indicates different kind of mutations (e.g. combination of H3K36Q and H3P38V).

Figure 3:

In vivo analysis of the biological effects of asymmetric H3 mutations. (A) Summary table of the effects of H3K36Q substitutions (Ichikawa *et al.* 2017). Schematic of wild-type, pseudo wild type, single and double H3K36Q mutants are shown on top of the table. Red asterisk indicates H3K36Q substitution. (-) indicates basal levels of cryptic transcripts or DNA damage sensitivity. (+) indicates significantly elevated levels of cryptic transcripts or DNA damage sensitivity. (B) A hypothesis about H3K36 methylation-dependent genome maintenance. In this model, H3K36-methyl recognition factors, such as the Rpd3S complex, maintain their interaction with chromatin when H3K36 methylation remains on either one or both H3 N-termini per nucleosome. This would help maintain genome stability during transient reductions in H3K36 methylation density. Such reductions could occur during RNA or DNA polymerase movement, or as a result of remodeling enzyme-driven histone exchange. (C) Crosstalk between H3S10 phosphorylation and H3K9 acetylation. When

H3S10 is phosphorylated, it stimulates H3K9 acetylation within the same tail. Although multiple acetyltransferases can acetylate H3K9 (Berndsen *et al.* 2008; Church *et al.* 2017), Gcn5 is pictured here because it is known to interact with the phosphorylated H3S10 residue (Lo *et al.* 2000; Cheung *et al.* 2000; Rossetto *et al.* 2012). “me”, “ac” and “ph” indicate methylation, acetylation and phosphorylation, respectively.

Figure 4:
Asymmetric centromeric nucleosomes in budding yeast. (A) Models of Cse4/CENP-A nucleosomes. Octamer (octasome) model is comprised of two H2A-H2B dimers and (Cse4-H4)₂ tetramer. Tetramer (hemisome) model is comprised of one (H2A-H2B) dimer and one (Cse4-H4) dimer. (B) Summary of the genetic analysis of heterodimeric Cse4X/Cse4Y pairs. Cells expressing Cse4X + Cse4Y are viable, whereas neither Cse4X alone nor Cse4Y alone support growth of yeast cells (Ichikawa *et al.* 2018).