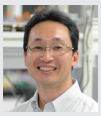
DIVISION OF CHROMATIN REGULATION



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The stable inheritance of gene expression or repression states is essential to cellular differentiation and development, and an epigenetic cellular-memory system derived from higher-order chromatin structure plays a fundamental role in this process. The assembly of higher-order chromatin structures has been linked to the covalent modifications of histone tails, chromatin-associated proteins, and non-coding RNAs. However, the exact means by which such chromatin-based epigenetic information is established and faithfully maintained across cell divisions and throughout development remains unclear. To try to gain a better understanding of the molecular mechanisms underlying chromatin-based epigenetic phenomena, our lab uses mammalian culture cells, fission yeast, *Schizosaccharomyces pombe*, and ciliate *Tetrahymena* as model systems for studying the molecular mechanisms of higher-order chromatin assembly. We are also attempting to determine the cellular functions of chromatin modifying factors so that we can develop a better understanding of complicated epigenetic phenomena in higher eukaryotic cells.

I. Establishment and maintenance of higher-order chromatin structures

In eukaryotic cells, the assembly of higher-order chromatin structures, known as heterochromatin, plays an important role in diverse chromosomal processes. We have previously shown that the specific methyl modification on lysine 9 of histone H3 (H3K9me) and the binding of the chromodomain (CD) proteins (such as HP1) to methylated histones are essential to the assembly of higher-order chromatin structures. However, the mechanism by which HP1 folds chromatin containing H3K9me into a higher order structure has not been elucidated yet. To understand the structural basis of heterochromatin, we prepared a dinucleosome containing H3K9me. The HP1α-dinucleosome complexes containing H3K9me were fixed with glutaraldehyde and subjected to a cryo-EM analysis. The HP1α-dinucleosome complexes were visualized in cryo-EM images (Figure 1A), particles were selected semi-automatically, and 2D class averages were obtained by unsupervised maximum likelihood classification (Figure 1B). This was followed by 3D reconstruction and classification. In the cryo-EM structure of the HP1α-dinucleosome complexes, the two nucleosomes do not directly interact with each other, and the HP1α dimer binds to two neighboring nucleosomes (Figure 1C). We ultimately confirmed that the treatment of micrococcal nuclease (MNase) preferentially cleaved the linker DNA, but not the nucleosomal DNA. The present structure of the HP1α-dinucleosome complexes matches best to the nucleosome-bridging model, in which two neighboring nucleosomes are connected by the HP1 dimer, and depicts the fundamental architecture of heterochromatin.

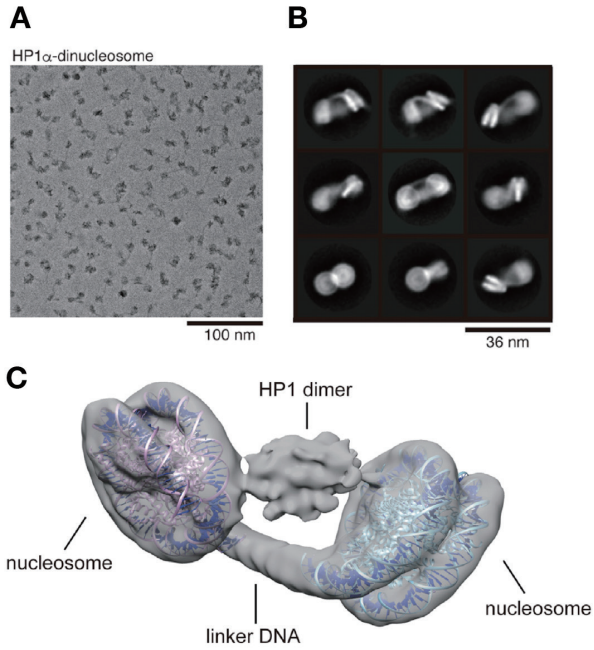

Figure 1. The structure of HP1α-dinucleosome complex. (A) Digital micrograph of HP1α-dinucleosome particles in amorphous ice; recorded in-focus with a Volta phase plate. Scale bar: 100 nm. (B) Selected 2D class average of 200 classes from 187,784 aliged single-particle images of the HP1α-dinucleosome complex. Box size: 36 nm. (C) Representation of the reconstituted three-dimensional electron potential of the HP1α-dinucleosome complex.

II. Roles of chromodomain proteins in assembling higher-order chromatin structure

2-1 Mitotic phosphorylation of HP1α regulates its cell cycle-dependent chromatin binding

HP1 is an evolutionarily conserved chromosomal protein that plays a crucial role in heterochromatin-mediated gene silencing. We previously showed that mammalian HP1α is constitutively phosphorylated at its N-terminal serine residues by casein kinase II (CK2), and that this phosphorylation enhances HP1α's binding specificity for nucleosomes containing H3K9me3. Although the presence of additional HP1α phosphorylation during mitosis was reported more than a decade ago, its biological significance remains largely elusive. To examine the roles played by HP1α's mitotic phosphorylation, we determined HP1α's mitotic phosphorylation sites and the cellular behavior of HP1α with mitotic phosphorylation. We found that S92 in the hinge region is the main mitotic phosphorylation site in human HP1α and that HP1α's S92 phosphorylation (S92ph) was regulated by

Aurora B kinase (AURKB) and two serine/threonine phosphatases. Immunoblotting analysis using cell cycle-synchronized cells demonstrated that HP1α S92ph precedes H3S10 phosphorylation; a major hallmark of mitotic chromatin. In addition, chromatin fractionation analyses revealed that hinge region-phosphorylated HP1α was preferentially dissociated from mitotic chromatin (Figure 2A). Furthermore, EMSA assays demonstrated that AURKB-mediated phosphorylation contributed to a decrease in HP1α's DNA-binding activity (Figure 2B). Although HP1α's mitotic behavior was previously linked to H3 serine 10 phosphorylation, which blocks the binding of HP1α's CD to H3K9me, our findings suggest that mitotic phosphorylation in HP1α's hinge region also contributes to changes in HP1α's association with mitotic chromatin.

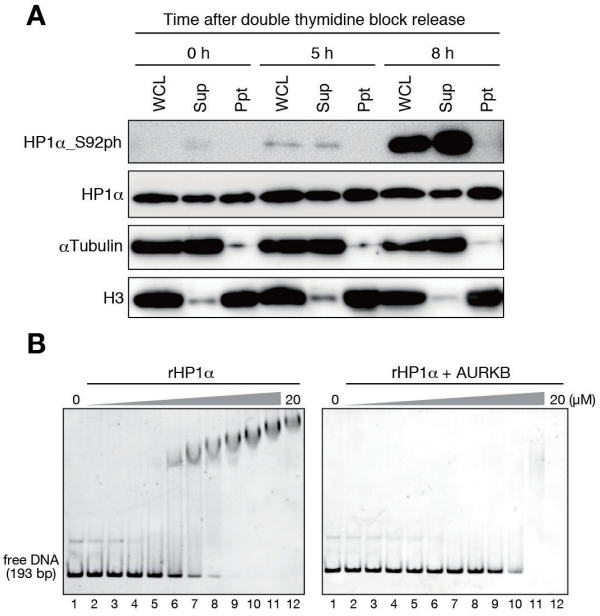

Figure 2. Mitotic phosphorylation of HP1α regulates its cell cycle-dependent chromatin binding. (A) Chromatin fractionation assays were performed using synchronized RPE-1 cells. Whole cell lysates (WCLs) and soluble (Sup) and insoluble chromatin-enriched (Ppt) fractions were resolved by SDS-PAGE and analyzed by immunoblotting. (B) Standard results of EMSAs that were performed with control or phosphorylated HP1α. Various concentrations of the mutant HP1α were incubated with 193-bp 601 DNA. The protein-DNA complexes were analyzed by 5% native-PAGE and SYBR Gold staining.

2-2 The binding of Chp2's CD to methylated H3K9 is essential for Chp2's role in heterochromatin assembly in fission yeast

The binding of HP1 to H3K9me is an essential step in heterochromatin assembly. Chp2, an HP1-family protein in the fission yeast *Schizosaccharomyces pombe*, is required for heterochromatic silencing. Chp2 recruits SHREC, a multifunctional protein complex containing the nucleosome remodeler Mit1 and the histone deacetylase Clr3 to perform this function. Although the targeting of SHREC to chromatin is thought to occur via two distinct modules regulated by the SHREC components Chp2 and Clr2, it is not clear how Chp2's chromatin binding regulates SHREC function. To investigate the role of H3K9me binding in Chp2's function, we created a strain that expressed a mutated Chp2 protein containing W199A (Chp2-W199A, in which tryptophan 199, one of the three conserved residues recognizing H3K9me, was changed to alanine). We then examined its effect on Chp2's silencing function and for SHREC's targeting to chromatin. Cells expressing Chp2-W199A have a silencing defect, with a phenotype similar to that of *chp2*-null cells (Figure 3). Genetic analysis using a synthetic silencing system revealed that a Chp2 mutant and SHREC-component mutants had similar phenotypes, suggesting that Chp2's function also affects SHREC's chromatin binding. Size-exclusion chromatography of native protein complexes showed that Chp2-CD's binding of H3K9me ensures Clr3's chromatin binding, and suggested that SHREC's chromatin binding is mediated by separable functional modules. Interestingly, we found that the stability of the Chp2 protein depended on the Clr3 protein's histone deacetylase activity. Our findings demonstrate that Chp2's H3K9me binding is critical for SHREC function and that the two modules within the SHREC complex are interdependent.

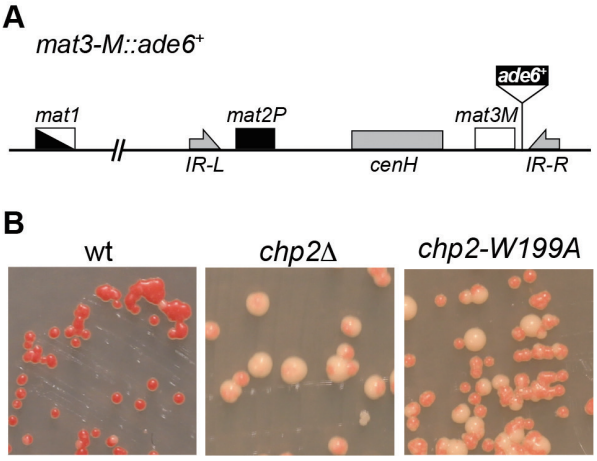

Figure 3. Chp2's H3K9me-binding ability is essential for its function in heterochromatin formation. (A) Schematic of the mating-type (*mat*) locus, showing the position of the silencing-reporter gene (*mat3-M::ade6*$^+$). (B) Wild-type and mutant strains with an *ade6*$^+$reporter gene inserted into the mating-type region were streaked on medium containing low amount of adenine (YE medium).

III. A proteomic approach to identifying chromatin-bound RNA-binding proteins

Various coding and non-coding transcripts are known to associate with chromatin and now there is accumulating evidence that interaction between RNA-binding proteins (RBPs) and RNA molecules regulate not only co-transcriptional mRNA processing, but also other biological processes within the nucleus. Although over a thousand RBPs have been identified through several mass spectrometry-based methods, it is still unclear which of these RBPs actually associate with chromatin, especially through interaction with RNAs. In addition, biological outcomes of such RBP-RNA-chromatin interactions are yet to be elucidated. In order to grasp the whole picture of physical or functional interactions between RNAs and RBPs in the nucleus, we have developed a simple proteomics-based method for systematic screening of RBPs that are anchored to chromatin and/or insoluble nuclear substructures by RNA molecules. We used RNase A to release said RBPs from the chromatin fraction (Figure

4A) and analyzed 'RNase A-solubilized' proteins by mass spectrometry. Using this method, we were able to identify 156 RNase A-solubilized proteins of which 144 were known RBPs/RBP candidates. Interestingly, several key players of the non-homologous end-joining (NHEJ) pathway were enriched in RNase A-solubilized fraction (Figure 4B) and the RNA-mediated chromatin association of these factors appeared to be dependent on transcriptional elongation. Furthermore, some enzymes involved in metabolic pathways were also released from chromatin and/or an insoluble nuclear structure by RNase A treatment. In summary, our methodology is highly versatile and is potentially a useful tool to unravel new biological functions for RBP-RNA-chromatin interactions.

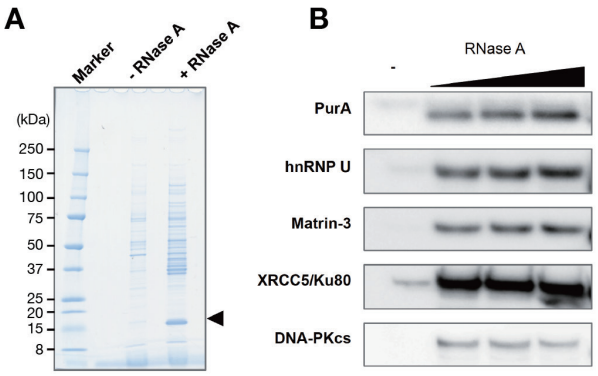

Figure 4. Proteomic analysis of RNA-dependent chromatin association of nuclear proteins. (A) Proteins solubilized after RNase A treatment were resolved by SDS-PAGE and visualized by staining with GelCode Blue dye. The black arrow indicates the position of RNase A. (B) A selection of chromatin-associated RBP candidates identified by mass spectrometry were subjected to western blot analysis.

IV. Dynamic chromatin reorganization during sexual reproduction of *Tetrahymena*

Tetrahymena, a unicellular protozoan, shows nuclear dimorphism, and contains the transcriptionally silent, germline micronucleus (MIC) and the transcriptionally active, somatic macronucleus (MAC) in a single cell. When the somatic MAC differentiates from the germline MIC, approximately 12,000 transposable element (TE)-related sequences are eliminated. In this process, TEs are heterochromatinized by the pathway related to RNAi/piRNA silencing and the heterochromatin acts as a signpost for their elimination. Although previous studies have identified more than 20 heterochromatin specific components, it remains unclear how they orchestrate DNA elimination. Using a yeast two-hybrid system, we analyzed the interactions between all known heterochromatin components and all predicted HP1 proteins, which recognize methylated histones and play important roles in heterochromatin formation. This attempt identified two protein complexes regulating the phosphorylation cycle of HP1-like protein Pdd1p, that is required for the assembly of multiple heterochromatin loci used to form heterochromatin bodies (Figure 5). Ectopic tethering of proteins composing Pdd1p phosphor-regulation complexes induced DNA elimination, thus indicating that the higher order heterochromatin assembly controlled by phosphorylation cycle of Pdd1p plays a central role in DNA elimination.

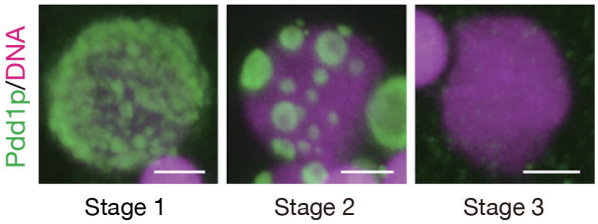

Figure 5. Three stages of heterochromatin body formation (stage 1. pre-heterochromatin body, stage 2. heterochromatin body, stage 3. post-heterochromatin body) visualized by Pdd1p (green) and DAPI (magenta). The scale bars represent 2 μm.

Publication List:

〔Original papers〕

- Hountondji, C., Crechet, J.B., Tanaka, M., Suzuki, M., Nakayama, J., Aguida, B., Bulygin, K., Karpova, G., and Baouz, S. (2018). Ribosomal protein eL42 contributes to the catalytic activity of the yeast ribosome at the elongation step of translation. Biochimie *158*, 20-33.
- Machida, S., Takizawa, Y., Ishimaru, M., Sugita, Y., Sekine, S., Nakayama, J., Wolf, M., and Kurumizaka, H. (2018). Structural basis of heterochromatin formation by human HP1. Mol. Cell *69*, 385-397.
- Maksimov, V., Oya, E., Tanaka, M., Kawaguchi, T., Hachisuka, A., Ekwall, K., Bjerling, P., and Nakayama, J. (2018). The binding of Chp2's chromodomain to methylated H3K9 is essential for Chp2's role in heterochromatin assembly in fission yeast. PLoS ONE *13*, e0201101.
- Nakayama, N., Sakashita, G., Nariai, Y., Kato, H., Shinmyozu, K., Nakayama, J., Kyo, S., Urano, T., and Nakayama, K. (2018). Cancer-related transcription regulator protein NAC1 forms a protein complex with CARM1 for ovarian cancer progression. Oncotarget *9*, 28408-28420.
- Okazaki, K., Kato, H., Iida, T., Shinmyozu, K., Nakayama, J., Murakami, Y., and Urano, T. (2018). RNAi-dependent heterochromatin assembly in fission yeast *Schizosaccharomyces pombe* requires heat-shock molecular chaperones Hsp90 and Mas5. Epigenetics Chromatin *11*, 26.

〔Original papers (E-publication ahead of print)〕

- Bommi, J.R., Prasada, Rao H.B.D.P., Challa, K., Higashide, M., Shinmyozu, K., Nakayama, J., Shinohara, M., and Shinohara, A. Meiosis-specific cohesion component, Rec8, promotes the localization of Mps3 SUN domain protein on the nuclear envelope. Genes Cells 2018 Dec 12.
- Nishibuchi, G., Machida, S., Nakagawa, R., Yoshimura, Y., Hiragami-Hamada, K., Abe, Y., Kurumizaka, H., Tagami, H., and Nakayama, J. Mitotic phosphorylation of HP1α regulates its cell cycle-dependent chromatin binding. J. Biochem. 2018 Dec 24.