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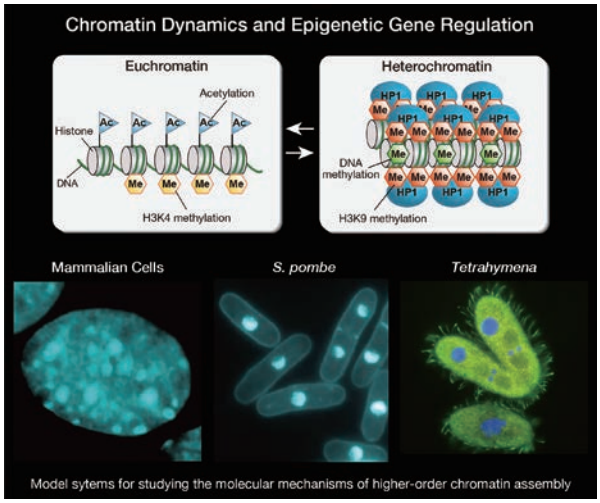

Model sytems for studying the molecular mechanisms of higher-order chromatin assembly

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 said 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 this 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* (*S. pombe*), in addition to 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

1-1 Mechanisms regulating Clr4 histone methyltransferase activity

In eukaryotic cells, the assembly of higher-order chromatin structures, known as heterochromatin, plays an important role in diverse chromosomal processes and epigenetic gene regulation. Heterochromatin is characterized by the methylation of histone H3 at lysine 9 (H3K9me). H3K9me is catalyzed by SUV39H-family histone methyltransferases. In fission yeast, H3K9me is catalyzed by histone methyltransferase Clr4. Clr4 has two functional domains, the N-terminal chromodomain (CD), which recognizes H3K9me, and the C-terminal SET domain responsible for Clr4's enzymatic activity (Figure 1A). Since Clr4's uncontrolled activity leads to inappropriate H3K9me and aberrant gene silencing, its enzymatic activity needs to be strictly controlled. A previous study showed that N-terminally deleted Clr4 mutant exhibits a stronger activity than full-length Clr4, suggesting that the Clr4 N-terminal region negatively regulates the activity of C-terminal SET domain. To examine the regulation mechanisms of Clr4's activity, we tested whether Clr4 N-terminal region interacts with its C-terminal SET domain. Pull-down assays using recombinant proteins reveal that the Clr4 N-terminal region can potentially interact with the SET domain and that this interaction requires both CD and its adjacent region (Figure 1B). To determine the regions/residues responsible for this interaction, we performed cross-linking mass spectrometry analyses and found that crosslinked residues were concentrated in a region near the C-terminal end of the SET domain (Figure 1C). These results support the idea that Clr4's enzymatic activity is regulated by inter- or intra-domain interactions.

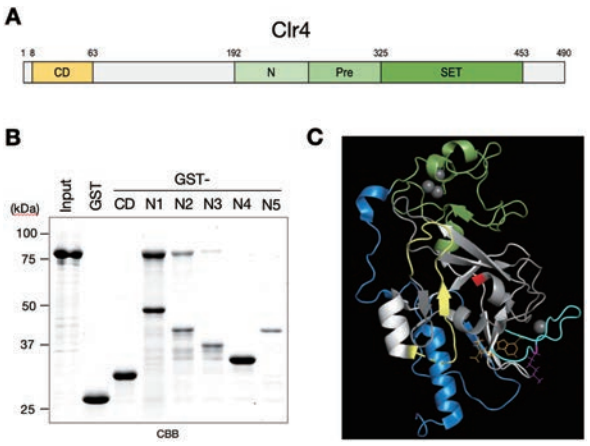

Figure 1. Characterization of inter- and/or intra-domain interactions of Clr4. (A) Domain organization of Clr4. The N-terminal chromodomain (CD) recognizes H3K9me, and the C-terminal SET domain is responsible for Clr4's enzymatic activity. (B) Pull-down assays of recombinant Clr4 to determine domains required for the interaction. Proteins were analyzed by SDS-PAGE and CBB staining. (C) Residues identified in the cross-linking mass spectrometry to be involved in the interaction are mapped on 3D structure of Clr4 SET domain.

1-2 Characterization of Clr4 methyltransferase complex (CLRC) in fission yeast

In fission yeast, Clr4 is the sole H3K9 methyltransferase and is essential for heterochromatin assembly. Biochemical and genetic analyses revealed that Clr4 forms a multiprotein complex called CLRC. CLRC consists of Cul4, Rik1, Raf1, Raf2, and Rbx1, and shows structural similarity to the CUL4-DDB1 complex involved in the repair of damaged DNA. We previously have demonstrated that affinity-purified CLRC ubiquitylates histone H3 and that H3 lysine 14 (H3K14) is the preferred target of the complex. We further demonstrated that CLRC-mediated H3 ubiquitylation promotes H3K9me by Clr4, suggesting a cross-talk mechanism between histone ubiquitylation and methylation that is involved in heterochromatin assembly (Oya *et al.* 2019). Although this study uncovered one of the physiological targets of CLRC, how Clr4 interacts with other components in CLRC remains unclear. To investigate the functional relationship between Clr4 and the CLRC, we expressed FLAG-tagged Clr4 in a strain expressing Rik1-myc and performed an IP-western experiment to confirm the interaction between Clr4 and Rik1 (CLRC). Using this assay, we expressed a series of Clr4 deletion mutants and successfully identified a domain which is necessary for the interaction with CLRC. Based on the above results, we performed a yeast two-hybrid assay and identified a candidate CLRC component responsible for the binding to Clr4. These results suggest that the interaction between Clr4 and CLRC is coupled with Clr4's intramolecular conformational change.

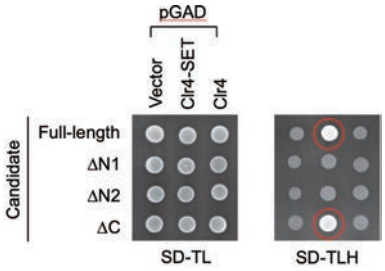

Figure 2. A representative result of Yeast two-hybrid assay using strains expressing full-length Clr4 or Clr4-SET in combination with full-length or truncated mutant of the candidate protein.

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

2-1 Mitotic phosphorylation of Swi6/HP1 regulates mitotic chromosome segregation

HP1 is a conserved chromosomal protein that plays important roles in heterochromatin assembly. There is growing evidence that HP1-family proteins undergo a variety of post-translational modifications, including phosphorylation, acetylation, methylation, and ubiquitination. Phosphorylation, the most extensively studied of these modifications, is widely implicated in HP1's dynamics and functions. We previously showed that Swi6, one of two HP1 isoforms in *S. pombe*, is constitutively phosphorylated by casein kinase II (CK2) and this phosphorylation is essential for its function in heterochromatin assembly. Several previous studies demonstrated that HP1 is subject to additional phosphorylation during mitosis. However, the functional importance of HP1's mitotic phosphorylation remains unclear. Using *E. coli* co-expression system, we demonstrated that Swi6 is phosphorylated by Ark1, a solo Aurora kinase in *S. pombe*. Subsequent mutation analyses revealed that serine residues in the N-terminal region of Swi6 are efficient targets for Ark1. We confirmed that these serine residues are phosphorylated during mitosis *in vivo*. Interestingly, expression of mutant Swi6 containing amino-acid substitutions at the serine residues differentially modulates temperature-sensitive growth of the mutations for Chromosome Passenger Complex (CPC) components. These results suggested that Ark1-mediated Swi6 phosphorylation regulates CPC's function during mitosis.

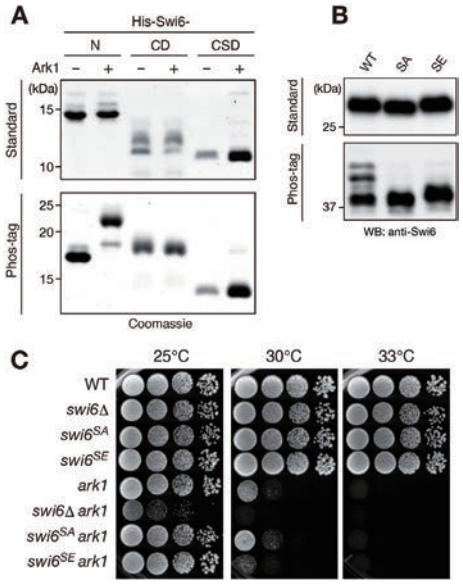

Figure 3. Mitotic phosphorylation of Swi6 regulates mitotic chromosome segregation. (A) Swi6 domains, N-terminal region (N), CD, and CSD, produced in control or control or Ark1-co-expressed *E. coli* cells were purified, resolved by standard or Phos-tag PAGE, and visualized by CBB staining. (B) Phosphorylation patterns of wild-type (wt) or mutant Swi6 proteins were analyzed by immunoblotting using anti-Swi6 antibody. (C) Serially diluted cells of the indicated strains were spotted on non-selective media and incubated at indicated temperatures.

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

3-1 Multiple HP1-like protein-containing complex regulates DNA elimination in *Tetrahymena*

Heterochromatin plays important roles in transposon (TE) silencing. A major type of heterochromatin contains chromatin, that is histone H3 methylated at lysine 9/27 (H3K9/27me), and its reader HP1 proteins, that recruit diverse proteins onto the chromatin to silence the TEs. Although multiple HP1 proteins are co-expressed in many eukaryotic cells, the interplay between these HP1 proteins has been elusive. Here, we show that subset of the HP1 proteins form a complex and play important roles to eliminate TE-related Internal Eliminated Sequences (IESs) from the somatic genome during macronuclear development in the ciliated protozoan *Tetrahymena*. We tethered 7 HP1-like proteins individually to the artificially created locus by the LexA-LexO system and found that only 4 of them includ-

ing Pdd1 induced the elimination of the tethered site (Figure 5). This ectopic DNA elimination was exclusively achieved by their chromoshadow domains (CSDs), indicating that the CSDs of the distinct type of HP1-like proteins recruits all the proteins that are required for DNA elimination. Immunoprecipitation of Pdd1 specifically enriched the other HP1-like proteins that were sufficient for the ectopic DNA elimination. The chromodomains of a subset of HP1-like proteins showed strong affinity to both H3K9me3 and H3K27me3. Overall, these results suggest that multiple HP1-like proteins cooperatively recognize the methylated histones and form core complex to recruit other effector proteins for DNA elimination.

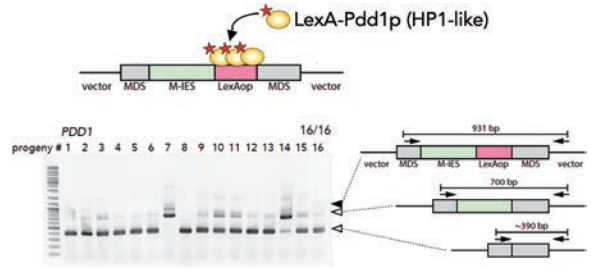

Figure 4. Identification of heterochromatin proteins sufficient to induce DNA elimination. Schematics of the tethering assay (top). A representative result of the tethering assay using Pdd1p (bottom).

3-2 The HP1-like protein Hpl8p is important for programmed DNA elimination in *Tetrahymena*

Transposon mobility represents a threat for the genome integrity in all living things. To counter this, the ciliated protozoan *Tetrahymena* uses heterochromatin to selectively eliminate over 10,000 transposon loci from its somatic nucleus in the developmentally programmed process of DNA elimination. To address how this process is coordinated by heterochromatin components, we focus on an HP1-like protein named Hpl8p that localizes to the developing macronucleus during heterochromatin formation and DNA elimination (Figure 5). The chromodomain of Hpl8p recognized both H3K9me3 and H3K27me3 *in vitro*. *HPL8* disrupted mutants showed defect in vegetative growth and in the progression of sexual reproduction. FISH analyses showed that more than 90% of *HPL8* disrupted cells did not complete its elimination, suggesting that Hpl8p plays important roles in DNA elimination. Altogether, these results suggest that Hpl8p binds the H3K9/27me marks over transposon loci and facilitates the heterochromatin formation by interacting with heterochromatin components required for DNA elimination. Furthermore, we propose that Hpl8p also plays important roles in suppression of unwanted genes during the vegetative growth and sexual reproduction process possibly by recognizing H3K27me3 in the transcriptionally active macronucleus.

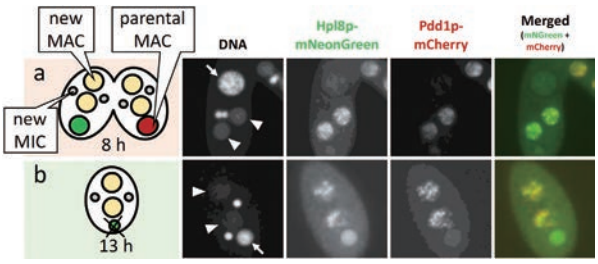

Figure 5. Cells expressing the Hpl8-mNeonGreen (green) and Pdd1p-mCherry (red) at 8 and 13 hours post induction of mating are shown in (a) and (b), respectively. DNA was counterstained with DAPI. Arrows and arrowheads indicate the parental and new MACs, respectively.

IV. Analysis of chromatin dynamics in spore nuclei of fission yeast

Gametogenesis is a crucial process for sexually reproducing organisms to produce haploid gametes from diploid cells. Generally, epigenetic memory is erased from zygote nucleus, a phenomenon known as reprogramming, resulting in increased totipotency. On the other hand, it has been demonstrated in some organisms that a part of epigenetic memory can be inherited transgenerationally. The balance between reprogramming and epigenetic inheritance plays a key role in the developmental processes of offspring. It has been also reported that some gametes, like plant sperm and mammalian spermatozoa, have highly compacted nuclei and have altered histone modifications. However, the mechanisms underlying how epigenetic marks are regulated during gametogenesis remain to be fully elucidated. To gain an insight into epigenetic inheritance and chromosome structural change during gamete formation, we analyzed chromatin dynamics in spore nucleus of fission yeast. We isolated fission yeast spores by density-gradient centrifugation and performed mass spectrometry analysis to determine spore-specific nuclear proteins. We further examined the change in histone modifications of spore nuclei. Chromatin immunoprecipitation analysis revealed that the levels of H3K9me at heterochromatic regions had not been noticeably changed, but the levels of H3K4me associated transcriptionally active regions had clearly increased in spore nuclei. These results imply that dynamic changes in active histone demonstrate a contribution to spore formation and epigenetic inheritance.

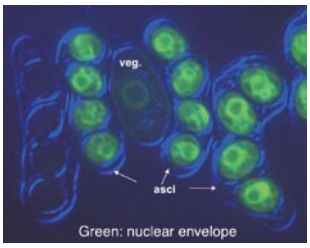

Figure 6. A representative image of vegetative cells (veg.) and spores in asci.

Publication List:

〔Original paper〕

- Dong, C., Nakagawa, R., Oyama, K., Yamamoto Y., Zhang, W., Dong, A., Li, Y., Yoshimura Yuriko and Kamiya, H., Nakayama, J., Ueda, J., and Min, J. (2020). Structural basis for histone variant H3tK27me3 recognition by PHF1 and PHF19. eLife *9*, e58675. DOI: 10.7554/eLife.58675

〔Book chapter〕

- Hiragami-Hamada, K., Tani, T., and Nakayama, J. (2020). Proteomics-based systematic identification of nuclear proteins anchored to chromatin via RNA. Methods Mol Biol *2161*, 88-99. DOI: 10.1007/978-1-0716-0680-3_8