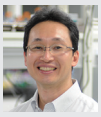
DIVISION OF CHROMATIN REGULATION



Professor
NAKAYAMA, Jun-ichi

Assistant Professor: *KATAOKA, Kensuke*
Postdoctoral Fellow: *HAYASHI, Aki*
SOKENDAI Graduate Student: *RAHAYU, Anisa*
VALENTIROVIC, Olivera
Visiting Undergraduate: *HACHISUKA, Aki*
Visiting Scientist: *HAMADA, Kyoko*
Technical Assistant: *YOSHIMURA, Yuriko*
ASAI, Yuriko
Secretary *KIYOHARA, Megumi*

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 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* (*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 H3K14 ubiquitylation promotes H3K9 methylation for heterochromatin assembly

In eukaryotic cells, the assembly of higher-order chromatin structures, known as heterochromatin, plays an important role in diverse chromosomal processes. Heterochromatin assembly is intimately associated with changes in post-translational histone-tail modifications. Histone H3-lysine 9 methylation (H3K9me), a hallmark of heterochromatin structure, is catalyzed by SUV39H-family histone methyltransferases, and functions as a binding site for recruiting heterochromatin protein 1 (HP1) family proteins. In the fission yeast *S. pombe*, Clr4, a homolog of mammalian SUV39H, plays a central role in heterochromatin assembly. Clr4 forms a multi-protein complex called the Clr4 methyltransferase complex (CLRC) with Cul4, Rik1, Raf1, and Raf2. Cul4, Rik1, and Raf1 display a strong structural resemblance to the conserved CUL4-DDB1-DDB2 E3 ubiquitin ligase, and it has been demonstrated that the CLRC exhibits ubiquitin ligase activity *in vitro*. However, whether the CLRC acts as an E3 ubiquitin ligase *in vivo* and how ubiquitylation modulates Clr4's activity remains unclear.

To identify the physiological substrate(s) ubiquitylated by CLRC, we affinity purified the CLRC (Figure 1A) and performed an *in vitro* ubiquitylation assay using histones as candidate substrates (Figure 1B). We discovered that the affinity-purified CLRC specifically ubiquitylates recombinant histone H3, and mass spectrometric and mutation analyses revealed that H3-lysine 14 (H3K14) is the preferred target of the complex (Figure 1C).

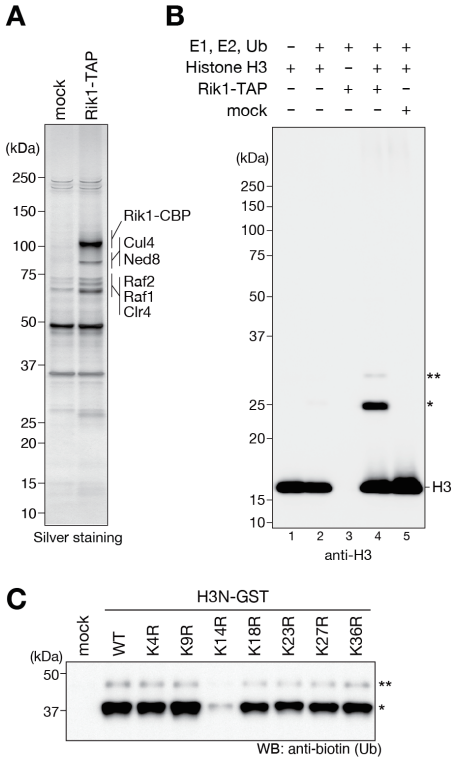


Figure 1. Histone H3 lysine 14 is ubiquitylated *in vitro* by CLRC. (A) Purified TAP-tagged Rik1-containing complexes analyzed by SDS-PAGE and silver staining. Proteins identified by LC-MS/MS are indicated on the right. (B) *In vitro* ubiquitylation assays using biotinylated ubiquitin, purified CLRC, and recombinant histone H3 as the substrate. Proteins were analyzed by Western blotting using the indicated antibodies. Asterisks indicate ubiquitylated histone H3 species. (C) Ubiquitylation assay using biotinylated ubiquitin and recombinant wild-type H3N-GST (WT) and arginine-substituted H3N-GST mutants as substrates. Proteins were analyzed by Western blotting. Asterisks indicate ubiquitylated H3N-GST proteins.

We next sought to assess the presence of K14-ubiquitylated H3 (H3K14ub) *in vivo*. We performed chromatin immunoprecipitation (ChIP) assays using antibodies against H3K9me2 and performed LC-MS/MS analyses. We confirmed that H3K14ub was detected exclusively in the H3K9me2-associated heterochromatin.

We then examined the possible connection between H3K14ub and H3K9me. To test whether H3K14ub modulates H3K9me, we performed *in vitro* histone methyltransferase (HMTase) assays using recombinant Clr4 and ubiquitylated H3N-GST as a substrate (Figure 2A). H3N-GST was first subjected to an *in vitro* ubiquitylation assay using affinity-purified CLRC. After the reaction, the resultant H3N-GST was purified and subjected to an *in vitro* HMTase assay. We found that even though only 10-15% of the H3N-GST was ubiquitylated by the CLRC under our assay condi-

tions and the rest remained unmodified, Clr4 exclusively methylated the ubiquitylated H3N-GST (Figure 2B). These results suggest that the CLRC-mediated H3 ubiquitylation promotes H3K9me by Clr4 and that H3 ubiquitylation is intimately linked to the establishment and/or maintenance of H3K9me. These findings also demonstrate a cross-talk mechanism between histone ubiquitylation and methylation that is involved in heterochromatin assembly (Figure 2C).

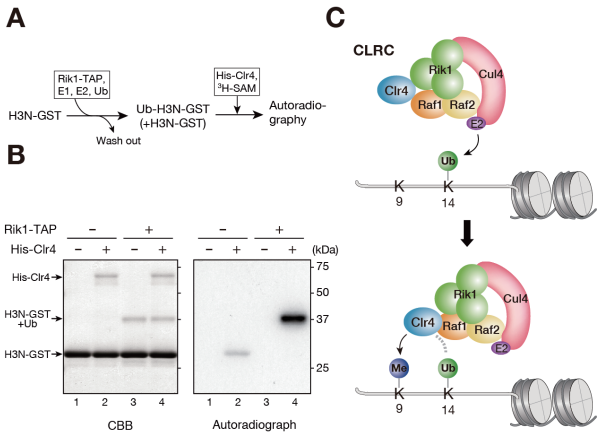


Figure 2. H3K14 ubiquitylation promotes H3K9 methylation. (A) Experimental scheme. (B) *In vitro* HMTase assays. Recombinant H3N-GST pre-ubiquitylated by the CLRC was purified and used in the HMTase assay with 6×His-tagged recombinant Clr4 (His-Clr4). Proteins were analyzed by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining (left) and autoradiography (right). (C) A cross-talk mechanism between histone ubiquitylation and methylation involved in heterochromatin assembly.

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 (Figure 3A). In addition, chromatin fractionation analyses revealed that hinge region-phosphorylated HP1α was preferentially dissociated from mitotic chromatin (Figure 3B). Furthermore, EMSA assays demonstrated that AURKB-mediated phosphorylation contributed to a decrease in HP1α's DNA-binding activity (Figure 3C). 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.

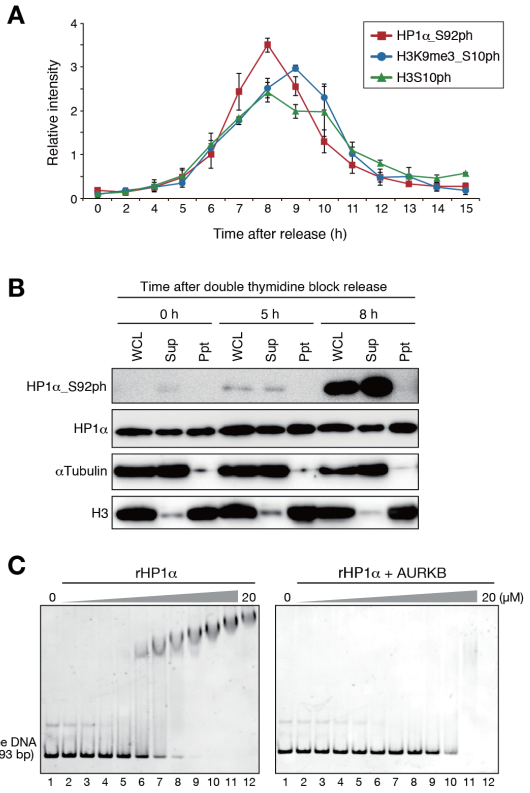


Figure 3. Mitotic phosphorylation of HP1α regulates its cell cycle-dependent chromatin binding. (A) The levels of HP1α_S92ph, H3K9me3_S10ph, and H3S10ph in synchronized PRE-1 cells. (B) Chromatin fractionation assays were performed using synchronized RPE-1 cells. Whole cell lysates (WCLs) and soluble (Sup) and insoluble chromatin-enriched (Ppt) fractions were resolved by SDS-PAGE and analyzed by immunoblotting. (C) Representative results of EMSAs that were performed with control or phosphorylated HP1α. Various concentrations of HP1α were incubated with 193-bp 601 DNA. The protein-DNA complexes were analyzed by 5% native-PAGE and SYBR Gold staining.

2-2 Identification of Aurora kinase-mediated phosphorylation sites in Swi6/HP1 regulating mitotic chromosome segregation

HP1 is a conserved chromosomal protein that plays important roles in heterochromatin assembly. We previously showed that Swi6, one of two HP1 isoforms in *S. pombe*, is multiplicatively phosphorylated by casein kinase II (CK2) and this phosphorylation is essential for its function in heterochromatin assembly. Several previous studies demonstrated that HP1 is subjected to additional phosphorylation during mitosis. However, the functional importance of HP1's mitotic phosphorylation remains unclear. We revealed that Swi6 mitotic phosphorylation is involved in mitotic chromosomal segregation. Using *E. coli* co-expression system, we demonstrated that Swi6 is phosphorylated by Ark1, a solo Aurora kinase in *S. pombe*, and 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.

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

The ciliated protozoan *Tetrahymena themophila* has two functionally distinct nuclei in a single cell: the transcriptionally silent germline micronucleus (MIC) and the transcriptionally active somatic macronucleus (MAC). When the somatic MAC differentiates from the germline MIC during sexual reproduction, approximately 12,000 transposable element (TE)-related sequences are eliminated from the MAC genome. In this process, TEs are heterochromatinized by the pathway related to RNAi/piRNA silencing and the heterochromatin acts as a mark for their elimination (Figure 4A). 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 (Figure 4B) and found several proteins that may act as 'hubs' to assemble heterochromatin components into functionally relevant chromatin structure for DNA elimination. Consequently, we are currently analyzing the roles of these hub proteins both *in vitro* and *in vivo* to understand spatiotemporal regulation of heterochromatin formation and DNA elimination.

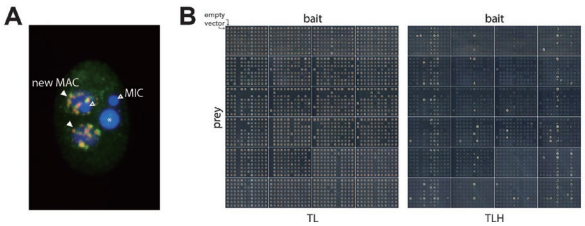


Figure 4. Heterochromatin formation in *Tetrahymena*. (A) *Tetrahymena* cell (at the late stage of sexual reproduction) expressing Pdd1p-mCherry (red) and HPL4-EGFP (red) were counter stained with DAPI (blue). The arrowheads indicate new MAC or MIC and the asterisk indicate old MAC undergoing degradation. (B) Analyses of all possible one-to-one interactions between heterochromatin proteins using yeast two hybrid assay. The yeast strain expressing the Gal4 binding domain fused to a heterochromatin protein (bait) were mated to strains expressing the Gal4 activation domain linked to a heterochromatin protein (prey). Diploid cells were plated on a 48 x 48 matrix on control plates containing all of the auxotrophic requirements (left: without threonine and leucine) and on test plates without histidine (right).

Publication List:

〔Original papers〕

- Bommi, J.R., Rao, H.B.D.P., Challa, K., Higashide, M., Shinmyozu, K., Nakayama, J.-I., Shinohara, M., and Shinohara, A. (2019). Meiosis-specific cohesin component, Rec8, promotes the localization of Mps3 SUN domain protein on the nuclear envelope. Genes Cells *24*, 94-106. doi: 10.1111/gtc.12653
- Ding, D.-Q., Okamasa, K., Katou, Y., Oya, E., Nakayama, J.-I., Chikashige, Y., Shirahige, K., Haraguchi, T., and Hiraoka, Y. (2019). Chromosome-associated RNA–protein complexes promote pairing of homologous chromosomes during meiosis in *Schizosaccharomyces pombe*. Nat. Commun. *10*, 5598. doi: 10.1038/s41467-019-13609-0
- Hountondji, C., Créchet, J.-B., Tanaka, M., Suzuki, M., Nakayama, J.-I., Aguida, B., Bulygin, K., Cognet, J., Karpova, G., and Baouz, S. (2019). Ribosomal protein eL42 contributes to the catalytic activity of the yeast ribosome at the elongation step of translation. Biochimie *158*, 20-33. doi: 10.1016/j.biochi.2018.12.005
- Nishibuchi, G., Machida, S., Nakagawa, R., Yoshimura, Y., Hiragami-Hamada, K., Abe, Y., Kurumizaka, H., Tagami, H., and Nakayama, J.-I. (2019). Mitotic phosphorylation of HP1α regulates its cell cycle-dependent chromatin binding. J. Biochem. *165*, 433-446. doi: 10.1093/jb/mvy117
- Okamoto, K., Tanaka, Y., Ogasawara, S., Obuse, C., Jun-Ichi, N., Yano, H., and Tsuneoka, M. (2019). KDM2A-dependent reduction of rRNA transcription on glucose starvation requires HP1 in cells, including triple-negative breast cancer cells. Oncotarget *10*, 4743-4760. doi: 10.18632/oncotarget.27092
- Oya, E., Durand-Dubief, M., Cohen, A., Maksimov, V., Schurra, C., Nakayama, J.-I., Weisman, R., Arcangioli, B., and Ekwall, K. (2019). Leo1 is essential for the dynamic regulation of heterochromatin and gene expression during cellular quiescence. Epigenet. Chromatin *12*, 45. doi: 10.1186/s13072-019-0292-7
- Oya, E., Nakagawa, R., Yoshimura, Y., Tanaka, M., Nishibuchi, G., Machida, S., Shirai, A., Ekwall, K., Kurumizaka, H., Tagami, H., and Nakayama, J.-I. (2019). H3K14 ubiquitylation promotes H3K9 methylation for heterochromatin assembly. EMBO Rep. *20*, e48111. doi: 10.15252/embr.201948111

〔Review article〕

- Hiragami-Hamada, K., and Nakayama, J. (2019). Do the charges matter? - Balancing the charges of the chromodomain proteins on the nucleosome. J. Biochem. *165*, 455-458. doi: 10.1093/jb/mvz004