学 位 論 文 の 要 旨

氏名　岡崎　宏亮

学 位 論 文 名　RNAi-dependent Heterochromatin Assembly in Fission Yeast *Schizosaccharomyces pombe* Requires Heat-shock Molecular Chaperones Hsp90 and Mas5

発 表 雑 誌 名（巻，初頁～終頁，年）　*Epigenetics & Chromatin* (11, 26, 2018)

著 者 名　Kosuke Okazaki, Hiroaki Kato, Tetsushi Iida, Kaori Shinmyozu, Jun-ichi Nakayama, Yota Murakami and Takeshi Urano

論 文 内 容 の 要 旨

INTRODUCTION

Assembly of heterochromatin, a dense chromatin structure that represses the expression of embedded genes, is vital for the establishment and maintenance of cell identity. A hallmark of heterochromatin is methylation of histone H3 at Lys-9 (H3K9me), a modification that is conserved from the fission yeast *Schizosaccharomyces pombe* to humans. Studies using *S. pombe* as a model organism have established the concept that the RNA interference (RNAi) pathway contributes to the assembly of heterochromatin.

In the *S. pombe* RNAi pathway, formation of the small interfering RNA (siRNA) -containing effector complex is coupled to heterochromatin assembly. siRNA is generated, by the Dicer family endoribonuclease Dcr1, from double-stranded non-coding RNA that is complementary to heterochromatin. The siRNA duplex is loaded onto a non-chromatin-associated complex called Argonaute small interfering RNA Chaperone (ARC), which contains the Argonaute family endoribonuclease Ago1. The loading of the siRNA duplex onto the Ago1 subunit requires the two ARC-specific subunits Arb1 and Arb2. This complex then changes its subunit composition to form a chromatin-associated effector complex called RNA-Induced Transcriptional Silencing (RITS). The RITS complex is composed of Ago1, now binding single-stranded siRNA as a guide for target recognition, and the two RITS-specific subunits Chp1 and Tas3.

The formation of small RNA-containing effector complexes is generally assisted by heat-shock molecular chaperones (Hsps). However, the heat-shock molecular chaperones

responsible for the RNAi-dependent heterochromatin assembly remain unidentified.

MATERIALS AND METHODS

General yeast manipulation methods and culture conditions were applied to this study. For N-terminal tagging of Ago1, p3FLAGago1N-natMX4 or p3MYCago1N-natMX4 was integrated into the *ago1* locus. Transformation was performed using a yeast transformation kit for *S. pombe* (Wako Pure Chemical Industries) according to the manufacturer's instructions. All of the tagged sequences were subjected to DNA sequencing to confirm that no additional mutation had been introduced during construction. Cells growing logarithmically in YES medium at 30°C were used for the analyses.

The genetic screen that identified a mutation in the *hsp90* gene was described previously. In order to identify candidate proteins that might act as silencing factors, we performed mass spectrometric analysis of the proteins co-immunoprecipitated with either RNA polymerase II or Spt6, both of which are involved in heterochromatic silencing.

Methods for immunoprecipitation, total RNA extraction, reverse transcription followed by quantitative PCR (RT-qPCR), northern blotting, and chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) were as documented elsewhere. M-280 anti-mouse sheep antibody-conjugated magnetic beads (112-02, Thermo Fisher Scientific) were used for immunoprecipitation. For northern blotting of RNA in FLAG-Ago1 complex, whole cell extracts were subjected to immunoprecipitation with mouse monoclonal anti-FLAG antibody M2. Ago1-bound RNA was extracted from the immunoprecipitates and subjected to northern blotting.

For western blotting analyses, mouse monoclonal anti-Myc antibody 9E10 (1:2000), anti-G196 ascites (1:2000), anti-α-tubulin antibody DM1A (1:2000), and anti-FLAG antibody M2 (1:2000) were used as primary antibodies. HRP-conjugated goat anti-mouse antibody (1:5000, Rockland Immunochemicals) was used as secondary antibody.

RESULTS AND DISCUSSION

In a forward genetic screen for factors that affect pericentromeric silencing, we isolated a missense mutation of the *hsp90* gene, which encodes the sole Hsp90-family protein in *S. pombe*. In parallel with the genetic screen, we conducted immuno-affinity purification of proteins that interact either with RNA polymerase II or Spt6, and identified an Hsp40-family protein, Mas5, as a silencing factor.

We evaluated the silencing state of mutant cells by monitoring the expression of *ade6* and *ura4* marker genes embedded in the pericentromere regions. In the absence of mutations, cells did not appreciably express *ade6* or *ura4*; thus, cells without a silencing defect formed red

colonies on a plate with a limited amount of adenine (due to the accumulation of an intermediate of adenine biosynthesis) and grew healthily on a plate containing 5-fluoroorotic acid (5-FOA) (a pyrimidine precursor analog that is toxic to *ura4*-expressing cells). We demonstrated that cells harboring a mutation in the genes encoding Hsp90 (*hsp90-A4*) or Mas5 (*mas5*Δ) formed near-white colonies and exhibited sensitivity to 5-FOA. Derepression of heterochromatic silencing was confirmed by means of strand-specific RT-qPCR.

To test whether Hsp90 and Mas5 are involved in the RNAi-dependent assembly of heterochromatin, we performed ChIP-qPCR to monitor the levels of dimethylation of histone H3 at Lys-9 (H3K9me2) and the Argonaute protein Ago1 at heterochromatic regions. In agreement with the results of RT-qPCR, *hsp90-A4* and *mas5*Δ mutations caused reductions in the levels of H3K9me2 and Ago1 at the RNAi-dependent pericentromeric hetrochromatin.

As molecular chaperone proteins, Hsp90 and Mas5 may contribute to effector complex formation in the RNAi pathway. To examine this possibility, we first tested whether the protein level of Ago1 is altered in the mutant cells. Western blotting with 2-fold serial dilutions indicated that the signal intensity of FLAG-Ago1 in *hsp90-A4* or *mas5*Δ cells was less than a half of that in wild-type cells. In *hsp90-A4* or *mas5*Δ cells, siRNA was undetectable by northern blotting. In addition, northern blotting of Ago1-associated RNA revealed that Ago1 did not bind siRNA in the mutant cells. Coimmunoprecipitation analyses indicated that the Hsp mutants have defects in ARC formation. Furthermore, we found that the RITS subunit Tas3 was difficult to detect in *mas5*Δ cells, which could lead to a dramatic reduction of functional RITS complex.

In this study, we demonstrated that the *S. pombe* molecular chaperones Hsp90 and Mas5 are required for the silencing, heterochromatin assembly, and chromatin localization of Ago1 in the pericentromere. In contrast, the RNAi-independent heterochromatin was not strongly affected by the mutations in Hsp90 or Mas5. We also showed that the *in vivo* generation of siRNA complementary to the pericentromeric repeats required these chaperones. Furthermore, we showed that Mas5 contributes to maintenance of protein levels of Tas3 in the cells. Together, these results indicated that Hsp90 and Mas5 are involved in RNAi-dependent heterochromatin assembly in *S. pombe*.

CONCLUSION

Based on the results presented in this study, we propose that molecular chaperones Hsp90 and Mas5 are required for RNAi-dependent heterochromatin assembly in *S. pombe*. Although the underlying molecular mechanism remains to be elucidated, mutations in the genes encoding these chaperones greatly decreased the levels of pericentromeric siRNA and H3K9me2 *in vivo*. Our results suggest that inhibition of the counterparts of these chaperones in other species may have similar destructive effects on chromatin regulation.