DEPARTMENT OF CELL BIOLOGY
LABORATORY OF SIGNAL TRANSDUCTION

Our group is interested in the regulation of higher order chromatin structure and its role in various chromosome functions. Currently this group focuses on the dynamic organization of heterochromatin that is important constituent of chromosome using fission yeast *Schizosaccharomyces pombe* as a model organism.

1) Fission Yeast Chromatin Assembly Factor 1 Assists in the Replication-Coupled Maintenance of Heterochromatin.: K. DOHKE, S. MIYAZAKI, K. TANAKA[1], T. URANO[2], S. I. S. GREWAL[3] AND Y. MURAKAMI ([1]Kwansei Gakuin Univ., [2]Shimane Univ., [3]NIH, USA)

Chromatin assembly factor-1 (CAF1) is a well-conserved histone chaperone that loads the histone H3-H4 complex onto newly synthesized DNA *in vitro* through interaction with the replication factor PCNA. CAF1 is thought to be involved in heterochromatin maintenance in several organisms, but functional details have not been established. We identified fission yeast CAF-1 (spCAF1), which interacts with PCNA. Depletion of spCAF1 caused defects in silencing at centromeric and mating locus heterochromatin, accompanied with a decrease in Swi6, the fission yeast HP1 homologue. Loss of spCAF1 destabilized both the silent and active states of heterochromatin at the meta-stable mating locus, with a more pronounced effect on the silent state, indicating that spCAF1 is involved in the maintenance of heterochromatin. Swi6 dissociated from heterochromatin during G1/S phase appears to associate with spCAF1. In early S phase, spCAF1 localized to replicating heterochromatin and remained associated with Swi6, and Swi6 then bound to heterochromatin. Taken together, we propose that spCAF1 functions in heterochromatin maintenance by recruiting dislocated Swi6 during replication to replicated heterochromatin at the replication fork.

2) Regulation of Transcriptional Gene Silencing by Phosphorylation of Swi6, a HP1 Homologue: A. SHIMADA, J-I. NAKAYAMA[1], M. SADAIE[1] AND Y. MURAKAMI ([1]Riken CDB)

In fission yeast, pericentromeric heterochromatin structure plays a multiple roles in nuclear functions including transcriptional genen silencing and sister chromatid cohesion. In fission yeast heterochromatin histone H3K9 is highly methylated and Swi6, a homologue of heterochromatin protein 1 (HP1), binds to the methylated histone. Swi6 then recruits “effector” complexes such as Clr3, a histone deacetylase for gene silencing and cohesin complex for sister

chromatid cohesion. However, the regulatory mechanism of multi-functions of Swi6 has not been clear yet. HP1 is a phospho-protein and some of the phosphorylation has been suggested to be functionally important, but the molecular details are not known. Here, we found that casein kinase 2 (CK2) directly phosphorylates Swi6 *in vivo* and the phosphorylation is specifically recuired for heterochromatic gene silencing. In attempts to identify genes involved in heterochromatin formation using newly developed reverse-genetical strategy, we identified *ckb1*, which encodes regulatory subunit of CK2. Deletion of *ckb1* caused the defect in heterochromatic gene silencing and also caused de-phosphorylation of Swi6. Fission yeast CK2 phosphorylated Swi6 *in vitro* and lack of Ckb1 resulted in inefficient phsphorylation. These results suggested Swi6 could be a target of CK2 *in vivo*. Indeed, substitutions of serine residues of putative CK2 phosphorylation sites with alanine resulted in decrease of Swi6 phosphrylation. Interestingly both the *ckb1* disruptant and the *swi6* mutants did not affect the heterochromatin structure, namely H3K9 methylation and Swi6 association. These mutants, however, showed a defect in the transcriptional gene silencing mediated by heterochromatin, while they did not show defect in chromosome segregation that is commonly observed in centromeric heterochromatin mutants or cohesion mutants. Consistent with these phenotypes, association of Clr3 with heterochromatin but not that of cohesin significantly decreased in the CK2 or Swi6 phsphorylation mutants. These results indicated that phosphorylation of Swi6 by CK2 differentially regulated the multi-function of Swi6 and suggests that unexpectedly dynamic regulatory mechanism of heterochromatin.

3) Centromeric non-coding RNAs Localize to Chromatin through DNA-RNA Hybridization:
M. NAKAMA, H. KATO[1], T. URANO[1], Y. MURAKAMI ([1]Shimane Univ.)

In the fission yeast *Schizosaccharomyces pombe*, heterochromatin assembly requires RNAi (RNA interference) machinery and initiated by small interference RNAs (siRNAs) derived from heterochromatic regions that is incorporated in the RNA-induced transcriptional silencing (RITS) complex. The model for RNAi-dependent heterochromatin assembly predicted that ncRNA transcribed at heterochormatin associated with chromatin and the assocation may be important for heterochromatin formation. To confirm this prediction, we tried to RNA-IP (RNA-immunoprecipitation) experiment to detect the interaction of ncRNA and histone H3. We used the antibody of anti-histone H3 for RNA-IP assay and detected the association of centromeric ncRNA with histone H3 in both wild-type and the mutants that has disturbed heterochromatin. In contrast, usual mRNA did not show such chromatin assoication. This result showed centromeric ncRNA tend to localize at chromatin independent of chromatin state. Furthermore, RNA-IP assay combined with RNase H treatment showed that centromeric ncRNA on chromatin hybridized with template DNA. Because DNA-RNA hybridization on chromatin could not be detected in *act-1* mRNA, hybridization was specific to centromeric ncRNA. To determine whether DNA-RNA

hybridization is required for heterochromatin conformation, we performed silencing assays with cells in which RNaseH was depleted or overexpressed. Deletion of the genes for fission yeast RNaseH did not affect the silencing of markers inserted at centromere, but disturbed the establishment of heterochromatin when the epigenetic silencing erased by the deacetylase inhibitor trichostatin A. On the other hand, overexpression of fission yeast RNase H affected the heterochromatic silencing. So the presence of the certain amount of DNA-RNA hybrid might be required for optimal heterochromatin formation. Our analysis indicates a link between ncRNA associated with histone and heterochromatin assembly.

4) Analysis of Swi6 in the RNAi-dependent heterochromatin formation: S. TSUNEMINE and Y. MURAKAMI

Swi6/HP1 that are bound to different nucleosomes is thought to mediate chromatin compaction through dimerization with chromo-shadow domain to establish heterochromatin structure. In addition, Swi6/HP1 functions as a recruiting platform for various “effectors” to control various heterochromatin activities (see (3) in this report). In centoromeric heterochromatin formation in fission yeast, the RNAi interference (RNAi) machinery converts pericentoromeric non-coding RNAs into small interfering RNAs (siRNA) and the siRNA is required for assembly of heterochromatin. Swi6 is required for the efficient generation of siRNA in this system, but its molecular mechanism is totally unknown. To analyze the molecular functions of Swi6, we have been trying to isolate various Swi6 mutations each of which has a defect in each function of Swi6. So far, we got several Swi6 mutants. Preliminary result suggests that one of the mutants showed specific defects in RNAi-dependent heterochromatin formation. We hope analysis of this mutant will reveal the role of Swi6 (or heterochromatin) in siRNA genereation.

5) Search for new factors indispensable to heterochromatin formation. : K.KAWAKAMI and Y.MURAKAMI

Heterochromatin is an important, higher-order chromatin structure that contributes various nuclear functions such as epigenetic regulation of gene expression. Recent analysis using fission yeast suggests that a large number of factors are involved in heterochromatin formation and its function and many of them are not identified yet. To further get the insights in the heterochromatin formation and function, we have been screening for mutants that affect pericentromeric heterochromatin formation. In the screening, we got six mutants whose pericentromeric heterochromatin was disturbed. Determination of a gene responsible for the defects in each mutants is in progress.

6) Mediator subunit Pmc6 regulates peri-centirc heterochromatin in fission yeast: E. OYA, H. KATO*, M.KAWAMUKAI*, Y. MURAKAMI (*Shimane Univ.)

In fission yeast *Schizosaccharomyces pombe*, peri-centromeric heterochromatin is formed in an RNAi-dependent manner. In this process, RNA polymerase II seems to couple the transcription from the target loci and generation of siRNAs (small interfering RNAs) to assemble the higher order chromatin structure (see (7) in this report).

We hypothesized that there were some RNA polymerase II-interacting factors that function in the RNAi pathway. Interestingly, we found that deletion of a gene encoding one of "Mediator" subunits, Pmc6, caused de-repression of the peri-centromeric silencing. Mediator complex is thought to "mediate" signals between transcription initiation complex and regulatory transcription factors in response to intra/inter cellular stimuli. In the Δ*pmc6* mutant cells, histone H3 methylation at Lys9 and localization of Swi6 that characterize heterochromatin, were diminished, whereas silencing at the mating locus was not affected as in the other RNAi mutants, suggesting that Pmc6 might function in the RNAi pathway. Accordingly, the centromeric non-coding RNAs that should be processed into siRNAs were accumulated in the mutant cells, suggesting that the Pmc6 is responsible for the generation of siRNA that is crucial for RNAi-dependent heterochromatin assembly pathway. We speculate that Pmc6-containing Mediator complex somehow determine the fate of the centromeric non-coding RNAs to generate siRNA through RNA polymerase II (see (7) in this report).

7) Role of RNA polymerase II in the RNAi-dependent Heterochromatin Formation: Y. MURAKAMI, H. KATO[1], A. SHIMADA, M. NAKAMA, F. BÜHLER[2], D. MOAZED[2] and K. TANAKA[3] ([1]Shimane Univ., [2]Harvard Medical School, [3]Kansei-Gakuin Univ.)

In heterochromatin formation at fission yeast centromere, the RNA interference (RNAi) machinery converts pericentromeric non-coding RNAs (ncRNA) into small interfering RNAs (siRNAs) and the siRNA is required for the assembly of heterochromatin. Specially, association of RNA induced transcriptional silencing (RITS) complex, which contains the siRNA and Argonaute homologue, to ncRNA appears to be a key step for RNAi-dependent heterochromatin formation, because artificial tethering of RITS complex to *ura4* RNA by a specific RNA binding protein induces *ura4*-siRNA formation and heterochromatin formation at *ura4* locus (Bühler et al, Cell, 125, 873-886, 2006). Previously we isolated a mutation of the second subunit of RNA polymerase II (*rpb2-m203*) that specifically inhibit RNAi-dependent heterochormatin formation but does not affect general transcription. Detailed analysis of this mutant indicated that RNA polymerase transcribes the ncRNA at centromeric repeats and couples transcription and subsequent siRNA formation (Kato et al. Science, 309, 467-469, 2005). Interestingly, We found that *rpb2-m203* did not

affect the heterochromatin formation or siRNA formation induced by the artifictial RITS tethering system. This indicates RNA polymerase II is involved in the siRNA-dependent binding of RITS complex to the non-coding RNA. Recently, RNA polymerase II in plant cells was shown to interact with one of the plant Argonaute homologues. Thus, direct recruitment of RITS complex to ncRNA by RNA polymerase II might be a basis for the coupling of transcription and siRNA processing. Now we are examining the interaction between RNA polymerase II and RITS complex in fission yeast.

List of Publication

Murakami, Y., Chen, L.-F., Sanechika, N., Kohzaki, H. and Ito, Y. (2007) Transscription factor, Runx1 recruits the polyomavirus replication origin to replication factories. J. Cell. Biochem. *100*, 1313-1323

Kohzaki H. and Murakami Y.: Faster and easier chromatin immunoprecipitation assay with high sensitivity. Proteomics, *4*, 10-14　2007

Yokoyama, M., Inoue H., Ishii, C., and Murakami Y.： The novel gene *mus7*$^{+}$ is involved in the repair of replication-associated DNA damage in fission yeast. DNA repair, *6*, 770-780, 2007

村上洋太：クロマチン高次構造と RNA　細胞　第 39 巻　20-23　2007

Murakami Y.: “Heterochromatin formation that depends on RNAi and non-coding RNA” Forth International fission yeast meeting, June 11-16, 2007, Copenhagen, Denmark

仲間美奈, 村上洋太：Non-coding RNAs associates with histone H3 RNA 若手の会 2007 年 9 月 10ー12 日　神戸

神崎秀嗣、村上洋太：染色体 DNA 複製開始点の位置決定のメカニズム　染色体ワークショップ　2007 年 1 月

仲間美奈, 村上洋太：ヘテロクロマチンにおける non-coding RNA の解析　第 30 回　日本分子生物学会　2007 年 12 月 11ー15 日　横浜

細胞生物学研究部門　　Department of Cell Biology
信号伝達学研究分野　　Laboratory of Signal Transduction

2007 年３月に生命科学研究科博士課程３年の横山美佳が無事学位を取り卒業した。われわれの研究室のメインテーマはヘテロクロマチンの形成機構であるが横山はそれとは少し離れたテーマ、DNA 損傷修復に関与する新規遺伝子 msu7 の解析を行なっていた。３月の学位取得に間に合わせるべく論文をまとめた結果,本当に期限ぎりぎりに論文が accept となった。驚いたことに、米国の損傷修復専門の研究室でも同じ遺伝子の解析を行ないほぼ同じデータを持っていて、本の少しの差で我々が先に論文発表にこぎつけたことが後からわかったのである。学位だけでなく論文そのものが期限ぎりぎりであった。過去の体験から常日頃「どんなにユニークな研究対象あるいはアイデアだと思っていても、かならず世界のどこかに二人同じことを考えている人がいると思え」と言っているのだが、またまたそれを実体験したのだ。今回は被害者（？）にならずにすんだが、今後もこのことは肝に銘じていきたい。

４月からは島根大学生物資源学部から川上慶が生命科学研究科の修士１年生として、また１０月から島根大学生物資源学部の修士１年の大屋恵梨子が特別研究学生として研究を始めた。また９月末で長らくポスドク（教務職員）として染色体複製研究をおこなっていた神崎秀嗣が去り、直接染色体複製研究を行うメンバーがいなくなった。過去長くこの研究に携わって来ただけに感慨深いものがある。神崎は現在も京都工業繊維大学の山口研を中心に同じテーマを追求している。

メインテーマでのヘテロクロマチンの研究であるが、まず生命科学研究科を単位取得の上退学し教務職員として研究をつづける道家康平のおこなうヘテロクロマチン維持機構の解析がほぼまとまった。複製依存的にクロマチンアセンブリーをおこなう CAF1 というヒストンシャペロンに着目し詳細な解析を続けた結果、この因子が実際に複製時にヘテロクロマチンの維持に機能し、その分子機構の一端を明らかにした。CAF1 がヘテロクロマチン維持に関わることはいくつかの生物で示唆されていたが、証拠はすべて間接的でそのメカニズムは不明であった。道家の結果はその点に光を当てるものであり、クロマチン構造のかかわるエピジェネティックな現象を考える上で重要なものと考えている。現在論文を投稿しているがどのような評価をうけるのか期待している。

博士２年のデータベース愛好家・島田篤はデータベースの情報をもとにシステマティックにヘテロクロマチン関連遺伝子の探索をおこなった。いくつかの興味深い遺伝子を見つけているが、今年はそのうちの一つ *ckb1* 遺伝子の解析が進んだ。Ckb1 は casein kinase II(CKII)の制御サブユニットに一つである。詳細な解析の結果、ヘテロクロマチンタンパ

ク質 HP1 の分裂酵母ホモログ Swi6 が CKII によりリン酸化をうけることにより、転写抑制活性が特異的に制御されていることがあきらかになった。このことはヘテロクロマチンが形成後、構成因子の化学修飾によりダイナミックに制御されていることを示しており大変興味深い。じつは同様の現象をアメリカの有力グループがみつけているという情報があり、同じスクリーニングで得た他の興味を引く因子の解析を休止して、最後のまとめに奮闘している。

分裂酵母のセントロメアヘテロクロマチン形成はヘテロクロマチン内で転写される non-coding RNA を契機とする RNAi 機構に依存している。博士２年の仲間美奈は、この ncRNA の動態解析をおこない、これが鋳型 DNA と hybrid を形成してクロマチンに結合しているという予想もしていなかった結果を得ている。この現象が実際のヘテロクロマチン形成にどう関わるかを示すことができれば大変面白い仕事になるだろう。

修士２年の常峰悟はヘテロクロマチン蛋白質 Swi6 がヘテロクロマチン形成やその機能において多彩な機能を果たすことに着目し、それぞれの機能特異的な変異体を取得することを試みている。実際、RNAi 依存性ヘテロクロマチン形成にのみ異常を生じていると思われる変異体が単離されつつあり、ヘテロクロマチンに依存する siRNA 合成という今まで謎であった問題に切り込むきっかけになりそうである。

修士１年の川上慶、特別研究学生の大屋恵梨子は、島根大からやってきたが、島根大在学時には、我々の研究室を 2006 年春に出て特別研究員として島根大で働く加藤太陽の薫陶を受けていた。おかげで、分裂酵母を扱う基本はできており、川上はヘテロクロマチン関連新規因子の探索、大屋は転写メディエーターの RNAi 依存性ヘテロクロマチン形成における役割、という発展が期待できるテーマに取り組んでいる。

「ヘテロクロマチン」という一つの対象でも実に多くの事象がかかわり、その全貌を明らかにするという目標はまだまだ遠いが、少しずつその目標への道筋が見えてきていると感じさせてもらえた 2007 年であった。