LABORATORY OF BIOLOGICAL DIVERSITY

KAMADA Group

Assistant Professor: *KAMADA,Yoshiaki*

Nutrients are indispensable for life. Thus, perception of the nutrient environment is also essential for cells. Eukaryotic cells employ Tor (target of rapamycin) protein kinase to recognize cellular nutrient conditions. Tor forms two distinct protein complexes, TORC (Tor complex) 1 and TORC2. TORC1 regulates rapamycin-sensitive branches of the TOR pathway, such as protein synthesis, cell cycle and autophagy. TORC1 is thought to act as a nutrient sensor, because rapamycin, a TORC1 inhibitor, mimics a starved condition. On the other hand, TORC2, whose function is insensitive to rapamycin, is responsible for actin organization and cell integrity. So far, it is not clear whether TORC2 also perceives nutrient signals.

The aim of our research group is to reveal the molecular mechanisms of how Tor receives nutrient signals and how the TORC1/2 pathways regulate each phenomenon. We have been studying Tor signaling in the budding yeast *Saccharomyces cerevisiae*, and have found three novel branches of the TOR signaling pathway (Figure 1).

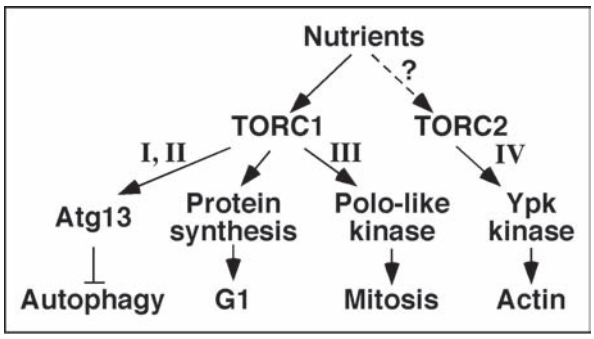

Figure 1. TOR signaling pathway of budding yeast. Our group has found three branches of the TOR pathway.

I. TORC1 phosphorylates Atg13, the molecular switch of autophagy.

Autophagy is mainly a response to nutrient starvation, and TORC1 negatively regulates autophagy. The Atg1 kinase and its regulators, i.e. Atg13, Atg17, Atg29, and Atg31 collaboratively function in the initial step of autophagy induction, downstream of TORC1. Atg1 is a Ser/Thr protein kinase, the activity of which is essential for autophagy and is largely enhanced following nutrient starvation or the addition of rapamycin. This regulation involves phosphorylation of Atg13.

We found that Atg13 is directly phosphorylated by TORC1. Phosphorylated Atg13 (during nutrient-rich conditions) loses its affinity to Atg1, resulting in repression of autophagy. On the other hand, under starvation conditions Atg13 is immediately dephosphorylated and binds to Atg1 to form Atg1 complex. Atg1 complex formation confers Atg1 activation and consequently induces autophagy. We determined phosphorylation sites of Atg13 and generated an unphosphorylatable Atg13 mutant (Atg13-8SA). Expression of Atg13-8SA induces autophagy bypassing inactivation of TORC1, such as starvation treatment or rapamycin. These results demonstrate that Atg13 acts as a molecular switch for autophagy induction.

II. Monitoring in vivo activity of TORC1 by phosphorylation state of Atg13.

Since Atg13 has turned out to be a substrate of TORC1, *in vivo* activity of TORC1 can be monitored by Phosphorylation state of Atg13. Various conditions and mutants have been examined to determine what kind of nutrients TORC1 recognizes and how nutrient signals are transmitted to TORC1.

III. Localization of Polo-like kinase is controlled by TORC1 to regulate mitotic entry.

It is well known that TORC1 regulates protein synthesis, which is important for promotion of the cell cycle at G1 (G0). Little is known, however, about whether or not TORC1 is involved in other stages of the cell cycle.

We generated a temperature-sensitive allele of *KOG1 (kog1-105)*, which encodes an essential component of TORC1. We found that this mutant, as well as yeast cells treated with rapamycin, exhibit mitotic delay with prolonged G2. We further demonstrated that this G2-arrest phenotype is due to mislocalization and resultant inactivation of Cdc5, the yeast polo-kinase. These results suggest that TORC1 mediates G2/M transition via regulating polo-kinase.

IV. Ypk2 kinase acts at the downstream of TORC2 to control actin organization.

Genetic studies have shown that TORC2 controls polarity of the actin cytoskeleton via the Rho1/Pkc1/MAPK cell integrity cascade. However, the target (substrate) of TORC1 was not yet identified. We found that Ypk2, an AGC-type of protein kinase whose overexpression can rescue lethality of TORC2 dysfunction, is directly phosphorylated by TORC2.

Publication List

〔Review article〕

● Klionsky, D.J., Abdalla, F.C., Abeliovich, H., Abraham, R.T., Acevedo-Arozena, A., Adeli, K., Agholme, L., Agnello, M., Agostinis, P., Aguirre-Ghiso, J.A. *et al.* (2012). Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy *8*, 445-544.

LABORATORY OF BIOLOGICAL DIVERSITY

OHNO Group

Assistant Professor: *OHNO, Kaoru*

The aim of this laboratory is to research reproductive hormones in invertebrates and to analyze the mechanisms by which they work. The comparisons of such molecules and mechanisms in various species are expected to provide insights into the evolution of reproductive hormone systems.

I. Gonadotropins in the starfish, *Asterina pectinifera*

Gonadotropins play important regulatory roles in reproduction in both vertebrates and invertebrates. The vertebrate gonadotropins, LH and FSH are structurally and functionally conserved across various species, whereas no such molecule has been identified in invertebrates. The insect parsin hormones are assumed to be the physiological counterpart of LH and FSH in mammals. Some gonadotropic hormones, such as the egg development neurosecretory hormone of the mosquito, the egg-laying hormone of the sea hare, and the androgenic gland hormone of the terrestrial isopod, have been found in invertebrate species. More recently, an insulin-like peptide was reported to be responsible for the regulation of egg maturation in the mosquito, *Aedes aegypti*, demonstrating the involvement of insulin signaling in egg maturation among invertebrates.

The gonad-stimulating substance (GSS) of an echinoderm, the starfish, was the very first gonadotropin to be identified in invertebrates. GSS mediates oocyte maturation in starfish by acting on the ovary to produce the maturation-inducing hormone (MIH), 1-methyladenine, which in turn induces the maturation of the oocytes. In this sense, GSS is functionally identical to vertebrate LH, especially piscine and amphibian LHs, acting on the ovarian follicle cells to produce MIH to induce the final maturation or meiotic resumption of the oocyte. Considering the functional similarity that GSS shares with vertebrate LH, it is very important from an evolutionary point of view to know the chemical and molecular structure of GSS. We cloned the gene encoding GSS referred to amino acid sequence of purified GSS from radial nerves of the starfish, *Asterina pectinifera*. Interestingly, phylogenetic analyses revealed that it belonged to the insulin/insulin-like growth factor (IGF)/relaxin superfamily and, more precisely, to the subclass of relaxin/insulin-like peptides (Figure 1).

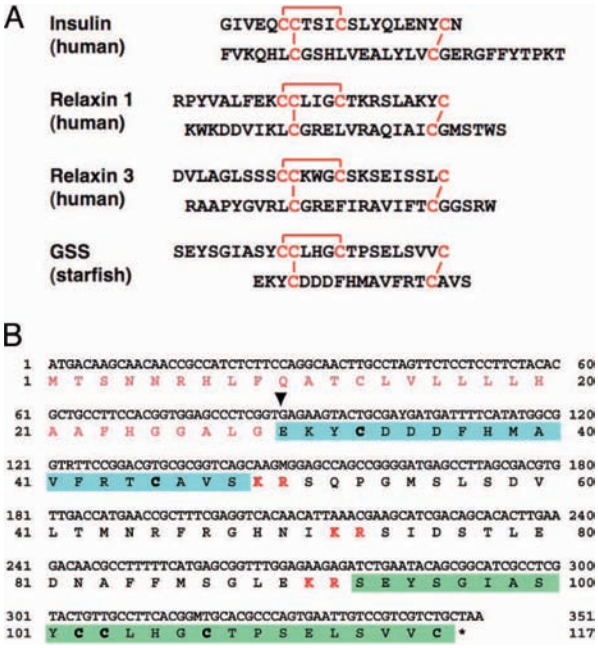

Figure 1. Amino acid sequence of starfish GSS. (A) Comparison of the heterodimeric structure of starfish GSS with those of various representative members of the insulin superfamily. The cysteine bridges are shown in red. (B) Coding DNA sequence and predicted amino acid sequences of GSS. Sequences of A and B chains are shown in green and blue boxes, respectively. Characters shown in red boldface indicate basic dipeptides that are the sites of proteolytic cleavage. Inverted triangle shows the deduced cleavage site of the signal peptide.

II. Search for reproductive hormones in invertebrates

In a collaborative effort with Prof. Yoshikuni's Laboratory of the Kyushu Univ. and Dr. Yamano and Dr. Awaji of the National Research Institute of Aquaculture, Fisheries Research Agency (NRIA), we are searching for reproductive hormones in invertebrates; sea urchin, sea cucumber, oyster, and shrimp. While the collaborators are partially purifying physiological materials which induce egg maturation from nerve extracts and analyzing them with a tandem mass spectrometer, we are creating and analyzing EST libraries from nerve tissues and developing a database of the mass analysis performed in this laboratory.

LABORATORY OF BIOLOGICAL DIVERSITY

HOSHINO Group

Assistant Professor: *HOSHINO, Atsushi*
Technical Assistant: *WATANABE, Seiko*
NAKAMURA, Ryoko
TAKEUCHI, Tomoyo

While genomic structures as well as their genetic information appear to stably transmit into daughter cells during cell division, and also into the next generation, they can actually vary genetically and/or epigenetically. Such variability has had a large impact on gene expression and evolution. To understand such genome dynamisms in eukaryotes, especially in plants, we are characterizing the flower pigmentation of morning glories.

I. Flower pigmentation patterns of the morning glories

Morning glories belong to the genus *Ipomoea* that is the largest group in the family *Convolvulaceae*. Of these, *I. nil* (Japanese morning glory), *I. purpurea* (the common morning glory), and *I. tricolor* have been domesticated well as floricultural plants, and their various spontaneous mutations have been isolated. The wild type morning glories produce flowers with uniformly pigmented corolla, whereas a number of mutants displaying particular pigmentation patterns have been collected (Figure 1). Because flower pigmentation patterns are easily observed, the molecular mechanisms underlying these phenomena provide fine model systems for investigating genome variability.

Margined, *Rayed* and *Blizzard* of *I. nil* are dominant mutations. While these mutants show distinct flower pigmentation patterns, the same pigmentation gene is repressed by non-coding small RNA in the whitish parts of the corolla. It is suggested that distinct regulation of small RNA cause the difference in pigmentation patterns. The recessive mutations, *duskish* of *I. nil* and *pearly-v* of *I. tricolor*, confer variegated flowers, and epigenetic mechanisms are thought to regulate flower pigmentation. We are currently characterizing detailed molecular mechanisms of these mutations.

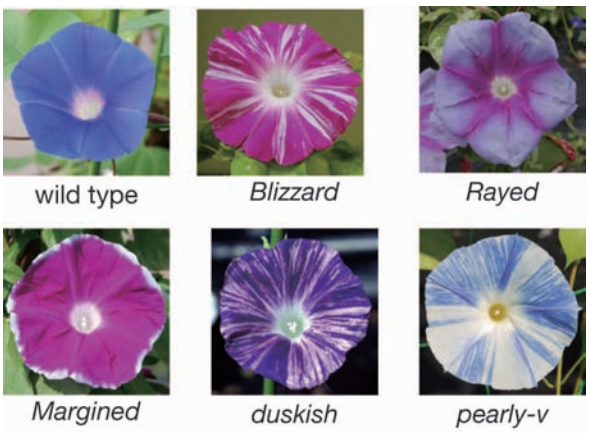

Figure 1. Flower phenotypes of the morning glories.

II. *de novo* sequencing of Japanese morning glory genome

Although morning glories are studied worldwide, especially in plant physiology and genetics, no whole nuclear genome sequences of any *Ipomoea* species are available. To facilitate the studies of our group as well as all morning glory researchers, we are conducting *de novo* genome sequencing of *I. nil*, having a genome of about 800 Mbp. We chose the Tokyo-kokei standard line for genome sequencing, and employed not only shotgun sequencing using high-throughput DNA sequencers but also BAC end sequencing. We are collaborating with several laboratories in Japan.

III. BioResource of morning glories

NIBB is the sub-center for National BioResource Project (NBRP) for morning glory. In this project, we are collecting, maintaining and distributing standard lines, mutant lines for flower pigmentation, and DNA clones from EST and BAC libraries of *I. nil* and its related species. *I. nil* has been one of the most popular floricultural plants since the late Edo era in Japan. It has extensive history of genetic studies and also has many advantages as a model plant; simple genome, large number of mutant lines, and efficient self-pollination. Our collection includes 220 lines and 117,000 DNA clones.

Figure 2. Novel spontaneous mutants isolated in NBRP. From left to right: delayed leaf senescence, morphological alteration of flowers and leaves, flower closure failing, and bushy.

Publication List

〔Original papers〕

- Park, K.I., and Hoshino, A. (2012). A WD40-repeat protein controls proanthocyanidin and phytomelanin pigmentation in the seed coats of the Japanese morning glory. J. Plant Physiol. *169*, 523-528.
- Tong, L., Fukuoka, H., Otaka, A., Hoshino, A., Iida, S., Nitasaka, E., Watanabe, N., and Kumoyama, T. (2012). Development of EST-SRR markers of *Ipomoea nil*. Breed. Sci. *62*, 99-104.

LABORATORY OF BIOLOGICAL DIVERSITY

TSUGANE Group

Assistant Professor: *TSUGANE, Kazuo*
Visiting Scientist: *HAYASHI-TSUGANE, Mika*

An active nonautonomous DNA transposon, *nDart1-0*, belonging to the *hAT* superfamily, was identified. The transpositions of *nDart1-0* were promoted by an active autonomous element, *aDart1-27*, on chromosome 6. By using the endogenous *nDart1*/*aDart1-27* system in rice, a large-scale *nDart*-inserted mutant population could be easily generated under normal field conditions, and the resulting tagging lines were free of somaclonal variation. The *nDart1* transposons tend to insert into the promoter, 5' UTR region, or exon of a gene, which suggests that the *nDart1*/*aDart1-27* system is a powerful tool for rice functional genomics. Furthermore, we are developing several *indica* lines bearing the active *nDart1*/*aDart1-27* system. These lines would effectively contribute to gene functional analysis and breeding for the *indica* rice varieties.

Activation and Epigenetic Regulation of DNA Transposon *nDart1* in Rice

A large part of the rice genome is composed of transposons. Since active excision/reintegration of these mobile elements may result in harmful genetic changes, many transposons are maintained in a genetically or epigenetically inactivated state. However, some non-autonomous DNA transposons of the *nDart1-3* subgroup, including *nDart1-0*, actively transpose in specific rice lines, such as *pyl-v* which carries an active autonomous element, *aDart1-27*, on chromosome 6. Although *nDart1-3* subgroup elements show considerable sequence identity, they display different excision frequencies. The most active element, *nDart1-0*, had a low cytosine methylation status (Figure1). The *aDart1-27* sequence showed conservation between *pyl-stb* (*pyl-v* derivative line) and Nipponbare, which both lack autonomous activity for transposition of *nDart1-3* subgroup elements. In *pyl-v* plants, the promoter region of the *aDart1-27* transposase gene was more hypomethylated than in other rice lines. Treatment with the methylation inhibitor 5-azacytidine (5-azaC) induced transposition of *nDart1-3* subgroup elements in both *pyl-stb* and Nipponbare plants; the new insertion sites were frequently located in genic regions. 5-azaC treatment principally induced expression of *Dart1-34* transposase rather than the other 38 *aDart1*-related elements in both *pyl-stb* and Nipponbare treatment groups. Our observations show that transposition of *nDart1-3* subgroup elements in the *nDart1*/*aDart1*-tagging system is correlated with the level of DNA methylation. Our system does not cause somaclonal variation due to an absence of transformed plants, offers the possibility of large-scale screening in the field, and can identify dominant mutants. We, therefore, propose that this tagging system provides a valuable addition to the tools available for rice functional genomics.

Publication List

〔Original paper〕

● Eun C.-H., Takagi, K., Park, K.I., Maekawa, M., Iida, S., and Tsugane, K. (2012). Activation and epigenetic regulation of DNA transposon *nDart1* in rice. Plant Cell Physiol. *53*, 857-868.

〔Review article〕

● Saze, H., Tsugane, K., Kanno, T., and Nishimura, T. (2012). DNA methylation in plants: Relationship with small RNAs and histone modifications, and functions in transposon inactivation. Plant Cell Physiol. *53*, 766-784.

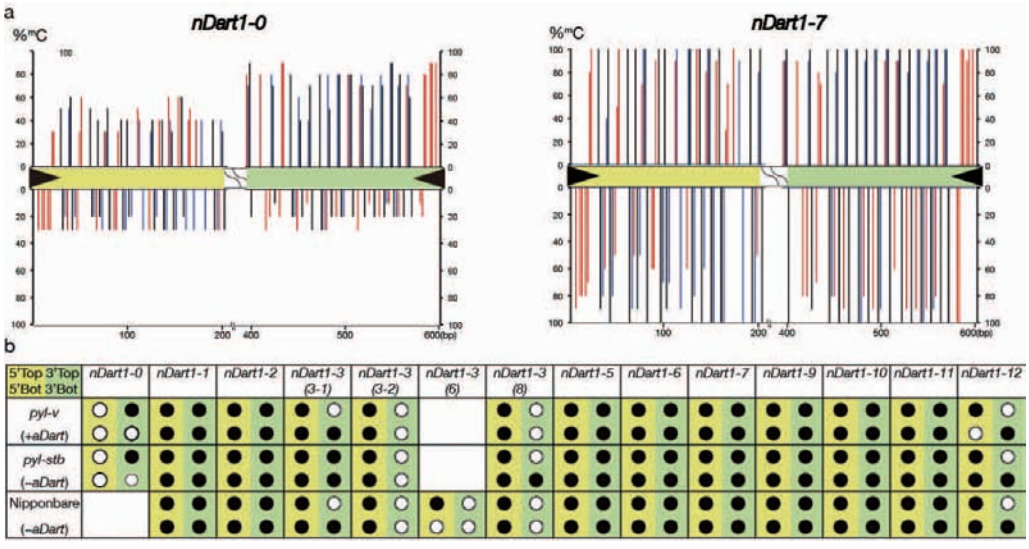

Figure 1. Bisulfite sequencing of *nDart1-3* subgroup elements in rice. (a) Methylation patterns of active *nDart1-0* and inactive *nDart1-7* elements in *pyl-stb* plants. Red, black and blue vertical lines indicate CG, CHG, and CHH, respectively. The top (upper regions) and bottom (lower regions) strands are indicated in the *nDart1-0* and *nDart1-7* boxes. (b) Summary of the bisulfite sequencing analysis of *nDart1-3* subgroup elements in rice. Open and closed circles define the hypomethylation or hypermethylation state of 10 sequenced clones, respectively.

LABORATORY OF BIOLOGICAL DIVERSITY

JOHZUKA Group

Assistant Professor: *JOHZUKA, Katsuki*
Technical Assistant: *ISHINE, Naomi*

Chromosome condensation is a basic cellular process that ensures the faithful segregation of chromosomes in both mitosis and meiosis. This process is required not only for shrinking the length of chromosome arms, but also for resolving entanglements between sister-chromatids that are created during DNA replication. Any abnormality in this process leads to segregation errors or aneuploidy, resulting in cell lethality. Studies in the past decade have demonstrated that chromosome condensation is mainly achieved by condensin, a hetero-pentameric protein complex, widely conserved from yeast to humans. Despite its conservation and importance for chromosome dynamics, how condensin works is not well understood. Recent studies reveal that condensin functions are not restricted to chromosome condensation and segregation during cell divisions. It is required for diverse DNA metabolism, such as genome stability, transcriptional regulation, and cell differentiation.

Our research interest is to understand the mechanisms and regulation of chromosome condensation. We have been studying the role of condensin in the budding yeast *Saccharomyces cerevisiae*. Microscopic observation indicated the nucleolar localization of condensin. Consistent with this, the ribosomal RNA gene (rDNA) repeat is the most condensed region in the genome during mitosis. We have found that condensin specifically binds to the RFB site located within the rDNA repeat. By genetic screening, we further discovered the multiple proteins interaction network recruits condensin to the RFB site.

I. Dynamic relocalization of condensin during meiosis

Our genetic screening indicated that two proteins, Csm1 and Lrs4, were required for condensin recruitment to the RFB site. Physical interactions between Csm1/Lrs4 and subunits of condensin are important for recruitment of condensin to the RFB site. These proteins are known as components of monopolin complex that are required for faithful segregation of homologous chromosomes during meiotic division I. During meiosis I, monopolin complex re-localizes from rDNA repeat to the centromere and acts for ensuring sister-chromatid co-orientation. Re-localization of Csm1/Lrs4 proteins suggested the re-localization of condensin from rDNA repeat to centromere. As expected, chromatin-IP experiments indicated that condensin re-localizes to the centromere during meiosis I. Condensin might clamp sister-chromatids together during meiosis I.

II. Condensin-dependent chromatin folding

The RFB site, which consists of a ~150bp DNA sequence, is functioning as a cis-element recruitment of condensin onto chromatin in the yeast genome. If the RFB site is inserted into an ectopic chromosomal locus, condensin can associate with the ectopic RFB site. To explore the role of condensin in chromosome organization, we have constructed a strain in which two RFB sites are inserted on an ectopic chromosome arm at an interval of 15kb in the cell with complete deletion of chromosomal rDNA repeat. Using this strain, condensin-dependent chromatin interaction between two RFBs was examined by chromosome conformation capture (3C) assay. We found condensin-dependent chromatin interaction between the two RFB sites on the chromosome arm. This chromatin interaction is already observed in interphase cells, and its frequency increased in metaphase arrested cells. These results indicate that condensin plays a role in chromatin interaction between condensin binding sites and this interaction lead to creation of a chromatin loop between those sites (Figure 2). It is thought that condensin-dependent chromatin folding is one of basic molecular processes of chromosome condensation. In addition, condensin-dependent chromatin folding observed during interphase cells suggest that other unknown reaction(s) are necessary for mitotic chromosome condensation, in addition to simple chromatin folding.

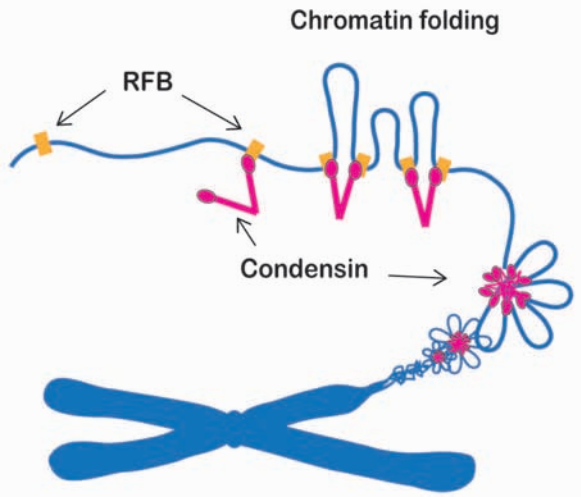

Figure 1. A Schematic model of chromosome condensation. Condensin makes chromatin interactions between adjacent binding sites (RFB, for example). This lead to a folding of chromatin fiber between the sites, as a basic process of chromosome condensation.

LABORATORY OF BIOLOGICAL DIVERSITY

WATANABE Group †

Assistant Professor: *WATANABE, Takaaki*
Technical Assistant: *YONEZAWA, Harumi*

Genomes have been dynamically evolving and are continually changing during development, and through diseases and environmental stress. One type of genome alteration, gene amplification, is involved in various biological phenomena, such as malignant progression of cancer, resistance to insecticides and anticancer drugs, and gene evolution. We are addressing the molecular mechanisms underlying gene amplification from a variety of perspectives.

I. Model systems for studying mechanisms for gene amplification

Long series of studies have shown that DNA double-strand breaks and inverted repeats play an important role in gene amplification. However, details of the molecular mechanisms remain to be determined. This is because previous approaches to understanding the mechanisms were based on the structural analysis of complex end products and very few model systems are available that allow chromosomal engineering and genetic analysis.

To better understand the molecular mechanisms, we have developed a new approach in which we design amplification processes and test whether the processes can produce the amplification seen in nature. Previously, we constructed a system designed to induce a rapid amplification mode, double rolling-circle replication, (DRCR, Figure 1A) via chromosomal breaks induced by site-specific endonuclease (EMBO J, 2005). This system produced intra-/extra-chromosomal products resembling those seen in mammalian cells; homogeneously staining regions (HSR) and double minutes (DMs). This result strongly suggested that amplification in mammalian cells involves DRCR.

We next examined whether recombinational processes coupled with replication can induce gene amplification via DRCR, using a distinct process, Cre-lox site-specific recombination. Here, we inferred that, if Cre recombination coupled with replication occurs, the replication fork makes an additional copy of the replicated region (Figure 1B); and that the processes from two pairs of lox sites could induce DRCR (Figure 1C). In this study, we successfully detected HSR/DM-type amplification products in yeast and Chinese hamster ovary (CHO) cells (Figure 1D and 1E). Surprisingly, over 10% of the Cre recombination-induced yeast cells undergo gene amplification. In addition, scattered-type products were also found (Figure 1F), which are frequently seen in cancer cells. From these results, we reasoned that DRCR and convergent replication are centrally involved in the amplification of drug-resistance genes and oncogene. This system can serve as a good model for amplification in mammalian cells and contribute to a better understanding of oncogene amplification and development of anticancer strategies in future.

II. Intensive rearrangement in amplified region

In amplified chromosomal regions intensive chromosome rearrangements are frequently observed, leading to an increase in the gene copy number and to a decrease in size of the amplification unit. In oncogene amplification, the complex patterns of amplification generated by the rearrangements are closely associated with poor prognosis in cancer. Interestingly, we have observed the rearrangement in all our DRCR systems.

To explore the link between the rearrangements and the DRCR process, we constructed a system that can turn on or off the occurrence of DRCR, using yeast 2μ plasmid. This system demonstrated that inversions, deletions, or duplications could be intensively induced in a DRCR-dependent manner. This result suggests that DRCR may cause the rearrangements in amplification in nature. We proposed a model in which DRCR markedly stimulates recombinational events.

III. A structural platform for gene amplification

Based on our results, we now focus on a type of genomic structure consisting of two sets of inverted repeats (IR), designated double IR. This structure is found in the human genome and can be observed in the early stages of gene amplification. The double IR constructed in yeast can induce gene amplification as seen in our DRCR systems, suggesting that double IR serves as a source or key intermediate of DRCR amplification in nature. To investigate the molecular mechanisms underlying double IR-based amplification, we have disrupted several genes involved in genome instability in a yeast strain carrying double IR, and constructed double IR in CHO cells.

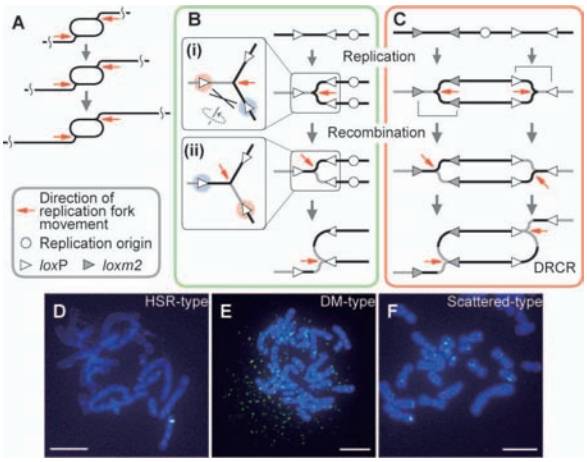

Figure 1. DRCR process, recombinational process coupled with replication, and amplification products in CHO cells.

†: This group was closed on 31 March, 2012.