Poster Abstracts
**Dynamic events of sister chromosomes in the cell cycle of* Escherichia coli**

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Various events involving partitioning of sister chromosomes were precisely analyzed in *Escherichia coli* cells. To examine the cohesion between sister chromosomes, we analyzed living cells growing under various conditions for the number of the replication origin (*oriC*) copies by flowcytometry and the foci of *oriC* by fluorescence microscopy. The average number of the *oriC* foci per cell was significantly smaller than the number of *oriC* copies per cell with few exceptions, indicating the cohesion between sister *oriC* copies. Sister *dif* copies were also found to cohesive.

MukB is a functional homologue of eucaryote SMC (structural maintenance of chromosomes). Analysis of the foci of MukB-GFP indicates that at least two MukB clusters existed when the chromosome initiated replication. Immunofluorescence microscopy for nascent DNA pulse-labeled by 5-bromo-2’-deoxyuridine (BrdU) indicated that paired replication forks acting in bidirectional replication could migrate toward opposite directions during ongoing replication. Additionally, immunofluorescent foci of ∆-subunit of DNA polymerase III holoenzyme showed different patterns through the BrdU pulse-labeled nascent DNA. Based on these results together with analysis of cell length (cell cycle), nucleoid morphology and septum formation in wild-type and *mukB* null mutant cells, we propose a model of sister chromosome partitioning.

**Medium C**

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Coordinated Requirements of Human Topo II and Cohesin for Metaphase Centromere Alignment under Mad2-dependent Spindle Checkpoint Surveillance

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Cohesin maintains sister chromatid cohesion until its Rad21/Scc1/Mcd1 is cleaved by separase during anaphase. DNA topoisomerase II (topo II) maintains the proper topology of chromatid DNAs, and is essential for chromosome segregation. Here we report direct observations of mitotic progression in individual HeLa cells after functional disruptions of hRad21, NIPBL, a loading factor for hRad21, and topo II [1,2] by RNAi and a topo II inhibitor, ICRF-193. Mitosis is delayed in a Mad2 dependent manner following disruption of either or both cohesin and topo II. In hRad21 depletion, pericentric DNA architecture becomes aberrant in interphase, and anaphase is virtually permanently delayed as pre-separated chromosomes are misaligned on the metaphase spindle. Topo II disruption perturbs centromere organization leading to intense Bub1, but no Mad2 on kinetochores and sustains a Mad2-dependent delay in anaphase onset with persisting securin signals. Thus topo II impinges upon centromere/kinetochore function. Disruption of topo II by RNAi or ICRF-193 overrides the mitotic delay induced by cohesin depletion. Sister centromeres are aligned and anaphase spindle movements occur. The ensuing accumulation of catenations in preseparated sister chromatids may overcome the reduced tension arising from cohesin depletion, causing an override. Cohesin and topo II have distinct, yet coordinated functions in metaphase alignment.
Construction of gene network based on comprehensive analysis of \textit{S.pombe} temperature sensitive mutant library

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Genome-wide analysis is a special feature of post-genomic molecular and cell biology. The availability of temperature-sensitive (ts) mutations in all the essential genes of the fission yeast \textit{Schizosaccharomyces pombe} would be of considerable use. \textit{S. pombe} has \textasciitilde 5,000 protein-coding genes, and \textasciitilde 900 (18\% of the total genes) are estimated to be essential. 1015 ts mutant strains were made by random mutagenesis. Their phenotypes were characterized and classified into subtypes. An attempt to clone genes responsible for all of these mutations has been made by transformation using a genomic DNA library. We can obtain information not just on the mutant genes, but also their multicopy suppressor genes at the same time by this method. Transformation to 1015 strains was completed and here we show results of 300 strains. For 239 strains we successfully recovered genomic DNA library plasmids, which suppress ts phenotypes and it became clear by sequencing that 628 species of plasmid was obtained. Genomic DNA library plasmid contains some ORF, so we are trying to identify mutant genes and multicopy suppressor genes by subcloning. Now we predict about 60\% of mutants are novel ts mutants and mutant genes are implicated in following diverse cellular functions: cell cycle control, transport, metabolism, transcription, RNA processing, cytoskeleton, signal transduction, ribosome biogenesis, translation and so on. We also found many novel genetic interactions. For example G protein related factors and farnesyl pyrophosphate synthetase show genetic interaction with many genes. Gene network will be constructed by comprehensive analysis of ts mutant library and finding many genetic interactions.
Involvement of Sds23 in nutrients consumption switching at G2/M transition

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Fission yeast cells elongate in interphase, and when the critical size is attained, growth ceases and M phase is initiated. At G2/M transition, nutrient consumption seems to be changed from cell growth to mitosis. Our interest is how this switching is regulated. If this switching is deficient, cells are expected to continue growing in M phase, and to show aberrantly elongated morphology. In our fission yeast temperature sensitive mutant library, there were some ts strains, which show such a phenotype. We found that many of them were suppressed by the multicopy sds23\(^+\) gene. We investigated 11 mutants of them whether the ts phenotype is genetically linked with genomic sds23\(^+\) locus, but no linkage was observed in all these strains. This indicates that sds23\(^+\) gene is multicopy suppressor of these mutants. 6 of these 11 strains were also suppressed by multicopy ssp1\(^+\) gene, and we found ts phenotypes of these were genetically linked with ssp1\(^+\) locus, which indicate that these 6 strains were ssp1 mutant. Although we have not yet identified the mutated gene of remaining 5 strains, these genes might be functionally related to sds23\(^+\). To identify proteins physically interacting with Sds23, we performed immunoprecipitation and analyzed by LC/MS/MS. We found that Type 2A family protein phosphatases, both catalytic subunit (Ppa1, Ppa2, Ppe1) and regulatory subunit (Ppa1, Ekl1), are coimmunoprecipitated with Sds23. The phenotype of ts strains investigated in this study is different from the known phenotype of mutants deficient in these phosphatases. Therefore, it is unlikely that the mutated gene of these ts strains is the gene coding these protein phosphatases. By LC/MS/MS, we also identified C-terminal phosphorylated peptides of Sds23, which contain MAP kinase or Cdc2 kinase consensus sequence. From these results, Sds23 might be the target of MAP kinase or Cdc2 kinase, and function in nutrient consumption switching in association with Type 2A family protein phosphatases.
Sty1/Spc1 MAP Kinase Enhances Securin / Separse Interaction that Facilitates Sister Chromatid Separation under Osmostress

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Separase-securin complex is essential for sister chromatid separation during mitotic anaphase. Separse is activated by destruction of associating securin protein, and enable sister chromatid separation by cleaving Rad21 protein. Rad21 is a component of cohesin complex, which is responsible for sister chromatid cohesion. The temperature sensitive mutants of fission yeast securin, cut2 and separase, cut1 have defect in sister chromatid separation, and show similar distinctive cut phenotype so-called "archery-bow". We found that the phenotypes of both of cut1 and cut2 mutants are suppressed when high concentration of KCl or sorbitol is added in the culture medium. In fission yeast, MAP kinase Spc1/Sty1 pathway is required for cell to react to most of the environmental stresses including osmostress. We have several genetic evidences that this suppression of cut1 and cut2 mutants requires Spc1/Sty1 MAP kinase pathway and also showed constitutive active form of MAPKK Wis1 can partly suppress these mutants. We also found that significant increase of Cut1/Cut2 protein complex was observed in cells under suppression by osmotic stress. We are thinking that this is caused by enhanced interaction between Cut1 and Cut2 protein. This increase of the complex is also dependent on the MAP kinase pathway. Mass spectrometric analysis of over expressed Cut2 protein indicates that modification state of Cut2 protein is modulated by addition of 1.2M sorbitol. Osmostress seems to facilitate separase and securin function directly because osmostress is not able to bypass anaphase-promoting proteolysis. We also found that artificial minichromosome became unstable in the cells under osmostress and that the toxicity of microtubule destabilizing drug TBZ was alleviated by osmostress. We want to elucidate how MAP kinase pathway is involved in regulation of sister chromatid separation, and the meaning of the connection between environmental stress and regulator of cell cycle.
Separase-mediated cleavage of cohesin at interphase is required for DNA repair.

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Sister chromatids are held together by cohesins. At anaphase, separase is activated by degradation of its inhibitory partner, securin. Separase then cleaves cohesins, thus allowing sister chromatid separation. Fission yeast securin (Cut2) has destruction boxes and a separase (Cut1) interaction site in the amino and carboxyl terminus, respectively. Here we show that securin is essential for separase stability and also for proper repair of DNA damaged by ultraviolet, X-ray and gamma-ray irradiation. The cut2-EA2 mutant is defective in the repair of ultraviolet damage lesions, although the DNA damage checkpoint is activated normally. In double mutant analysis of ultraviolet sensitivity, checkpoint kinase and excision repair rad13 mutants were additive with cut2-EA2, whereas recombination repair rhp51 and cohesin subunit rad21 mutants were not. Experiments using either mutant cohesin that cannot be cleaved by separase or a protease-dead separase provide evidence that this DNA repair function of securin-separase acts through the cleavage of cohesin. Cohesin was hyper-phosphorylated on ultraviolet irradiation in a Rad3 kinase-dependent way. The rad21 mutant whose Rad3 target sites were all changed to alanine (rad21-5A) showed synthetic growth defect when crossed with cut1 mutant. We propose that the securin-separase complex might aid DNA repair by removing local cohesin in interphase cells, and the Rad3 dependent phosphorylation on Rad21 may facilitate the cleavage of cohesin by separase on DNA damage.
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Cdc48 Is Required for the Stability of Cut1/Separase in Mitotic Anaphase

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Separase, a large protease essential for sister chromatid separation, cleaves the cohesin subunit Scc1/Rad21 during anaphase and leads to dissociation of the link between sister chromatids. Securin, a chaperone and inhibitor of separase, is ubiquitinated by APC/cyclosome, and degraded by 26S proteasome in anaphase. Cdc48/VCP/p97, an AAA ATPase, is involved in a variety of cellular activities, many of which are implicated in the proteasome-mediated degradation. We previously reported that temperature-sensitive (ts) fission yeast Schizosaccharomyces pombe cdc48 mutants were suppressed by plasmid carrying the cut1/separase gene and that the defective mitotic phenotypes of cut1 and cdc48 were similar. We here describe characterizations of Cdc48 mutant protein and the role of Cdc48 in sister chromatid separation. Mutant residue resides in the conserved D1 domain within the central hole of hexamer, while Cdc48 mutant protein possesses the ATPase activity. Consistent with the phenotypic similarity and the rescue of cdc48 mutant by overproduced Cut1/separase, the level of Cut1 is greatly diminished in cdc48 mutant. We show that the stability of Cut1 during anaphase requires Cdc48. Cells lose viability during the traverse of anaphase in cdc48 mutant cells. Cdc48 may protect Cut1/separase from the instability upon ubiquitin-mediated Cut2/securin degradation.
A novel interphase phenotype of *S. pombe* condensin SMC subunit cut14/smc2 mutant

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Condensin is a highly conserved protein complex composed of five subunits, all of which are essential for chromosome condensation and segregation in mitosis. Cut3 and Cut14 belong to the SMC (structural maintenance of chromosomes) family. We previously showed that Cnd2, one of the three non-SMC Cnd subunits, is required for DNA repair and its defect was recognized by DNA checkpoint system: *cnd2-1* is hypersensitive to UV irradiation and hydroxyurea (HU) at the permissive temperature, and abolished the activation of Cds1, a DNA checkpoint kinase, in the presence of HU. To examine whether condensin mutants other than *cnd2-1* may have similar phenotype, we investigated the UV and HU sensitivity of other condensin alleles, and found that one *smc2* mutant allele *cut14-tsY566* was hypersensitive to HU and UV. The *cnd2-1* phenotype might thus be not restricted to the non-SMC trimeric subcomplex, and instead the whole condensin holo-complex might be involved in the interphase role.

The *cut14-tsY566* mutation site resides in a conserved amino acid in the hinge of Cut14. It was reported that the chromatin isolated from *cut14-208*, another *cut14* mutant identified previously, showed hypersensitivity to S1 nuclease. S1 nuclease is specific to single-strand DNA. The chromatin isolated from *cut14-tsY566* also showed the sensitivity to S1 nuclease. For more detailed analysis, we made the double mutant of *cut14-tsY566* and *ssb1-418*, the mutant of RPA large subunit. The temperature sensitivity of *cut14-tsY566* was partially suppressed by the mutation of *ssb1-418*. In addition, the accumulation of Rad 22-YFP foci in *cut14-tsY566* at permissive temperature was also suppressed by the *ssb1-418* mutation. The DNA damage sensitivity of *cut14-tsY566* might be due to the recognition of the aberrant chromatin structure by RPA. Condensin complex might have an important role for the genome stability even in interphase.
Condensin localizes to kinetochores at early M phase and is required for chromosome segregation even in anaphase

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Condensin is a conserved protein complex that plays a central role in mitotic chromosome condensation. In this study, we investigated condensin localization and its roles in late mitosis using fission yeast *S. pombe* through detailed time-lapse imaging of living cells. Condensin subunits, Cut14/SMC2 and Cnd1, which is one of non-SMC subunits, tagged with GFP co-localized with a conserved kinetochore protein Mis12 during early M phase. We also detected Cut14 association to inner centromeric DNA by chromatin immunoprecipitation (ChIP). This kinetochore localization of condensin subunits is significant because that was diminished in *cut14-208* temperature sensitive (ts) mutant and altered in topoisomerase-I mutant. In *cut14-208* mutant, the movement of SpCENP-A protein became abnormal and both sister centromeric DNAs tended to remain in close proximity to one of the separating SPBs at early M phase. These results indicated that condensin functions at kinetochores in early to mid mitosis. Furthermore, *nda3* *cut14-208* mutant cells were arrested at prophase at the cold temperature 20°C, then transferred to 36°C to specifically inactivate Cut14 protein only in anaphase. The double mutant showed aberrant, “streaked” chromosomes and subsequently showed fragmented chromosomes or unequal segregation. Therefore, we propose that condensin maintains chromosome condensation even in anaphase for faithful segregation of sister chromatids.
The Amino Terminal Region of the Mis6 Complex Component Mis17 is Hyper-Phosphorylated and Plays a Role in Kinetochore Function

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Fission yeast centromeres contain specialized chromatin that includes the centromere-specific histone H3 variant in Cnp1/spCENP-A. The kinetochore protein, Mis6/CENP-I is also required for maintaining the central domain of the centromeres. Mis17 is a very serine rich kinetochore protein composed of 441 amino acids identified through temperature sensitive mutant mass-screening, and has no homologous gene in other species. Similarly to other kinetochore mutants, mis17-362 ts mutant exhibits unequal chromosome segregation, and its viability dramatically decreased at the restrictive temperature. Mis17 likewise Mis15 is a component of the Mis6 complex. Therefore, we started characterization of Mis17 in order to clarify the molecular function of the complex.

Mis17 was hyper-phosphorylated, and the phosphorylation bands observed by immuno blotting declined in mis17-362 mutant at the restrictive temperature. The reduction of Mis17 phosphorylation was also observed in cnp1-1, mis6-302, mis15-68, mis16-53 and mis18-262 mutants. Through truncation analysis, the phosphorylation was proved to concentrate on the N-terminal half region of Mis17. When the Mis17 N-terminus overproduced in wild type cells, unequal chromosome segregation was observed. In this condition, Mis15-GFP, Cnp1-GFP still localized to kinetochores of missegregated chromosomes. According to ChIP analysis, Mis6-HA also associated central domain of the centromeres in the Mis17 N-terminus overproduction background. These maintained localizations of Mis6, Mis15 and Cnp1 on kinetochores were unexpected and very interesting, because in mis17-362 mutant background, the kinetochore localizations of Mis6, Mis15 and Cnp1 were dramatically diminished at the restrictive temperature. At the present, whether Mis17 phosphorylation plays a role in the kinetochore function and how overproduced N-terminus Mis17 effects on endogenous Mis17 are under investigation.
Mis16 and Mis18 are required for CENP-A loading and histone deacetylation at centromeres

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Centromeres contain specialized chromatin required for equal segregation of chromosomes. A centromere specific histone H3 variant, spCENP-A/Cnp1, and other proteins (Mis6/CENP-I/Ctf3 and Mis12/Mtw1) are essential for forming the central element of the fission yeast centromere. We report the identification of five fission yeast centromere proteins, Mis14-Mis18. Mis14 interacts physically with the known kinetochore component Mis12. Recruitment of Mis14 and Mis12 to centromeres does not depend upon the prior recruitment of CENP-A and, conversely, CENP-A does not require Mis14 or Mis12 to associate with centromeres. In contrast, Mis6, Mis15, Mis16 (strong similarity with human RbAp48 and RbAp46), Mis17 and Mis18 are all part of the CENP-A recruitment pathway. Mis6, Mis15 and Mis17 form an evolutionarily conserved complex. Mis16 and Mis18 form a complex and maintain the deacetylated state of histones specifically in the central core of centromeres. Mis16 and Mis18 are the most upstream factors in centromere assembly as they can associate with centromeres in all centromere protein mutants except for mis18 and mis16, respectively. RNAi knock down in human cells shows that Mis16 function is conserved as RbAp48 and RbAp46 are both required for localization of human CENP-A.
Permission of CENP-A Loading Requires Telophase Centromere Protein Complex of Mis18 that Guides Hat1-binding RbAp46/48/Mis16

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Centromere is chromosomal site that joins to microtubules during mitosis for proper segregation. CENP-A, a histone H3 variant, is a component of centromere-specific nucleosome. We show that three human proteins, hMis18[1], hMis18[2] and M18BP1, form the complex and act as essential upstream loading factors for CENP-A. These proteins neither bind to CENP-A nor to mitotic centromeres but are accumulated in telophase centromeres, prior to DNA replication. The same behaviour is found for S. pombe that contains only single spMis18. Substitution mutations in the conserved, functionally essential residues abolish centromere localization of spMis18 and hMis18[1]. Evidence is provided that human Mis18 complex plays a role for guiding or shuttling nuclear protein RbAp46/48 to centromere. RbAp46 and its fission yeast homologue Mis16 are bound to histone acetylase Hat1 and may remodel chromatin structure appropriate for CENP-A loading. Mis18 and RbAp46/48/Mis16 are physically interacted in human and fission yeast, and they permit CENP-A loading, through modifying specifically pre-replicative centromere chromatin possibly at the level of protein acetylation.
Conserved Mis12 Centromere Complex: Link to Heterochromatin HP1 and Outer Kinetochore Zwint-1

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Kinetochore chromatin protein Mis12 is essential for equal chromosome segregation and evolutionarily conserved from yeast to human. In fission yeast and human cells, loading of Mis12 to kinetochore is independent of CENP-A that is a histone H3 variant specific to centromere. To identify the gene products interacted with Mis12, we investigated and identified components of the Mis12 complex, first in fission yeast by genetic interactions followed by immunoprecipitation and then in human cells by immunoprecipitation followed by mass spectrometry. We found five conserved core components, spMis12/hMis12, Mis13/c20orf172, Mis14/DC8, Nnf1/PMF1 and Spc7/AF15q14. In human cells, HP1 (heterochromatin protein-1) and Zwint-1, Hec1 (reported as a outer kinetochore protein) were bound to the hMis12 complex. The chromoshadow domain of HP1 was essential for the interaction with hMis12 core complex. The interaction between hMis12 and HP-1 are functionally significant, because double HP1 RNAi abolishes kinetochore localization of hMis12 and DC8. Centromeric HP1 may be the base to anchor the hMis12 core complex that is enriched with coiled coil and extends to outer Zwint-1 and Hec1 during mitosis.
hMis12 RNAi causes spindle rotation in HeLa cells.

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Kinetochore protein Mis12 is essential for equal chromosome segregation and evolutionarily conserved from yeast to human. The human homologue hMis12 located at the kinetochore region indistinguishable from CENP-A, a centromeric histoneH3 variant, during mitosis. The reduction of hMis12 by RNAi led to chromosome misalignment at metaphase, chromosome lagging in anaphase, and micronuclei formation in interphase, while CENP-A was located at kinetochore. The similar mitotic phenotype was found in CENP-A knockdown cells, but hMis12 was maintained at the kinetochore. These results suggest that there are at least two independent loading pathways for kinetochore proteins, one dependent on CENP-A and the other for hMis12 (Goshima et al., JCB 2003). Mass spectrometric analysis reveals that hMis12 forms protein complex with c20orf172, DC8, PMF1, KIAA1570, Zwint1, HEC1, and HP1 (obuse et al., NCB 2004). Here we report that hMis12 locates at cell cortex as well as at kinetochore, and is required for the stable positioning of metaphase spindle in HeLa cells. We observed Histone-H2B-CFP and tubulin-YFP in living cells after RNAi and found that WT cells maintain constant metaphase spindle position throughout mitosis, but hMis12 RNAi cells cause severe spindle rotation. Such spindle rotation phenotype was also observed in DC8 or PMF1 RNAi cells, but not in CENP-A RNAi cells. These results suggest that hMis12 complex is specifically required for the spindle positioning in HeLa cells. Next we used some drugs to investigate the factor related to the spindle rotation after hMis12 RNAi. CytochalasinD, inhibitor of actin polymerization, and low dose of Nocodazole, inhibitor of microtubule polymerization, suppressed the spindle rotation. These results suggest that the spindle rotation after hMis12 RNAi was dependent on actin on the cell cortex and aster MTs. Through the live imaging, we found that GFP-hMis12 locates at cell cortex as well as kinetochore in mitosis. hMis12 complex may stabilize the association not only between centromere and kMTs, but also between cell cortex and asterMTs.
Conserved inner region of histone H2B required for monoubiquitination, centromere function, silencing and chromosome segregation

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To dissect the role of histones in chromatin dynamics, the reiterated nature of histone genes has hampered genetic approach. We here report isolation of three temperature-sensitive (ts) *S. pombe* strains, containing amino acid substitutions in the sole histone H2B gene (*htb1*). The mutation sites reside in the highly conserved, non-helical residues of H2B, which are implicated in DNA-protein or protein-protein interactions in the nucleosome. In the allele of *htb1-72*, the substitution (G52D) occurs at the DNA binding loop L1, causing disruption of the gene silencing in heterochromatic regions and lagging chromosomes in anaphase. In another allele *htb1-223* (P102L) locating in the junction between 3 and C, the mutant residue is in contact with H2A and other histones, leading to structural aberrations in the central centromere chromatin and unequal chromosome segregation in anaphase. The third allele *htb1-442* (E34K) near 1 displayed little defect. Evidence is provided that mono-ubiquitinated H2B is greatly unstable in P102L mutant, possibly due to proteasome-independent destruction or enhanced deubiquitination. Functional implications of DNA-H2B and H2B-other histone interactions are thus quite distinct.
Cdc2 phosphorylation of fission yeast Dis1 similar to XMAP215/TOG improves segregation accuracy via metaphase kinetochore localization

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Spindle formation and microtubule dynamics are vital for chromosome segregation. Kinetochore microtubules are shortened in anaphase to bring separated chromatids toward the opposite spindle poles. Fission yeast Dis1, similar to frog XMAP215, human Tog, fly mini spindles, nematode Zyg-9 and budding yeast Stu2 that are required for proper microtubule dynamics, is immuno-coprecipitated with the central centromere DNAs in mitosis. We here report that Dis1 is hyper phosphorylated by Cdc2 in mitotic metaphase and rapidly dephosphorylated in anaphase. Whilst Dis1-6A mutant cells that substitute all of the six Cdc2 sites can produce colonies at 22-36°C, they lose minichromosome at a high frequency, become hypersensitive to a tubulin poison and synthetic lethal with mis12-537, a centromere defective mutant. Cdc2 phosphorylation of Dis1 improves the fidelity of chromosome segregation through conferring on Dis1 the ability to locate at metaphase kinetochores. The signals of GFP-tagged unphosphorylatable Dis1-6A are greatly diminished in the metaphase kinetochores, but instead present along the short spindle. The wild type Dis1 is enriched at the plus and minus ends of microtubules, respectively, in metaphase and anaphase. The transition is regulated by Cdc2 phosphorylation and dephosphorylation. Dis1-6A resembles and partly substitutes Alp14/Mtc1, another XMAP215-like protein in fission yeast.
Toward identification of a factor involved in silencing the spindle checkpoint in Fission yeast

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The spindle checkpoint plays a crucial role in ensuring the fidelity of chromosome segregation. It delays the onset of anaphase until all the kinetochores are attached to the spindle. Mad2, which is one of the key components, binds to Slp1/Cdc20 and prevents it from promoting destruction of an anaphase inhibitor, Cut2/securin.

In contrast to the activation of the checkpoint, it has been far less understood how this signal is silenced. Though it has been reported that p31 comet is required for silencing this checkpoint in mammalian cells, homology search showed that S. pombe doesn’t have homologue of p31 comet. However, this checkpoint mechanism is well-conserved. Thereby, we speculate that there is an unknown factor which functions like p31 comet.

In order to examine the above possibility, we attempted to isolate a mutant defective in silencing the spindle checkpoint. We constructed a strain in which expression of Mad2 depends on the absence of Thiamine. When Mad2 is not expressed, this strain can bypass the requirement for the checkpoint silencer. After mutagenesis of the strain, we obtained 28 mutants which exhibit a temperature sensitivity only when Mad2 is expressed. Out of obtained 28 clones, 19 mutants, which displayed mitotic abnormal phenotypes under the restrictive temperature, were further examined for accumulation of Slp1, a protein which is normally degraded at the onset of anaphase. If a mutant is defective in the silencer of the spindle checkpoint, the accumulated Slp1 should remain as a complex with Mad2. By immunoprecipitation, we are currently investigating whether Slp1 binds to Mad2 in each strain. After biochemical characterization, we will select candidates for a mutant defective in the silencer of the checkpoint and attempt to identify the responsible gene.
Role of Mal3, the fission yeast EB1 homologue, in mitosis

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Accurate segregation of sister chromatids into two daughter cells is essential process in every eukaryotic cell cycle and is central to genome stability. In this process the spindle must be attached to all kinetochores that is assembled on centromeric DNA. The spindle checkpoint monitors spindle-kinetochore interaction, thereby delaying onset of anaphase until bipolar attachment is completed.

Fission yeast Mal3 is a homologue of the human EB1, which was identified as a protein interacting with tumor suppressor protein APC (adenomatous polyposis coli), belongs to the conserved microtubule plus-end-tracking protein family. EB1 is believed to play a role in stabilization of microtubules by promoting growth and/or in regulation of microtubule dynamics. However, physiological role for mitotic EB1, including Mal3, at the growing end of the microtubule remains largely elusive.

In this study, to investigate the function of Mal3 in mitosis, we generated a mal3 deletion mutant. Cells containing a mal3 null allele were viable but showed several phenotypes which were TBZ hypersensitivity, abnormal cell shape and defect of microtubules array. Furthermore, upon expression of this mal3 dominant negative mutant generated by error-prone PCR in the wild type background, chromosomes mis-segregate at 36°C but not at 26°C. By analyzing phenotype of this mutant in detail, we want to show the function of mitotic Mal3. Furthermore, we would like to discuss the relationship between Mal3 and spindle checkpoint together with our upcoming results.
P31

Purification of human kinetochores for unraveling a switching mechanism of the spindle checkpoint

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Kinetochores are attachment sites of spindle microtubules emanated from both opposite spindle poles. The correct bipolar attachment between kinetochores and spindle microtubules is essential for accurate chromosome segregation. Kinetochores are a huge proteinaceous machinery which assembles on a centromeric DNA region, which is constituted of highly repetitive sequences from 0.5 to a few megabases (α-satellite DNA repeat) in mammalian cells. To date, several centromeric components were identified. Among these, the centromere protein A (CENP-A), B (CENP-B), and C (CENP-C) were showed to cooperatively form the centromeric chromatin complex. CENP-A is the centromere-specific histone H3 variant and it has been widely accepted that formation of CENP-A nucleosomes defines the active centromere region. CENP-B specifically binds a 17-base pair sequence (the CENP-B box), which appears within every other α-satellite DNA repeat. And CENP-C associates with the centromeric DNA sequences and localizes to the inner kinetochores of plates. In addition, a variety of proteins are recruited to kinetochores at their specific timing during mitosis. Especially, we focus on the components related to the spindle checkpoint: Mad2, Cdc20, and Cmt2/p31<sub>C<sub>om</sub>et</sub>. The spindle checkpoint is a surveillance system that ensures accurate chromosome segregation by delaying the onset of anaphase until all the kinetochores are attached to the spindle microtubules. Mad2 is a key component of the spindle checkpoint. Upon checkpoint activation, Mad2 binds to and inhibits Cdc20, an activator of anaphase promoting complex or cyclosome (APC/C) and thus, prevents securin from being degraded. On the other hand, Cmt2/p31<sub>C<sub>om</sub>et</sub> is required to inactivate the checkpoint. Cmt2/p31<sub>C<sub>om</sub>et</sub> also binds to Mad2 and promotes the release of Cdc20 from Mad2. This process of switching on and off of the spindle checkpoint is thought to be regulated by kinetochores according to whether spindle microtubule is connected to every kinetochore. Because information of molecular basis of kinetochores at each phase during mitosis will provide powerful tips for elucidating a mechanism of controlling the spindle checkpoint, we are trying to purify intact kinetochores following MNase digestion and glycerol gradient sedimentation, to begin with.
Integrin-mediated cell adhesion orients the mitotic spindle parallel to the cell-substrate adhesion plane

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The orientation of mitotic spindles is tightly regulated in polarized cells, but it has been unclear whether there is a mechanism regulating spindle orientation in non-polarized cells. Here we show that integrin-dependent cell adhesion to the substrate orients the mitotic spindle of non-polarized cultured cells parallel to the substrate plane. The spindle is properly oriented in cells plated on fibronectin or collagen, but misoriented in cells on poly-L-lysine or treated with the RGD peptide or anti-αI-integrin antibody, indicating requirement of integrin-mediated cell adhesion for this mechanism. Remarkably, this mechanism is independent of gravitation or cell-cell adhesion, but requires actin cytoskeleton and astral microtubules. Furthermore, myosin X and the microtubule plus-end-tracking protein EB1 are shown to play a role in this mechanism through remodeling of actin cytoskeleton and stabilization of astral microtubules, respectively. Our results thus uncover the existence of a mechanism that orients the spindle parallel to the cell-substrate adhesion plane, and identify crucial factors involved in this novel mechanism.
Myt1 is essential for Golgi reassembly during mitotic exit

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Myt1 was originally identified in Xenopus oocytes as an inhibitory kinase for Cdc2 (Cdk1), the master engine of mitosis, and has been thought to function, together with Wee1, as a negative regulator of mitotic entry. However, the function of Myt1 in somatic cell cycle has remained unclear. Here we report an unexpected finding that Myt1 is not involved in the regulation of mitotic entry timing but rather is essential for the reassembly of the Golgi apparatus during telophase in mammalian cells. Myt1 localizes to membranous structures, which are closely associated with the Golgi apparatus and endoplasmic reticulum. Myt1 protein levels increase dramatically during G2/M phase and decreases after telophase. Inhibition of Myt1 expression by siRNA in HeLa cells does not change the timing of mitotic entry but rather induces severe defects in Golgi reassembly during telophase, resulting in the disappearance of normally stacked Golgi. Importantly, add-back of wild-type but not kinase-inactive Myt1, rescues the Golgi reassembly defect. Moreover, cyclin B2, which associates with the Golgi apparatus, binds to Myt1. Remarkably, inhibition of cyclin B2 expression by siRNA markedly rescues the Myt1 siRNA-induced Golgi reassembly defect. Thus, we demonstrate that Myt1 functions to facilitate Golgi reassembly during telophase, and this function of Myt1 is mediated, at least in part, by its ability to suppress cyclin B2/Cdc2 activity.
Plk1 facilitates chromosome alignment during prometaphase through BubR1

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Plk1, an evolutionarily conserved M phase kinase, associates with not only spindle poles but also kinetochores during prometaphase. However, the role of Plk1 at kinetochores has been poorly understood. Here we show that BubR1 mediates the action of Plk1 at kinetochores for proper chromosome alignment. Our results show that BubR1 co-localizes with Plk1 at kinetochores of unaligned chromosomes, and physically interacts with Plk1 in prometaphase cells. Downregulation of Plk1 by siRNA abolished the mobility-shifted, hyperphosphorylated form of BubR1 in the prometaphase arrested cells. In addition, BubR1 was phosphorylated by Plk1 in vitro at two Plk1 consensus sites in the kinase domain of BubR1. The add-back of either wild-type BubR1 or BubR1 2E, in which the two Plk1 phosphorylation sites were replaced by glutamic acids, but not that of BubR1 2A, an unphosphorylatable mutant, rescued the chromosome alignment defects in BubR1-deficient cells. Moreover, when both Plk1 and BubR1 were downregulated, the add-back of BubR1 2E, but not that of wild-type BubR1, rescued the chromosome alignment defects. These results taken together suggest that Plk1 facilitates chromosome alignment during prometaphase through BubR1.
Stable inheritance of telomere chromatin structure and function in the absence of telomeric repeats

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It is generally believed that telomeric repeats are a necessary and sufficient cis-element for telomere function. Here we show that telomere structure and meiotic function are stably inherited in fission yeast circular chromosomes that have lost all telomeric repeats. We found that the telomeric repeat binding protein, Taz1, and the heterochromatin protein, Swi6, remain associated with subtelomeres in the absence of telomeric repeats. We also found that the fusion point of circular chromosomes that lack telomeric repeats associates with SPB (the yeast counterpart of the centrosome) in the premeiotic horsetail stage, similarly to wild-type telomeres. However, a taz1+ deletion/reintroduction experiment revealed that the maintenance of Taz1 binding and premeiotic function is achieved via different strategies. Taz1 is recruited to subtelomeres by an autonomous element present in subtelomeric DNA, thus in a genetic mechanism. In contrast, the premeiotic subtelomere-SPB association is maintained in an epigenetic manner. These results shed light on the previously unrecognized role played by the subtelomere and underscore the robust nature of the functional telomere complex that is maintained by both genetic and epigenetic mechanisms. Furthermore, we suggest that the establishment and the maintenance of the functional telomere complex are mechanistically distinguishable.
Fission yeast Tel2 is required for replication checkpoint and prevention of DNA breaks

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Tel2/Rad-5/Clk-2 family proteins are conserved among species, and are involved in various cellular processes, such as cell proliferation, telomere maintenance, biological clock and DNA damage checkpoint. Their molecular functions, however, remain unclear. Here we report studies of fission yeast Tel2. Our studies have shown that Tel2 is essential for cell proliferation. Tel2 is required for the phosphorylation of Mrc1, a mediator of replication checkpoint signaling, and for the activation of Cds1, a replication checkpoint kinase, when DNA replication was blocked by hydroxy urea (HU). These data indicate that Tel2 acts at the early step of replication checkpoint. Additionally, Tel2 has another important role in genome stability. Rad22 DNA repair foci were observed more frequently in the Tel2-depleted cells than in the wild-type cells in the absence of DNA damaging agents. Furthermore, Swi1, a component of a replication fork protection complex, is vital in the Tel2-depleted cells. A high amount of DNA breaks was observed in the Tel2-depleted swi1Δ cells. We propose that fission yeast Tel2 plays important roles in replication checkpoint and stability of genome DNA.
Replication checkpoint kinase Cds1 is involved in the response to oxidative damage of mitochondria

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Mitochondria are the tiny but vital organelle specialized for energy production, and a large amount of oxygen is consumed there. During respiration, reactive oxygen species (ROS), highly reactive molecules that destroy a variety of cellular components, are generated from oxygen. Because ROS is largely produced at mitochondria and its half-life is short, mitochondria are the primary target of ROS. Therefore, accumulation of ROS-induced damages inhibits mitochondrial functions, leading to pathological states such as aging. It is supposed that cells possess a mechanism monitoring the decline of mitochondrial functions, however, experimental evidence supporting such hypothesis is lacking.

Paraquat produces superoxide (O$_2^-$) at mitochondria, preferentially damaging mitochondria components. In contrast, H$_2$O$_2$ diffuses throughout the cell to act on various cellular components, and DEM (diethylmaleate) depletes GSH (glutathione), the major anti-oxidant molecule, from cells. Thus, the H$_2$O$_2$ and DEM treatment cause cellular damages not restricted to mitochondria. We found that the fission yeast cells deleted for cds1*, the gene function in DNA replication checkpoint, is moderately sensitive to paraquat, but not to H$_2$O$_2$ or DEM. Moreover, the Cds1-EGFP protein became accumulated in nuclei when cells were treated with paraquat. Interestingly, deletion of chk1*, which functions in DNA damage checkpoint, did not cause such paraquat-specific sensitivity. These results suggested the possibility that the replication checkpoint kinase Cds1 is involved in the response to mitochondrial damage as well as to chromosomal DNA damage for cell survival.
Molecular mechanism of cross-tolerance to environmental stress in fission yeast

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Cells continuously monitor changes of their external environment. Appropriate response to environmental stress (defined as changes of environment) is essential for their survival. It is known that treatment of cells with low dose of stress (priming stress) leads to transient resistance to high dose of the same stress (acquired tolerance). Furthermore, priming stress frequently induces transient resistance to other forms of stress (cross-tolerance). However, molecular mechanisms of such adaptive responses remain largely unknown.

It has been proposed that expression of a set of common stress-responsive genes is induced by priming stress, and that it confers acquired and cross tolerance. We found that the cross-tolerance was proficient even when de novo protein synthesis was inhibited during the priming stress, or when genes involved in the transcription of stress-responsive genes were deleted. These results suggest that general stress response of stress-responsive genes is not essential for cross-tolerance. We then set out a genetic screen in order to identify mutants defective in cross-tolerance. Among 100,000 MNNG-mutagenized cells, we isolated 708 mutants that were sensitive to oxidative stress after primed with heat stress. Six of them did not show cross-tolerance between oxidative and osmotic stresses, suggesting that these mutants have defects in a common pathway for cross-tolerance to various forms of stress.
Chromatin structure modification during the stress response in fission yeast

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Cells regulate gene expression in response to various forms of stress, such as a change of nutritional condition. It is believed that transcription factors play the primary role in such responses through regulating their target genes. However, expressions of neighboring genes are frequently regulated simultaneously, suggesting the presence of a mechanism that controls gene expression through modulating chromatin structure.

Chromatin is classified into euchromatin and heterochromatin according to the degree of chromatin packing. Fission yeast contains constitutive heterochromatin, such as centromeres, telomeres, the silent mating type locus and the rDNA repeats. In addition to such constitutive heterochromatin, the recent report (Cam et al. Nat. Genet. 2005) suggests the presence of facultative heterochromatin as in the case of higher eukaryotes. We have analyzed whether the structure of the heterochromatin in fission yeast is modified in response to stress.
The Pot1 complex maintains telomere integrity in fission yeast

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The telomere refers to the functional domain at the end of linear chromosomes, and is essential for protecting DNA ends from hazardous reactions, such as end-ligation and nucleolytic attack, and preventing excessive telomere shortening due to the end replication problem. To achieve these functions, telomeres comprise a large DNA-protein complex, one of important component of which is Pot1 (Protection of telomeres) in fission yeast. Fission yeast Pot1 prevents DNA degradation and end-to-end fusion at telomeres. However, the mechanism of end protective roles of Pot1 is poorly understood. To further elucidate the functions of Pot1, we performed affinity purification of the Pot1 complex in fission yeast. By the mass spectrometry analysis, we identified two putative Pot1-associating proteins, an SMC domain protein, Ccq1 (Coiled-coil protein quantitatively enriched) and an unknown protein. When we disrupted these genes individually, the single mutants exhibited the significant loss of telomere length control and the collapse of telomeric heterochromatin. This result indicated that these proteins are bona fide components of telomeres, and suggest that the Pot1 complex regulates not only telomere protection, but also telomere length control and heterochromatin maintenance.
The mechanism of the conventional DNA replication at telomeres in fission yeast

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The eukaryotes have linear chromosomes and the ends of the chromosomes have a specific structure called telomeres, which consist of proteins and specific repeat sequences of DNA. Telomere repeats recruit many proteins and form nucleoprotein complexes which contribute to the end-protective role of chromosomes. Although these telomere specific complexes generate specific heterochromatic structures, the structures become obstacles and make it difficult for replication forks to progress the higher-order hindrance in DNA replication. Previously, our group reported that human TRF1 and TRF2, both of which are telomeric DNA-binding proteins, regulate fork progression at telomere (Ohki et al., 2004). Fission yeast Taz1, an ortholog of human TRF1 and TRF2, plays central roles in telomere protection and telomeric heterochromatin formation. In this study, to understand the mechanism of the conventional DNA replication at telomeres through the analysis of the telomere specific nucleoprotein complex in fission yeasts, we are trying to demonstrate the roles of Taz1 in telomere replication and to identify the proteins interacting with Taz1 by the affinity purification method using the FLAG-tagged Taz1 expressing cells.

Ref.
Analysis of the interaction between DNA methyltransferase and DNA replication machinery using the SV40 in vitro DNA replication system

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Genomic information is encoded not only by DNA sequences but also by epigenetic states, such as DNA methylation. Both genetic and epigenetic information must be faithfully replicated when cells proliferate. DNA methylation in vertebrates occurs at cytosine residue present in the context of CpG. DNMT1 (DNA methyltransferase 1) is considered to maintain the cytosine methylation by methylating hemi-methylated DNA to full-methylated DNA. It was reported that DNMT1 was localized at the replication foci in S phase, suggesting the intimate relationship between DNA replication and methylation maintenance. However, the molecular mechanism in this process remains to be elucidated. To address this issue, we are exploiting the SV40 in vitro DNA replication system that is extremely amenable to biochemical analysis.

To analyze the methylation states of DNA, it was necessary to develop a method to distinguish between full- and hemi-methylated DNA. The restriction enzyme Cla I digests hemi- and null-methylated DNA, but does not full-methylated DNA, a unique property enabling us to analyze the methylation maintenance in a simple manner. When full-methylated DNA was replicated, DNA methylation was maintained only when the recombinant DNMT1 was added in this system. Both ChIP assay and the analysis of replicated DNA in which biotin-dUTP was incorporated, revealed that DNMT1 was bound to replicated DNA specifically. Moreover, this interaction was independent of methylated DNA and the methyl donor SAM. These results indicate that DNMT1 is not simply recruited to hemi-methylated DNA, rather to replication machineries at replication forks. Furthermore, we found that DNMT1 or its several mutant fragments promoted DNA replication efficiently. In contrast, this effect was not observed in the C-terminal deletion fragment, suggesting that the C-terminal region of DNMT1 have an important role in DNA replication. Our results suggest the molecular and functional interaction between DNMT1 and replication machineries.
Analysis of MKK6 in the human senescent cells and the human telomere heterochromatin

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The normal human fibroblast cells approach the irreversible arrest of cell growth, called the replicative senescence, after the multiple rounds of cell divisions. The various types of the external stresses also induce the cellular senescence as well. The importance of the constitutive activation of the MAPK p38 for the cellular senescence has been suggested, but it remains unclear how the different types of stresses result in the common phenotypes of the senescent cells and what is the molecular basis on this response. In this work, we investigated whether the MKK6, the upstream MAPK kinase of the p38, is comprised in the specific protein complex to senescent cells. At first, we examined the preparation method for the MKK6-containing complex. The interactions were determined by the mass spectrometric analyses of the preparations from the coimmunoprecipitation experiment. The interaction between the MKK6 and the TAK1, the upstream MAPKKK for MKK6, could be observed in the extracts of the 293T cells expressing the large ectopic amounts of those two proteins. The association of the MKK6 and the p38, on the other hands, was detected only in the extract of the cells fixed by formalin prior to the cell lysis. These results indicate that the formalin fixation is useful for stabilizing the given labile interactions of proteins, and the fixed samples can be applied to the mass spectrometry for protein identification. Next we analyze the MKK6 in the senescent cells. The localization of the MKK6 is pancellular in both the young and the senescent cells, suggesting that the regulation mechanism of the MKK6 is distinct from its localization. We also demonstrated that the MKK6 is included into the large complex by the analysis of gel filtration chromatography.

Heterochromatin is required for the various kinds of the nuclear functions. The telomere is a remarkable example of constitutive heterochromatin, but the mechanism of the telomere heterochromatin formation is unknown. We tried to construct the model system, where the TRF1, a telomere DNA binding protein, could form the heterochromatin structures on the episomal DNA. We demonstrate that the plasmid replicated in a target cell synchronized with its cell cycle. The cell line harboring this plasmid as an episome and the nucleosomes are formed on the plasmid, suggesting the basic structure of chromatin on the model plasmid.
Function of the human telomere protein TRF1 on transcriptional repression

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Telomere functions are essential for genome stability in all eukaryotes and have been implicated in human aging and cancer. Telomeres are composed of the repetitive sequences and the various specific proteins, which are required for the telomere functions. TRF1, one of the major components of vertebrate telomeres, acts as a negative regulator of telomere length maintenance.

Telomeres also play a role in heterochromatin formation. In yeast and fruit fly, the insertion of telomeric DNA sequences to the given chromosomal region causes the variegated expression of the genes around. The molecular details for the formation of telomeric heterochromatin remains to be clarified. In this study, we examined the function of the human TRF1 (hTRF1) in heterochromatin formation. We firstly established the assay system for the effect of any proteins on the transcription of the plasmid. A reporter plasmid in which the promoter of luciferase gene was positioned in vicinity of the lacO repeat sequences, the binding sites for the lacI repressor protein, was constructed. This reporter plasmid was cotransfected with the effector plasmid into the 293T cells, which contains the gene encoding the lacI-hTRF1 protein. The activity of luciferase was remarkably reduced in the hTRF1-expressing cell extract, compare to that in the control cell extract. This result suggests that the tethering of hTRF1 to the target sequence makes a heterochromatin-like structure, which can repress the transcription of genes nearby. The N-terminus region of hTRF1 (the amino acid numbers 72-94) is required for the transcriptional repression, suggesting the function in heterochromatin formation of this region. We next tried to isolate the binding proteins of this region by coimmunoprecipitation and identify by mass spectrometric analysis. The several proteins of the COP I complex were identified, which has been characterized as the machinery for the intracellular membrane traffic. From immunofluorescent observation, the ectopically expressed N-terminus region of hTRF1 was localized to the nuclear periphery. These results suggest that the repression mechanism by hTRF1 be related with a specific partitioning of the chromatin in the nucleus.
**Human Sir2-related protein SIR2/SirT1 associates with the bHLH repressors HES1 and HEY2 and involves in HES1- and HEY2-mediated transcriptional repression**

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In budding yeast, SIR (silent information regulator) 1-4 genes were identified as the repressors of the mating type loci. The proteins encoded by the SIR2, SIR3, and SIR4 genes form a protein complex, Sir complex. This complex associates with underacetylated N-termini of histones H3 and H4, as well as with the sequence-specific DNA-binding factors that recruit and nucleate its binding. Among the SIR genes, SIR2 is evolutionarily conserved from bacteria to mammals. Interestingly, the SIR2 family genes encode an NAD-dependent histone/protein deacetylase. In addition, *Drosophila* homologue of SIR2 (dSir2) plays an important role in early development of embryo and interacts genetically and physically with the members of the bHLH transcriptional repressors, hairy and deadpan, which are conserved from fly to mammals like SIR2.

In order to reveal the molecular mechanisms of Sir2-mediated transcriptional repression in human, I cloned the human SIR2_ gene, which has the highest homology to the yeast SIR2 gene. Northern blot analyses showed that SIR2_ codes a 4.0 kb transcript which is expressed in various human tissues. Next, I generated a rabbit anti-hSIR2_ polyclonal antibody which recognized a single band of ~140 kD in whole cell extracts by Western blot. The indirect immunofluorescence analyses provided that hSIR2_ localized at nucleus, but not at nucleolus. Furthermore, I showed that recombinant SIR2_ protein has both ADP-ribosyltransferase and NAD-dependent protein deacetylase activities *in vitro*.

I demonstrated that hSIR2_ physically associates with the human bHLH repressor proteins, HES1 and HEY2, both *in vitro* and *in vivo*. Moreover, using the reporter assay, I showed that both hSIR2_ dependent and -independent deacetylase pathways are involved in the transcriptional repressions mediated by these bHLH repressors. These results indicate that the molecular association between bHLH proteins and Sir2-related proteins are conserved among metazoan, from *Drosophila* to human, and suggest that the Sir2-bHLH interaction also plays important roles in human cells.
Structure of telomeric DNA and localization of damage response proteins in telomerase-independent human immortalized cell lines

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Telomeres are the special structure at eukaryotic linear chromosome ends. They are essential for the protection of ends from noxious reactions, such as end-fusions and nucleolytic attacks, and prevent excessive shortening due to the end replication problem. Most cancer cells express telomerase to counteract the telomere shortening. A subset of cancer cells, however, compensates shortening in a telomerase-independent manner, a mechanism referred to alternative lengthening of telomeres (ALT). Telomeres in ALT cells are widely heterogeneous in length, and frequently clustered at a nuclear substructure called ALT-associated PML body (APB), together with damage-responsive and recombination proteins, suggesting the involvement of DNA recombination in the ALT pathway. We have already reported that the damage checkpoint proteins hRad9, hHus1, hRad1 (9-1-1) and hRad17 are constitutive component of APBs, defined by colocalization of telomeric DNA and PML. We have also found that formation of BrdU foci at APBs is sensitive to caffeine, suggesting that ATM and/or ATR kinases could play a role in de novo synthesis of telomeric DNA in ALT cells.

Recently we identified that unusual DNA structures exist in ALT telomeric DNA. First, we found that ALT genomic telomeric DNAs run in a denaturing gel much faster than in a native gel, suggesting the presence of gaps in both strands of telomeric DNAs. 2D-gel analyses revealed that the gaps are present in the linear genomic DNA, but not in the extrachromosomal circular telomeric DNAs that have been frequently observed in ALT cells. Importantly, we found that telomeric DNAs in ALT cells are more sensitive to the T7 endonuclease I, which preferentially digests non-perfectly matched DNA, such as Holliday junctions and heteroduplex DNAs, than those from telomerase-positive control cells. We propose that the gaps observed in ALT telomeres be derived from unusual DNA structures related to recombination activities in ALT cells.
Histone H1 dynamics in stress-applied living cells

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Cells exhibit their characteristic changes in the morphology, physiology and pattern of gene expression in response to environmental changes. Senescence, a state of irreversible growth arrest, is induced by mild stresses, and accompanies a drastic changes in chromatin structure to form a highly condensed state called senescence-associated heterochromatic foci (SAFHs).

Chromatin is comprised by a number of proteins bound to DNA. Histone is by far the most abundant chromatin protein and is classified into two groups, octamer histones and the linker histone H1. Although it appears that histones associate with chromatin very stably, recent studies using the FRAP (fluorescence recovery after photobleaching) technique indicated that they dynamically dissociate and associate with chromatin with short turnover periods. H1 is particularly dynamic, presumably reflecting its binding to the linker regions that are exposed to the outside of chromatin.

In this study, we are analyzing the turnover of H1 in stress-applied cells. We visualized the C-terminally GFP-tagged histone H1 (H1-GFP) expressed in HeLa cells and observed the FRAP. It has been previously reported that heterochromatin regions contain a higher fraction of immobile H1 than euchromatin regions. We also observed similar results using H1-GFP-expressing HeLa cells (the recovery levels were 85.2% and 95.8% in heterochromatin and euchromatin, respectively), suggesting the FRAP system we are using faithfully measures the protein dynamics. We are now investigating the behavior of H1 in stress-applied cells.
Association of the TRF1 with the telomeric DNA through the cell cycle in human cells

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Telomere is the special structure of nucleoproteins at the end of eukaryotic chromosomes. After DNA replication, telomeric DNA shortens from its lagging-strand end. To counteract this shortening, the telomerase elongates the telomeric DNA at the termini. In the budding yeast, its action is limited to the late S phase, suggesting the coordinated activity with DNA replication. TRF1, a negative regulator for telomere length in mammals, is of interest among the telomere-specific proteins. First, *Xenopus* TRF1 can bind to the telomeric DNA only in the M phase extract, where the telomerase activity is strongly repressed (Nishiyama et. al., 2006). Second, TRF1 can inhibit the progression of replication fork at the telomeric DNA, as shown by the SV40-based *in vitro* replication system (Ohki and Ishikawa, 2004). We therefore analyze the behavior of human TRF1 protein *in vivo* through S phase, at the stage for *de novo* synthesis of telomeric DNA.

We analyzed the localization of TRF1 at the telomere in respect to the replication foci, which were visualized as the BrdU-incorporation signals. We calculated the colocalization frequency of TRF1 with the telomeric DNA plus or minus the replication foci. We found little difference in the frequencies between two categories of telomere signals. The result suggests that TRF1 localizes at each of telomeres constitutively during replication. Otherwise TRF1 may dissociate very transiently from the given telomere, which could not be detected by this cytological observation.
Roles of the mammalian OBFC1 protein, a putative homologue of yeast Stn1, in telomere maintenance

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Telomere maintenance is essential for the protection of chromosome ends, and changes in telomere length have been implicated in ageing and cancer. Telomeric DNA can be maintained by telomerase, which uses the 3’ end of the telomeric overhang as a substrate. In budding yeast, the Cdc13 protein binds to this single-stranded DNA. It recruits telomeric proteins, Stn1 and Ten1, which protect the chromosome end and regulate the length of telomeres. Stn1 also coordinates the telomeric G-strand synthesis by telomerase and C-strand synthesis by replication machineries including DNA polymerase alpha. Vertebrate contains a single-strand binding protein, Pot1, as a counterpart of Cdc13 in telomere. However, vertebrate orthologs of Stn1 and Ten1 have not been found so far.

We identified human and mouse OBFC1 (OB-fold-containing 1) on the basis of sequence similarities to the STN1 gene from budding yeast. OBFC1 possesses one predicted OB-fold domain, which is shared by Stn1 as a domain responsible for single-stranded DNA binding. Indirect immunofluorescence revealed that OBFC1 signals colocalized with some of the TRF1 signals, suggesting that OBFC1 is a telomere component. *In vitro* pull-down assay demonstrated that OBFC1 interacts with Pot1 through its N-terminal region. OBFC1 mutant lacking N-terminal domain did not localize to telomeres. In an electrophoretic mobility shift assay, OBFC1 did not bind to the G-rich single-stranded telomeric DNA efficiently while Pot1 did. Mass spectrometry analysis implicated that OBFC1 associated with not only Pot1 but also all DNA polymerase alpha subunits. These results suggest that OBFC1 localizes to telomeres through the interaction with Pot1, but not through the direct binding to single-stranded DNA. It is possible that OBFC1 coordinates *de novo* synthesis of both strands of the telomere mediated by telomerase and DNA polymerase alpha. Since OBFC1 has both structural and functional similarity to yeast Stn1, we conclude that OBFC1 is a putative homologue of yeast Stn1. We will discuss the possibility whether OBFC1 is involved in telomere length regulation.
P50

Purification of the human telomerase complex

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The ends of eukaryotic chromosomes are capped by nucleoprotein structures termed telomeres. Telomeres protect the chromosomal ends from degradation and fusion events. Telomerase is a ribonucleoprotein complex that prevents telomere erosion caused by the end-replication problem. The catalytic activity of this enzyme can be reconstituted with only two components in vitro. One is the reverse transcriptase subunit, TERT, another is the RNA template subunit, TR. However, although some human factors associated with telomerase have been reported, the regulation of telomerase activity in vivo is still not clear. Such regulatory factors may be included in a telomerase complex, and be identified by purifying the complex.

To understand the control mechanism of telomerase activity, we have tried to purify the complex containing TERT using affinity tags and to identify proteins bound to TERT or TR. We firstly constructed a retroviral vector expressing TERT fused with three-tandem HA and FLAG tags to its N-terminal. Using retroviral gene transfer technique, a sub-line stably expressing the recombinant TERT was obtained from HeLa cells. Theoretically, TERT-containing complexes can exist as distinct forms undergoing different processes, such as complex maturation and catalytic action at telomeres. These forms may be partitioned in different compartments in cells. Accordingly, the cell extracts were fractionated into three fractions, cytoplasm, chromatin and insoluble materials, which were monitored for TERT and telomerase activity. Recombinant TERT was largely recovered in the cytoplasmic fraction, in which the telomerase activity was very low, however. In contrast to the cytoplasmic fraction, we found that a significant telomerase activity was present in the chromatin fraction, although the amount of TERT was relatively small. We have tried to purify this TERT complex with a high specific activity using the anti-FLAG beads and FLAG peptide elution.
Dynamic changes of SUMO proteins in their abundance and localization in cellular senescence

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Primary cultured cells cannot divide indefinitely due to the process termed cellular senescence. Cellular senescence is characterized by irreversible exit from the cell cycle, enlarged morphologies and some molecular markers. In addition, the number and size of the PML NBs (promyelocytic leukemia nuclear bodies), a body-like structure normally present in the nucleus, are increased in senescent cells. In PML NBs, it is known that many proteins are modified by sumoylation. Sumoylation involves the iso-peptide conjugation of a family of small peptides (SUMO-1, -2 and -3) to target proteins. However, it is not clear whether the morphological changes of PML NBs are accompanied by alteration in the sumoylation metabolism, which in turn causes senescence.

To investigate the potential role of sumoylation in senescence, we have used the system in which the primary human fibroblast WI-38 cells are induced to senesce by ectopic expression of activated Ras by retrovirus transfer. WI-38 cells stably expressing N-terminally tagged SUMO-1, -2 or -3 individually were established. Then Ras was retrovirally introduced to induce senescence and the behavior of SUMO proteins were analyzed using anti-tag antibodies or anti-SUMO-1 or anti-SUMO-2/3 antibodies. RanGAP1 (Ran GTPase-activating protein 1) is the major target of SUMO-1 in most cells. Sumoylated RanGAP1 associates with nuclear pores and localizes with the nuclear membrane. Interestingly, in senescent cells, RanGAP1 was not observed at the nuclear envelope in senescent cells, concomitantly with the reduction of the amounts of free SUMO-1 and SUMO-1-conjugated RanGAP1. SUMO-2/3 exists throughout the nucleus and at PML NBs, in a detergent-sensitive and -resistant manner, respectively, presumably representing the free and conjugated form of SUMO-2/3. We found that the detergent-sensitive SUMO-2/3 changed its distribution from intra nuclear to both nuclear and cytoplasmic in senescent cells. The amount of the detergent-resistant SUMO-2/3 was increased as PML NBs were enlarged in senescent cells. These results together suggest that both the quantitative and qualitative (conjugation and localization) changes of SUMO proteins specifically occur in senescent cells. Future studies are necessary to elucidate the potential causative roles of these changes.
Efficient formation of the ternary complex containing Oct-3/4 and Sox2 and target DNAs in vitro

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Expressions of the transcription factors, Oct-3/4 and Sox2, overlap spatially and chronologically during early embryogenesis, and both are essential for the maintenance of pluripotency in early embryos. It was reported that the precise amount of Oct-3/4 is important for the maintenance of pluripotency: Expressions of Oct-3/4 in either lower or higher amount of a narrow window led to differentiation in ES cells. We have reported that the Oct-3/4 enhancer elements required for expression in ES cells contains an Oct-3/4- and a Sox2-binding elements in a close vicinity (bipartite element). Such Oct-3/4- and Sox2-bipartite elements have been reported in several other genes, notably those involved in pluripotency including the Sox2 gene itself. The fact that Oct-3/4 and Sox2 autoregulate their own expressions suggests the presence of a positive feedback loop to maintain the expressions. In some cases, it has been shown that Oct-3/4, Sox2 and the bipartite elements form a ternary complex in vitro, suggesting that Oct-3/4 and Sox2 recognizes the elements cooperatively. However, it is not known how the Oct-3/4-Sox2 regulatory loop is controlled to express the genes within the narrow window of expression level in pluripotent cells and is inactivated in differentiating cells.

To understand the molecular mechanism of Oct-3/4 and Sox2 expressions, we have tried to identify an associated protein(s) in the ternary complex containing Oct-3/4, Sox2 and target DNAs. Oct-3/4 and Sox2 proteins ectopically expressed in COS-7 cells were used for electrophoretic mobility shift assays (EMSAs). A battery of oligonucleotides containing the Oct-3/4- and Sox2-biding sites derived from reported genes containing the bipartite elements were assayed for the ternary complex. A consensus nucleotide sequences showing the highest ability to produce the complex was deduced. Isolation of proteins derived from ES cells that bind to this consensus sequences is in progress.

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Oct-3/4 is a key transcriptional factor whose expression level governs the fate of primitive inner cell mass and embryonic stem (ES) cells. Previously, an upstream 3.3-kb distal enhancer (DE) fragment was identified to be responsible for the specific expression of mouse Oct-3/4 in the inner cell mass and ES cells. However, little is known about the cis-elements and trans-factors required for DE activity.

In this study, we identified a novel cis-element, called Site 2B here, located approximately 30 bp downstream from Site 2A, which was previously revealed in DE by an in vivo chemical modification experiment. Using the luciferase reporter assay, we demonstrated that both Site 2A and Site 2B are necessary and sufficient for activating DE in the contexts of both the native Oct-3/4 promoter and the heterologous thymidine kinase minimal promoter. In an electrophoretic mobility shift assay we showed that Site 2B specifically binds to Oct-3/4 and Sox2 when ES-derived cell extracts were used, whereas Site 2A binds to a factor(s) present in both ES and NIH 3T3 cells. Furthermore, we showed that the physiological level of Oct-3/4 in ES cells is required for Site 2B-mediated DE activity using the inducible knock-out system of Oct-3/4 in ES cells. These results indicate that Oct-3/4 is a member of the gene family regulated by Oct-3/4 and Sox2, as reported before for the FGF-4, UTF1, Sox2, and Fbx15 genes. Thus, Oct-3/4 and Sox2 comprise a regulatory complex that controls the expression of genes important for the maintenance of the primitive state, including themselves. This autoregulatory circuit of the Sox2-Oct-3/4 complex may contribute to maintaining robustly the precise expression level of Oct-3/4 in primitive cells.
Mass spectrometric analyses for post-translational modification of core histones

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Recent studies revealed that post-translational modifications of core histones play important roles in controlling gene expression. In addition, histone modification is involved in determining the higher structure of chromatin in the non-coding region such as heterochromatin. To date, although a number of histone modifications have been reported, our understanding of the role of the modification in chromatin regulation is far from comprehensive. Histone modifications have been routinely analyzed by exploiting antibodies that specifically recognizes individual modifications. However, preparing specific antibodies is time-consuming and has not been always successful. Mass spectrometry is an alternative method to detect known histone modifications. Moreover, the method is potentially able to identify unknown modifications by measuring the mass differences between the modified and unmodified peptides. However, there has been no report to analyze the histone modifications in a systematic and comprehensive manner.

Histones were isolated from human normal fibroblast OUMS36, and further purified by SDS-PAGE. The histone peptides recovered by in-gel digestion technique were analyzed using tandem mass spectrometry. First, we have established the method to detect already known histone modifications as extensively as possible. Trypsin, the most commonly used protease to produce peptides, digests histones too frequently because histones are rich in basic amino acids. Using other proteases, Arg-C and V8, as well as trypsin, improved the fraction of peptides among the total length of histones that could be analyzed by the mass spectrometry to over 95% for H2B and H4, and 74% for H3. This improvement significantly increased the chance of identifying known histone modifications. We then extended our investigation to identify unknown histone modifications. A total of 16 modifications including phosphorylation, acetylation and methylation was detected as candidate novel histone modifications. One of the drawbacks of mass spectrometry analyses is the difficulty of detecting peptides in a quantitative manner. This is due to variable ionization efficiencies among different molecules. To overcome this problem, we measured the relative abundance of modified peptides by calibrating them to the unmodified internal control peptides. Applying this method, we showed that the acetylation level of H3 lysine79, one of the novel modifications, increased after treatment with Trichostatin A, an inhibitor of histone deacetylases. These results indicate the usefulness of the mass spectrometry technique in analyzing histone modification.
C-strand resection of reconstituted telomeres in *Xenopus* egg extracts

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The chromosomal end, telomeres, consists of G-rich repetitive sequences (G-strand) and its complementary sequences (C-strand). One of the most important roles of telomeres is end-protection to prevent chromosomal ends from nucleolytic attack or end-to-end fusion event. G-tail, a 3′-overhang on G-strand, is thought to have pivotal roles for end-protection. However, the molecular mechanism how G-tail is formed *in vivo* is still unknown.

To investigate this issue, we have analyzed the behaviors of linearized DNAs carrying telomeric-repeat sequences at the both ends in the *in vitro* *Xenopus* egg extracts. We found that linear DNAs with 3′-overhangs, irrespective of telomeric or non-telomeric sequences, did not undergo end-to-end ligation, suggesting that the presence of 3′-overhangs by itself is sufficient for protection of ends from ligation. In contrast to the relaxed sequence requirement of 3′-overhangs, the double-stranded (ds) DNA showed a specific requirement of telomeric repeats, since non-telomeric ds-DNAs underwent rapid degradation. The vertebrate telomere DNA binding protein TRF2 is required for this telomeric DNA-dependent protection, since telomeric ds-DNAs were no longer stable in TRF2-depleted extracts. Although telomeric ds-DNAs are largely intact in extracts, detailed analyses revealed that they were processed in a highly regulated manner so that the first 5′-terminal nucleotide of C-strand became to be 5′-CTAA..., irrespective of the terminal sequence of the input DNAs. The cytosine is exactly the nucleotide found in most frequently (~80%) in C-strand *in vivo* (Sfeir AJ et al., Mol. Cell, 2005). These results suggest the presence of processing activities to form the terminal nucleotide sequence of C-strand and the *Xenopus* egg extract system recapitulate the activity.
Binding modes of full-length *Xenopus* POT1 (xPOT1) to sperm chromatin reconstituted in *Xenopus* egg extracts and oligonucleotide DNAs *in vitro*

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Eukaryotic chromosome ends, telomeres, consist of G-rich repetitive DNA sequences (TTAGGG repeats in vertebrates, G-strand), and its complementary sequences (C-strand). The most 3’-end of G-strand is single-stranded (ss), an overhang called G-tail. POT1 (protection of telomeres 1) binds to G-tail through their two OB folds (oligonucleotide/oligosaccharide-binding folds), and is required for the G-tail integrity. However, the precise functions of POT1, especially during the cell cycle, are not known. We are studying the behavior of xPOT1 (*Xenopus* POT1) at telomeres using the *Xenopus* egg extract system.

[$^{35}$S]-methionine-labeled xPOT1 together with demembranated *Xenopus* sperm were incubated in mitotic or interphase egg extracts. Recombinant xPOT1 was associated with the sperm chromatin and no obvious change in binding efficiencies was observed between in mitotic and interphase extracts, suggesting that xPOT1 associates with chromatin in a relatively constitutive manner.

It was reported that POT1 associates with telomeres via interaction with another telomeric protein TRF2. Therefore, at least of two binding modes of POT1 to telomeres can be envisioned: through direct binding to G-tail and via protein-protein interactions. Moreover, due to the difficulty in preparing full-length POT1 recombinant proteins, most *in vitro* studies have been done using POT1 recombinant proteins encompassing the OB-folds only. We have succeeded in preparing full-length xPOT1 recombinant protein. Using the protein we have found that xPOT1 specifically binds to single-stranded G-strand *in vitro* as previously reported. However, we also found that xPOT1 preferentially bind to the junction region of the double- and single-stranded telomeric DNAs. These results suggest that although xPOT1 appears to associate with telomeres constitutively, its binding modes are complex and regulated during the cell cycle. Future studies are necessary to analyze the precise behaviors of xPOT1 at telomeres.
Cell-cycle-dependent regulation of Xenopus Telomeric DNA binding protein TRF2

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Telomeres are the end of linear chromosomes, and in vertebrates, they contain tandem arrays of duplex TTAGGG repeats and a 3’ TTAGGG overhang. TRF1 (TTAGGG Repeat binding Factor 1) and TRF2, which carry highly conserved TRFH (TRF-Homology) domain and Myb-related DNA binding motif, bind exclusively to double-stranded telomeric DNA. TRF2 plays an important role in protecting chromosomal ends, thus human telomeres become dysfunctional when TRF2 is removed and associated with DNA damage response factors such as □H2AX, ATM and Mre11. However, it is not still clear how TRF2 protects chromosome ends, especially through cell cycle progression. To better understand the cell-cycle-dependent regulation of TRF2, we have exploited the Xenopus egg extracts system.

First, we cloned cDNA of Xenopus homolog of TRF2 (xTRF2) and raised specific antibodies against recombinant xTRF2. We found that xTRF2 formed homodimer and specifically bound to the TTAGGG repeats in vitro. Immunodepletion of xTRF2 from interphase extracts induced accumulation of □H2AX and NBS1 to telomere ends. This suggests that xTRF2, like human TRF2, plays key roles in telomere end protection in Xenopus egg extracts system, too. Using immunofluorescence analysis and chromatin binding assay, we found that xTRF2 localized to the telomere throughout the cell cycle. Interestingly, xTRF2 derived from mitotic chromatin migrate more slowly than that derived from interphase chromatin on SDS-PAGE. When immunopurified xTRF2 were treated with lambda phosphatase, not only mitotic xTRF2 but also interphase xTRF2 showed increase in mobility in a manner sensitive to phosphatase inhibitors, indicating that xTRF2 in mitotic and interphase extracts are hyperphosphorylated and phosphorylated, respectively. Furthermore, fractionation of mitotic or interphase egg extracts by Superose 6 gel filtration column suggested that xTRF2 might form distinct protein complexes through cell cycle. These results raise the possibility that xTRF2 function could be regulated through cell-cycle-dependent modification and protein-protein interactions.
Cell-cycle-dependent *Xenopus* TERT recruitment to telomeric DNA

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Telomeres are specialized nucleoprotein complexes at the ends of eukaryotic chromosomes that are essential to protect chromosome ends from degradation and end-to-end fusions. Telomeres also enable the complete replication of chromosome ends, which cannot be accomplished by conventional DNA polymerases. Synthesis of telomeric DNA is carried out by telomerase, a specialized reverse transcriptase consisting of an RNA template, the catalytic protein subunit (TERT) and several accessory proteins. It has been reported that telomere replication is regulated cell-cycle-dependently in the budding yeast, *Saccharomyces cerevisiae*. The semiconservative replication of telomeres occurs in late S phase, and lengthening of telomeres by telomerase is also limited to late S phase. In addition, Est2p, the TERT homolog in the budding yeast, associates with telomere throughout the cell cycle, including G1 and early S phase, when telomere addition does not occur. However, in higher eukaryotes, although it is thought that both telomere replication and elongation also occur in S phase, when TERT is recruited to telomeres is unclear. To examine how TERT recruitment to telomere ends is regulated, we used *Xenopus* egg extracts system.

We first measured the total telomerase activity present in *Xenopus* M phase and interphase extracts. We next analyzed the telomerase activity associated with sperm chromatin reconstructed in M phase and interphase extracts. Isolated sperm chromatin derived from either extract was directly analyzed by the stretch PCR assay. This analysis revealed that a significantly higher level of telomerase activity was detected in association with interphase chromatin than with mitotic chromatin. Experiments are underway to examine direct interaction of telomerase with telomere ends using ChIP analysis.
A novel experimental system for analysis of telomere replication

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Telomeres are specialized nucleoprotein structures at the ends of linear eukaryotic chromosomes that are composed of short repetitive sequence (TTAGGG in vertebrates) and a variety of associated telomeric binding proteins. Telomeres protect chromosome ends from degradation and inappropriate recombination. In proliferating cells, telomeres gradually shorten because the semiconservative replication mechanism fails to replicate the very ends of a linear chromosome (the end replication problem). In many eukaryotes, a telomere-specific reverse transcriptase, telomerase, counteracts the loss of terminal sequences during DNA replication. Although the functions of telomeres and telomerase are well understood in a biological context, we know little about the structures and dynamics of telomere during replication.

*Xenopus* egg extracts are among the most powerful experimental approaches to study DNA replication, since they recapitulate a complete round of cell cycle regulated chromosomal DNA replication *in vitro*. Although small circular plasmids replicate efficiently in the recently developed nucleus free system (the NPE system), attempts to replicate small linear DNAs have not been successful because the ends of linear DNAs are vulnerable to end joining reactions or degradation in the extracts. We found that addition of telomeric sequence and G-tail (single stranded overhangs at 3’ ends, which are thought to be essential for telomere functions) at the ends of a small linear DNA significantly increased the stability of the ends, and showed that this telomeric linear DNA could be a good substrate for DNA replication. We also showed that a circular plasmid containing telomeric sequence replicated as efficiently as pBluescript, a circular plasmid that does not contain telomeric sequence, suggesting that the egg extracts are highly competent for telomeric replication.

This is the first study of showing the possibility for small linear DNAs to replicate in *Xenopus* egg extracts. Given the high activity of telomerase in the egg extracts, our novel system is very promising for analysis of telomere replication.
Analysis of the mechanism of maintenance methylation by Xenopus Dnmt1 during DNA replication

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In higher eukaryotes, DNA methylation predominantly occurs at cytosine residues in CpG dinucleotide sites, entailing repressive effects on gene expression in most cases. Tissue-specific patterns of methylated deoxycytidine residues in the genome are preserved by postreplicative methylation of newly synthesized DNA. It is proposed that Dnmt1 (DNA cytosine methyltransferase 1) is the maintenance methyltransferase and that its association with replication sites during S phase ensures the faithful duplication of the DNA methylation patterns postreplicatively. However, it is presently unclear how Dnmt1 is recruited to the replication fork or when Dnmt1 functions during S phase. We have, therefore, undertaken a detailed analysis of the molecular basis for the association of Dnmt1 with the replication machinery and with chromatin during S phase using Xenopus egg extracts.

Our results show that Xenopus Dnmt1 (xDnmt1) transiently associates with chromatin at the same time with pre-initiation complex formation on the chromatin. Interestingly, when DNA replication was inhibited by aphidicolin, an inhibitor of B-type polymerase, replication fork components accumulated on chromatin, but xDnmt1 did not. We also found that the xDnmt1 dissociation from chromatin after DNA replication was significantly inhibited by the addition of a chemical inhibitor of DNA methylation, even after the dissociation of MCM from chromatin. These results suggest that the loading of Dnmt1 to replication fork is regulated independently of the chromatin binding of replication fork proteins.
Nuclear reprogramming of *Xenopus* epithelial cells in *Xenopus* egg extract

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Epigenetic marks confer flexibility and stability of gene expression during mammalian development. These epigenetic states can be broadly divided into three categories: euchromatin, constitutive heterochromatin and facultative heterochromatin. Although the static snapshots of the epigenetic marks in such chromatin states have been relatively well characterized, less is known about how to erase the epigenetic marks. Recent success in producing clone animals by nuclear transfer highlighted the presence of an erasure mechanism of epigenetic marks. As nuclear transplantation experiments have shown that cytoplasm of *Xenopus* oocyte or egg can extensively reverse the genetic and epigenetic programs of a somatic cell to an embryonic state (reprogram), it is expected that cytoplasm of *Xenopus* egg has an activity to erases the epigenetic marks of differentiated cells. To identify a mechanism that erases the epigenetic marks, we exploited the *Xenopus* egg extracts system.

To simplify nuclear reprogramming assays by eliminating nuclear transport steps, we permeabilized the plasma membrane of *Xenopus* epithelial cells (henceforth called nuclei). The nuclei were incubated in *Xenopus* M phase extracts for 2 hr at 22°C and isolated nuclei was analyzed by immunoblot. After incubation, the nuclei showed the distinct pattern of protein composition; e.g., linker histone H1 is replaced with the egg type linker histone B4, indicating that reprogramming was successful. In contrast, core histones of these nuclei were not degraded or released in the extract. Now, we are investigating the changes in the protein composition induced by reprogramming, such as in histone modification, chromatin binding proteins or chromatin remodeling complexes.
Mitogen-activated protein kinase p38 defines the common senescence-signaling pathway

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Cellular senescence is defined by such terminal phenotypes as irreversible growth arrest, large flat cytoplasm, up-regulated cyclin-cdk inhibitors (p21ⁱ⁴¹⁴ and p16⁰⁶⁰⁶) and activated p53 and pRb. It is believed that senescence has evolved adaptively as a mechanism to prevent cancer development. Diverse stimuli, such as telomere shortening and sustained expression of oncogenic Ras, induce senescence. It is not known, however, how these apparently unrelated signals converge to produce the common senescence phenotypes. Here we show that the mitogen-activated protein kinase p38 plays a key role in the senescent signaling pathway in three distinct systems, namely, replicative senescence, Ras-induced senescence and “culture shock”. We suggest that Ras-induced senescence is a p38-mediated cellular response to stress conditions caused by Ras activation. We also show that papilloma virus E7, but not E6, abolishes the p38-induced cell cycle arrest, suggesting that pRb plays an important role in the downstream of p38. We propose that p38 is the common senescence transducer that integrates the total load of various cellular stresses, and when the load reaches a critical level, it induces senescence phenotypes.
ESE-3 Up-regulation in Stress-induced Senescence

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It has been established that stress-activated protein kinase p38 plays a key role in cellular senescence by our and other laboratories (Please refer to the poster “Mitogen-activated protein kinase p38 defines the common senescence-signaling pathway” in this conference). It remains unclear how the activation of p38 contributes molecularly to senescent signal transduction, however. To better understand the downstream of p38 pathway in cellular senescence, we conducted a microarray analysis with M KK6-EE-induced senescent cells. M KK6-EE is a constitutively active form of M KK6 that is a specific upstream kinase for p38. Among genes found to be up-regulated by M KK6-EE, we focused on a gene, ESE-3 (Epithelial-specific ets), in this study. By the RT-PCR analysis, ESE-3 gene was found to be strongly up-regulated in Ras V12- and M KK6-EE-induced senescence but not in serum starvation and contact inhibition at all. In addition, ESE-3 protein was expressed in Ras V12- and M KK6-EE-induced senescence at a high level almost equal to that found in exogenously ESE-3 expressing cells. The up-regulation of ESE-3 in Ras V12-induced senescence at least partially depends on the activation of p38 because adding SB203580 (a p38-inhibitor) suppresses the expression of ESE-3. When ESE-3 was exogenously expressed in normal human fibroblast WI-38 cells, p16INK4a protein was up-regulated. Using the luciferase reporter assay, we demonstrated that ESE-3 activate the p16 promoter depending on EBS (Ets-binding site). Furthermore, the electrophoretic mobility shift assay demonstrated that ESE-3 binds DNA oligonucleotides containing the EBS sequence derived from the p16 promoter. Our results suggest that ESE-3 is a senescent-related downstream component of the p38 pathway, being involved in the induction of p16 transcription.
Analysis of ATM-dependent protein phosphorylations in the absence of DNA DSBs

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ATM (Ataxia-Telangiectasia Mutated) is a protein kinase that is mutated in the autosomal recessive disease, ataxia-telangiectasia. ATM and other related kinases have essential roles in DNA double-strand breaks (DSBs) repair. In response to DNA DSBs, ATM is phosphorylated at Ser1981 by itself and thereby activated. Activated ATM phosphorylates downstream substrates to induce cell cycle arrest and activate DNA repairing machinery. These substrates include p53 in G1 checkpoint; Brca1, and SMC1 in DSBs-induced S-phase arrest; Chk2 in G2 checkpoint; and Nbs1 for DNA repair. It has been recently reported that ATM is also activated by stress treatments including Trichostatin A, Chloroquine and hypotonic treatment, that are suggested to alter global chromatin structure, without inducing DNA DSBs. However, little is known about the roles of ATM and downstream substrates that are phosphorylated by ATM in the absence of DNA DSBs.

In this study, we are trying to identify proteins that are phosphorylated by ATM in the absence of DNA DSBs. In consistent with a previous report, ATM and p53 are phosphorylated by Chloroquine treatment in telomerase-immortalized WI-38 human fibroblasts. To identify ATM-dependent protein phosphorylations, we use proteomic approaches such as two-dimensional gel electrophoresis of proteins and mass spectrometry. The roles of ATM in stress response without DNA DSBs will be discussed.
Analysis of post-translational modifications of histones in cellular senescence

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Cellular senescence is an extremely stable form of growth arrest that is induced by various types of stress stimuli including DNA damages, oxidative stress and activation of oncogenes. It has been suggested that cellular senescence plays a role in tumor suppression *in vivo* via restricting the capacity of tumor growth. In addition to growth arrest, senescent cells are also characterized by a flat and enlarged morphology, increase in β-galactosidase activity at acidic pH and formation of highly condensed chromatin (Senescence-Associated Heterochromatic Foci, SAHF). It has been argued that the SAHF formation may be important for the irreversible nature of the senescence state. Structural and functional properties of chromatin are regulated by post-translational modifications of histones, such as methylation, acetylation and phosphorylation. However, post-translational modifications specific to SAHFs in senescent cells are remained unclear.

In this study, we are trying to identify post-translational modifications of histones specific to SAHFs. Cellular senescence was induced by expression of an oncogenic Ras (RasG12V) in normal human fibroblasts WI-38 cells. SDS-PAGE and Coomassie staining analyses of histones proteins revealed that histone H4 migrated slightly slower in Ras-induced senescent cells compared to mock-transfected young cells. Immunoblotting analysis showed that histone H4 was hypo-acetylated at Lys8 in senescent cells. Similar result was also confirmed by immunofluorescence analysis. Furthermore, to identify other kinds of modifications in histones, we use a two-dimensional gel electrophoresis system (Radical-Free Highly Reducing 2D, RFHR-2D). This system is suitable for the separation of basic proteins such as histones at higher resolution. The functional role of hypo-acetylation of histone H4 in cellular senescence and the results of RFHR-2D analysis will be discussed.
Loss of linker histone H1 in cellular senescence

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Cellular senescence was originally described as a cell cycle arrest after a finite number of cell divisions in vitro. Senescent state is extremely stable and can be maintained in culture for several years. Cellular senescence is implicated in vivo as a tumor suppressor mechanism via restricting the capacity of tumor growth. Senescent phenotypes are further characterized by large and flat cellular morphology, a positive stain of β-galactosidase activity at pH 6.0, and the formation of heterochromatin (Senescence-Associated Heterochromatic Foci, SAHF). It has been argued that the SAHF formation may be responsible, at least in part, for the irreversible nature of the senescence state. However, little is known about the molecular mechanism governing the SAHF formation.

In this study, we examined chromosome and chromatin structures specific to SAHFs. We demonstrated that SAHFs are condensed chromosome territories. SAHFs do not show histone modifications that are known to characterize other types of chromatin condensation, including phosphorylated histone H3 (S10 and S28), phosphorylated histone H2B (S14), and methylated histone H3 (K9) that are responsible for mitotic chromosome condensation, apoptotic chromatin condensation and transcriptionally inactive heterochromatin, respectively. Moreover, we found that linker histone H1 is lost from senescent cell chromatin. Loss of histone H1 is not merely due to the repression of de novo histone H1 synthesis but post-translationally regulated. Furthermore, the expression of N-terminally EGFP-tagged histone H1 induces decrease in endogenous histone H1 protein level and premature senescence in WI-38 cells. These results suggest that SAHFs are a novel type of chromatin condensation lacking linker histone H1, and that the compromised histone H1 function is sufficient for induction of senescent phenotypes. This is the first demonstration that somatic cell chromatin is condensed with the loss of linker histone H1.
Roles of MAPKKK TAK1 in cellular senescence

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Normal human fibroblasts irreversibly enter a growth-arrested state, called cellular senescence, after a limited number of cell divisions \textit{in vitro}. Cellular senescence is implicated \textit{in vivo} as a tumor suppressor mechanism via restricting the capacity of tumor growth. This process accompanies a series of changes including enlargement of cell size, increase in acidic β-galactosidase activity and formation of heterochromatin. Apparently indistinguishable phenotypes are also induced by various types of stress stimuli such as exposure to hydrogen peroxide. The stress-induced senescence is called premature senescence. It has been reported that phosphorylation and subsequent activation of p38, a stress-responsive mitogen-activated protein kinase (MAPK), is important for induction of senescent phenotypes. However, little is known about upstream kinases governing phosphorylation of p38 in senescent cells.

In this study, we investigated a possible role of TGF-β-activated kinase 1 (TAK1), a member of MAPKKK, in cellular senescence. Serial transfections of TAK1 and its regulator TAK1-binding protein 1 (TAB1) induced senescent phenotypes including growth arrest and morphological changes in WI-38 normal human fibroblast cells, while TAK1 alone did not. Because TAB1 is implicated in the regulation of TAK1 kinase activity, this result suggested that kinase activity of TAK1 might be involved in induction of senescent phenotypes. However, compromised TAK1 function induced by expression of a dominant-negative form of TAK1 (TAK1K63W) and RNA interference did not affect the ability of WI-38 cells to show senescent phenotypes in response to expression of a oncogenic Ras (RasG12V) and exposure to hydrogen peroxide. TAK1 may be specifically involved in induction of senescent phenotypes induced by other types of stress stimuli.
Differential and collaborative actions of Rad51 paralog proteins in cellular response to DNA damage

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Metazoan Rad51 plays a central role in homologous DNA recombination, and its activity is controlled by a number of Rad51 cofactors. These include five Rad51 paralogs, Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3. We previously hypothesized that all five paralogs participate collaboratively in repair. However, this idea was challenged by the biochemical identification of two independent complexes composed of either Rad51B/C/D/XRCC2 or Rad51C/XRCC3. To investigate if this biochemical finding is matched by genetic interactions, we made double mutants in either the same complex (rad51b/rad51d) or in both complexes (xrcc3/rad51d). In agreement with the biochemical findings the double deletion involving both complexes had an additive effect on the sensitivity to camptothecin and cisplatin. The double deletion of genes in the same complex, on the other hand, did not further increase the sensitivity to these agents. Conversely, all mutants tested displayed comparatively mild sensitivity to \(\alpha\)-irradiation and attenuated \(\alpha\)-irradiation-induced Rad51 foci formation. Thus, in accord with our previous conclusion, all paralogs appear to collaboratively facilitate Rad51 action. In conclusion, our detailed genetic study reveals a complex interplay between the five Rad51 paralogs and suggests that some of the Rad51 paralogs can separately operate in later step of homologous recombination.
Similar effects of Brca2 truncation and Rad51 paralog deficiency on immunoglobulin V gene diversification in DT40 cells support an early role for Rad51 paralogs in homologous recombination

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BRCA2 is a tumor suppressor gene that is linked to hereditary breast and ovarian cancer. Although the Brca2 protein participates in homologous DNA recombination (HR), its precise role remains unclear. From chicken DT40 cells, we generated BRCA2 gene-deficient cells which harbor a truncation at the 3' end of the BRC3 repeat (brca2tr). Comparison of the characteristics of brca2tr cells with those of other HR-deficient DT40 clones revealed marked similarities with rad51 paralog mutants (rad51b, rad51c, rad51d, xrc2, or xrc3 cells). The phenotypic similarities include a shift from HR-mediated diversification to single-nucleotide substitutions in the immunoglobulin variable gene segment and the partial reversion of this shift by overexpression of Rad51. Although recent evidence supports at least Xrcc3 and Rad51C playing a role late in HR, our data suggest that Brca2 and the Rad51 paralogs may also contribute to HR at the same early step, with their loss resulting in the stimulation of an alternative, error-prone repair pathway.
Repair of crosslink DNA damage by the Fanconi pathway requires BRCA2, a gene responsible for familial breast and ovarian cancer

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The human chromosome breakage syndrome, Fanconi Anemia (FA) is characterized by genomic instability and marked sensitivity, specifically to chemical DNA cross linking agents, such as cisplatin. Most FA genes encode the components of an E3 monoubiquitin ligase complex, and its downstream substrate. A novel exception is FANC-D1, mutations in a gene encoding BRCA2, which facilitates homologous recombination (HR) by promoting assembly of Rad51 recombination factor at damaged DNA. This finding is surprising, because the cellular phenotype of FA is distinctively different from that of BRCA2 deficient cells, which are hypersensitive to a variety of DNA damaging agents. To understand the functional interactions between the FA pathway and BRCA2, we inactivated the FANC-C gene in brca2 null cells. Remarkably, the resulting fanc-c/brca2 mutant showed the same level of cisplatin sensitivity as brca2 and fanc-c single mutant cells. In contrast, deletion of FANC-C in another HR mutant, rad54, caused an increase in cisplatin sensitivity when compared to either type of single mutants. These genetic data reveal that BRCA2 dependent HR is required for the FA dependent DNA cross link repair.
Identification of HR-related proteins interacting with Rad51, Brca2, or Rad54 in chicken DT40 cells

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Inheriting accurately our genetic information to daughter cells is important, while DNA is continually damaged and the presence of DNA damage activates a cell cycle checkpoint. This allows time for the cell to correct the damage so as not to propagate the defect of affect normal cellular functions. Therefore, highly conserved repair mechanisms exist in all organisms to recognize and repair DNA damages. Among these DNA damages, double-stranded DNA breaks (DSBs) are potentially lethal lesions that are efficiently repaired in eukaryota by homologous recombination (HR) or non-homologous end joining (NHEJ). HR typically results in accurate repair, while defects in HR proteins cause genome instability. By contrast, NHEJ often results in imprecise repair, yielding deletions or insertions.

Chicken B cell line DT40 possesses the high efficiency of gene targeting, but little is known about the details of the molecular mechanism. Accumulating reports have allowed us to predict that the feature of DT40 cells may be explained by following three protein complexes. Rad51 plays a pivotal role in HR and RAD51 mutant cells are lethal at cellular level. Brca2 seems to provide HR machinery with a scaffold. RAD54 mutant cells show the dramatic reduction of gene targeting. In this study, to deepen the understanding of HR in DT40 cells, we have tried to identify new proteins interacting with these three key players by immunoaffinity purification method. Two different epitope tags tandemly (FLAG and HA) were put on either the N or the C terminus of the protein of interest and each tagged-protein was expressed in corresponding mutant cell. To activate HR pathway, cells were exposed to ionizing radiation before such a protein complex was purified rapidly by immunoprecipitation with antibodies against epitopes. As a result, we fortunately identified three proteins. One of them shares a common domain with Rad54 and appears to remodel compact chromatin before HR machinery localizes to the sites of DNA damage. To expect HR-related phenotype, mutant cells are under the construction.
A role for RAD18 in double strand break repair by homologous recombination

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The S. cerevisiae RAD18 gene is essential for all types of postreplication repair (PRR) but appears not to be required for the two major double strand break (DSB) repair pathways, homologous recombination (HR) and nonhomologous end joining (NHEJ). To investigate the role of vertebrate Rad18 in DSB repair, we analyzed rad18-deficient chicken DT40 cells. rad18 DT40 cells exhibited a significant reduction in HR-dependent repair of DSBs induced by ionizing radiation (IR), a topoisomerase1 inhibitor, camptothecin, and I-Sce1 nuclease expression. A defect in the repair of IR induced DSBs was also observed in Rad18 depleted human (Hela) cells. Remarkably, deletion of Ku70, an essential early component of NHEJ, suppressed these defects in DSB repair. These observations suggest that, in vertebrate cells, loss of Rad18 results in the accumulation of DNA ends that when bound by Ku70 lead to unrepairable intermediates causing cell death.
UBC13, a ubiquitin ligase, is essential for maintaining chromosomal stability in vertebrates

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Ubc13 is a unique E2 ubiquitin ligase that mediates poly-ubiquitination through lysine 63 and has been implicated in the RAD6/RAD18 dependent post-replication repair pathway in *Saccharomyces cerevisiae*. However, the function of Ubc13 in higher eukaryotes is poorly understood. Here, we report that UBC13 deficient DT40 cells displayed hypersensitivity to UV, CPT, CDDP, IR, H$_2$O$_2$ and MMS, indicating that Ubc13 is indispensable for DNA damage tolerance. Ubc13-depleted human cell exhibited severe defects in DSB-induced HR repair and recruitment of Rad51 after IR. The focus formation of both conjugated ubiquitin molecule and Brca1, a potential E3 enzyme, were abolished in UBC13 deficient cells. Our observations strongly suggest that UBC13 is a key E2 enzyme for maintaining chromosomal stability by regulating multiple repair pathways.
Ubiquitin-proteasome pathway promotes homologous DNA repair

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The ubiquitin-proteasome pathway regulates a wide range of biological functions such as cell cycle control, immune responses and apoptosis, thus being considered to be a potential target for chemotherapy. Recently, Bortezomib, an inhibitor of proteasome, is found to be surprisingly effective against certain lymphoid malignancies such as multiple myeloma and non-Hogkin's lymphoma.

In this report, we firstly demonstrate that ubiquitin-proteasome system promotes double strand break repair mediated by homologous recombination (HR). The treatment of cells with MG132, a proteasome inhibitor, sensitized cells to DSB-inducing genotoxic stresses such as ionizing radiation and cisplatin, and abolished the IR-induced Rad51 focus formation, suggesting that MG132 inhibits HR. The impaired HR in the MG132-treated cells was also confirmed by the DR-GFP reporter assay. To explore the target of proteasome in HR, we examined the recruitment of various checkpoint and repair proteins to the site of the DNA damage. We found that although DSB recognition by ATM and MRN complex was normal, the subsequent activation of downstream checkpoint and repair proteins such as RPA, ATR and BRCA1 was severely impaired. These results suggest that proteasomal degradation at an early phase is critical for the proper assembly of HR repair machinery.
The function of the FBH1, novel helicase which has F-box domain

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FBH1 (F-box DNA helicase 1) gene belongs to members of the superfamily 1 helicase which contains seven conserved helicase motifs, and has both DNA-dependent ATPase and DNA unwinding activity. The F-box proteins constitute one of the four subunits of ubiquitin protein ligase complex called SCFs (SKP1/Cullin/F-box), which displayed ubiquitin ligase activity. Here, we established FBH1 null mutant DT40 cell by disrupting both helicase, F-box domain and then investigated the function of FBH1.

FBH1 null mutant cell showed normal growth rate. This mutant cell did not show significant sensitivity against several DNA damaging reagents. However, the level of spontaneous sister chromatid exchange (SCE) was about three times higher in mutant cell than WT.

To investigate precise function of FBH1, we created \textit{FBH1/RAD54} and \textit{FBH1/BLM} double mutants in chicken DT40 cells. Unlike the \textit{FBH1} single mutant, \textit{FBH1/RAD54} was hypersensitive to camptothecin(CPT), which preferentially induces fork block in replicating cells. The reconstitution of chicken FBH1 transgene in \textit{FBH1/RAD54} cells reversed CPT sensitivity , which indicated that the loss of FBH1 function induced CPT hyper-sensitivity.

These data suggest that FBH1 is involved in homologous recombination (HR) mediated repair during replication, thus maintain the genome stability in vertebrate cells.
Vertebrate PolQ, a PolA family DNA polymerase, is involved in base excision repair in parallel with Polβ

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The Drosophila melanogaster MUS308 gene is a unique DNA polymerase containing a helicase domain. The human ortholog of MUS308, designated POLQ, which encodes 2592 amino acid residues, and belongs to the A type family of DNA polymerases. In addition, two vertebrate POLQ paralogs were identified; HEL308 shows homology to the helicase domain of POLQ but has no polymerase domain, while POLN shows homology to the polymerase domain of POLQ but has no helicase domain. Subsequently, biochemical studies showed that both PolQ and PolN have DNA polymerase activity while only Hel308 exhibits helicase activity. Furthermore, PolQ can perform translesion DNA synthesis (TLS) past AP sites. However, the function of these PolQ paralogs in vivo has not been identified.

To investigate their function, we targeted each of these genes and created polq/HEL308 and polq/PolN double mutants in chicken DT40 cells. Unlike the mus308 mutant, these mutants are not hypersensitive to DNA crosslinking agents, but polq cells exhibit hypersensitivity to H2O2, implying a role for PolQ in base excision repair (BER). Accordingly, PolQ, similar to Polβ, accumulates rapidly at sites of laser-induced oxidative base damage and polq/Polβ double mutants show synergistic sensitivities to base damage inducing agents.
DNA polymerase [pol] plays an important role in homologous DNA recombination

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Homologous recombination (HR) is involved in the shuffling of allelic chromosomes during meiosis, the repair of DNA double-strand breaks (DSBs), and is also essential for the releasing of DNA replication blocks. Furthermore, some vertebrate species diversify their immunoglobulin variable (IgV) genes through HR mediated Ig gene conversion (GC). For example, chicken B lymphocyte precursors and DT40 cells diversify their IgV genes through HR mediated GC. HR is a multi-step process involving DNA synthesis. However, it has been unclear which DNA polymerase performs DNA synthesis in HR.

The release of DNA replication blocks is carried out, not only by HR, but also by error-prone translesion DNA synthesis (TLS). A number of TLS polymerases such as DNA polymerase [polI] and DNA polymerase [pol][polII] have been identified in yeasts and mammals, and polI, and polII are conserved between species. A defect in pol[polII] is responsible for variant form of xeroderma pigmentosum (XP-V), which is characterized by a predisposition to skin cancer and elevated UV sensitivity.

To investigate the role of pol[polII] in HR, we generated POL[polII]/- (hereafter called pol[polII]) clones in the DT40 line. Like XP-V cells, pol[polII] clones exhibited hypersensitivity to UV. Remarkably, pol[polII] cells showed a significant decrease in the frequency of both GC and DSB-induced HR, when compared to wild-type cells. Furthermore, human POL,[polII], but not its mutant gene defective in polymerase activity, reversed the rate of GC. These observations provide evidence that pol[polII] can promote DNA synthesis during HR. Our findings firstly identify a DNA polymerase that carries out DNA synthesis for physiological HR.
A major role for vertebrate 53BP1 in non-homologous end joining of DNA breaks

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53BP1 (p53 binding protein) is a BRCT domain-containing protein that is rapidly recruited to DNA double strand breaks (DSBs). To investigate the role of 53BP1 in the DNA damage response, we generated 53BP1⁻/⁻ cells from the chicken DT40 cell line. As in mammalian cells, mutation of 53BP1 increased cellular sensitivity to ionizing radiation. Although depletion of 53BP1 resulted in checkpoint defects in mammalian cells, DT40 53BP1⁻/⁻ cells had normal intra-S-phase and G2/M checkpoints. G1 specific radiosensitivity and a higher sensitivity to topoisomerase II suggested defective non-homologous end joining (NHEJ) defects in DT40 53BP1⁻/⁻ cells. Genetic analyses confirm this suggestion as we have demonstrated an epistatic relationship between 53BP1 and the NHEJ genes, Ku70 and Artemis, but not with Rad54, a gene essential for repair of DSBs by homologous recombination. We conclude that the major role of 53BP1 in supporting survival of DT40 cells that have suffered DNA DSBs is in facilitating repair by NHEJ.
Collaborative roles of $\mathbb{H}H2AX$ and the Rad51 paralog Xrcc3 in homologous recombinational repair

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One of the earliest events in the signal transduction cascade that initiates a DNA damage checkpoint is the phosphorylation on serine 139 of histone H2AX ($\mathbb{H}H2AX$) at DNA double-strand breaks (DSBs). However, the role of $\mathbb{H}H2AX$ in DNA repair is poorly understood. To address this question, we generated chicken DT40 cells carrying a serine to alanine mutation at position 139 of H2AX ($H2AX^{139A}$) and examined their DNA repair capacity. $H2AX^{139A}$ cells exhibited defective homologous recombinational repair (HR) as manifested by delayed Rad51 focus formation following ionizing radiation (IR) and hypersensitivity to the topoisomerase I inhibitor, camptothecin (CPT), which causes DSBs at replication blockage. Deletion of the Rad51 paralog gene, XRCC3, also delays Rad51 focus formation. To test the interaction of Xrcc3 and $\mathbb{H}H2AX$, we disrupted XRCC3 in $H2AX^{139A}$ cells. XRCC3$^{-/-}$/ $H2AX^{139A}$ mutants were not viable, although this synthetic lethality was reversed by inserting a transgene that conditionally expressed wild-type H2AX. Upon repression of the wild-type H2AX transgene, XRCC3$^{-/-}$/ $H2AX^{139A}$ cells failed to form Rad51 foci and exhibited markedly increased levels of chromosomal aberrations after CPT treatment. These results indicate that parallel pathways involving $\mathbb{H}H2AX$ and the Rad51 paralogs maintain genome stability by facilitating Rad51 assembly during HR.
Structure-specific endonuclease Xpf promotes homologous recombination associated with crossover

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Homologous recombination (HR) is associated with crossover particularly during meiosis. Crossover involves the extensive strand exchange and resolution of two homologous DNAs. No nucleases involved in the later step of HR have been elucidated so far in vertebrate cells. We observed that Xpf depletion in chicken DT40 cells strongly reduced sister chromatid exchange (SCE), crossover type HR, but not non-crossover type HR, and eventually resulted in lethality exhibiting mitotic chromosome breaks at two sister chromatids at the same sites, while Rad51 focus formation was not affected by the Xpf deletion. This phenotype appears to be caused by the absence of the Mus81-Eme1 endonuclease, the ortholog of which acts as a resolvase in fission yeast, because no chicken Mus81-Eme1 ortholog genes are present and ectopic expression of human Mus81-Eme1 significantly suppressed every phenotype of Xpf depleted DT40 cells. Moreover, the defective phenotype was also partially suppressed by E. Coli RusA resolvase but not by nuclease deficient Xpf transgenes. In conclusion, Xpf is required for HR with crossover.
Generation of p53-deficient medakafish by ENU-driven mutagenesis

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Medaka has long been used as a model organism. Recently, the molecular technology has been applied to medaka, enabling the detailed analyses of various biological processes. We report here the inactivation medaka gene function by random mutagenesis with N-ethyl-N-nitrosourea (ENU) followed by selection of mutations by PCR/sequencing. The p53 gene was analyzed in 5760 F1 fish for heterozygous mutations. Among 6 induced mutations found in the selected regions, two resulted in a premature stop codon. The fish that carry the p53 mutation showed impaired target gene activation in response to UV irradiation. As the cryopreserved medaka sperm is very stable in contrast to that of zebrafish, the frozen stock of mutagenized fish provides the valuable source for prompt production of knockout medaka.
Multiple repair pathways mediate tolerance to chemotherapeutic cross-linking agents in vertebrate cells

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Cross-linking agents, which induce DNA interstrand cross-links (ICLs), are widely used in anti-cancer chemotherapy. Yeast genetic studies show that nucleotide excision repair (NER), Rad6/Rad18 dependent postreplication repair (PRR), homologous recombination (HR) and cell cycle checkpoint pathway are involved in ICL repair. To study the contribution of DNA damage response pathways in tolerance to cross-linking agents in vertebrates, we made a panel of gene disrupted clones from chicken DT40 cells, each defective in a particular DNA repair or checkpoint pathway, and measured the sensitivities to cross-linking agents, including cisplatin, mitomycin C (MMC), and melphalan. We found that cells harboring defects in translesion DNA synthesis (TLS), Fanconi anemia complementation groups (FANC), or HR displayed marked hypersensitivity to all the cross-linking agents, while NER appeared to play only a minor role. This impact of replication-dependent repair pathways is distinctively different from the situation in yeast, where NER appears to play a major role in dealing with ICL. Cells deficient in Rev3, the catalytic subunit of TLS polymerase pol[δ] showed the highest sensitivity to cisplatin, followed by fanc-c. Furthermore epistasis analysis revealed that these two mutants work in the same pathway. Our genetic comprehensive study reveals a critical role for DNA repair pathways that release DNA replication block at ICLs in cellular tolerance to cross-linking agents, and could be directly exploited in designing an effective chemotherapy.
Critical Roles for Polymerase[] in Cellular Tolerance to Nitric Oxide–Induced DNA Damage

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Nitric oxide (NO), a signal transmitter involved in inflammation and regulation of smooth muscle and neurons, seems to cause mutagenesis, but its mechanisms have remained elusive. To gain an insight into NO-induced genotoxicity, we analyzed the effect of NO on a panel of chicken DT40 clones deficient in DNA repair pathways, including base and nucleotide excision repair, double-strand break repair, and translesion DNA synthesis (TLS). Our results show that cells deficient in Rev1 and Rev3, a subunit essential for DNA polymerase [] (Pol[]), are hypersensitive to killing by two chemical NO donors, spermine NONOate and S-nitroso-N-acetyl-penicillamine. Mitotic chromosomal analysis indicates that the hypersensitivity is caused by a significant increase in the level of induced chromosomal breaks. The data reveal the critical role of TLS polymerases in cellular tolerance to NO-induced DNA damage and suggest the contribution of these error-prone polymerases to accumulation of single base substitutions.
Extensive chromosomal breaks are induced by tamoxifen and estrogen in DNA repair-deficient cells

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Tamoxifen (TAM) possesses antiestrogen activity and is widely used for the treatment or prevention of breast cancer. However, it is also carcinogenic in human uterus and rat liver, highlighting the profound complexity of its actions. To explore the molecular mechanisms of TAM-induced mutagenesis, we analyzed the effects of this drug on gene-disrupted chicken B lymphocyte (DT40) clones deficient in various DNA repair pathways. Rad18, Rev3, and Pol[ ] are involved in translesion DNA synthesis (TLS), which facilitates recovery from replication blocks on damaged template strands. DT40 cells deficient in TLS were found to be hypersensitive to TAM, exhibiting an increase in chromosomal breaks. Furthermore, these mutants were also hypersensitive to 4-hydroxyestradiol, a physiological metabolite of estrogen. These data suggest a contribution of TLS to the prevention of chromosomal breaks by TAM and estrogen, and they therefore indicate that such error-prone DNA synthesis underlies mutagenesis induced by these agents.