DEPARTEMENT OF GENETICS AND MOLECULAR BIOLOGY
LABORATORY OF GENE INFORMATION ANALYSIS

1) Binding specificity of hREV7 protein: T. HANAFUSA, E. OHASHI, K. HARA, H. HASHIMOTO[1], and H. OHMORI ([1]Yokohama City University)

Human cell has at least 15 different DNA polymerases (Pols). Among them, only a few enzymes (such as Polδ and Polε) essential for chromosomal DNA replication have proof-reading function due to intrinsic 3'-5' exonuclease activity and exhibit high processivity in the presence of PCNA, the sliding clamp. Most of human Pols lack such proof-reading activity and hence are error-prone. However, such error-prone Pols are required for bypassing through sites of damaged DNA. Four Y-family Pols (Polη, Polι, Polκ and REV1) and one B-family (Polζ) have been shown to participate for translesion DNA synthesis (TLS). While the four Y-family enzymes are composed of a single subunit, Polζ contains two subunits; the REV3 catalytic subunit and the REV7 accessory subunit of unknown function. The human REV7 (hereafter hREV7) has a weak similarity (23% identity) to the human MAD2 protein, which plays a crucial role in spindle assembly checkpoint via interactions with MAD1 or CDC20. For the reason, hREV7 is also called hMAD2B (hMAD2L2 or hMAD2β). hREV7 has also been reported to interact with multiple human proteins of various function, such as ADAM9 (a metalloprotease disintegrin), PRCC (papillary renal cell carcinoma) or ELK-1 (transcriptional factor involved in JNK MAP kinase pathway). Moreover, a recent study has reported that hREV7 interacts with the *Shigella* IpaB protein, which is one of the promoting effectors for the bacterial inversion into host cells. Thus, hREV7 seems to play a critical role in linking TLS to cell cycle regulation or gene expression changes.

Previously, hREV7 was shown to interact with a C-terminal domain (CTD) of hREV1 (approximately 120 aa in the total 1251 residues) and a central region of hREV3 (approximately 50 aa in the total 3130 residues). More recently, we and other groups reported that the same hREV1 CTD interacts also with Polκ, Polι and Polη via short motif sequences containing two consecutive phenylalanine (FF) residues. However, since hREV7 does not contain any FF sequence in the entire region (211 residues), we expected that hREV7 should interact with the REV1 CTD differently from the three Pols.

To investigate how hREV7 interacts with hREV1 or hREV3, we first analyzed which region of hREV1 or hREV3 were required for the binding to hREV7. Our obtained by yeast two-hybrid assay revealed that while approximately 90 residues in the hREV1 CTD is required for its interaction to hREV7, a short (9 aa) sequence of hREV3 (1877-ILKPLMSPP-1885) is sufficient for interacting with hREV7. Further analyses by alanine scanning mutagenesis revealed that the P1880A and P1885A mutations significantly reduced the hREV7-interacting activity and that the P1880A/P1885A double mutation completely abolished the activity. Similar analyses with ADAM9 and ELK1 also revealed that the presence of two Proline residues is crucial for the hREV7 interaction. Thus, we concluded that ϕLxPxxxxP (ϕ is a hydrophobic residue and x is any amino acid residue) is the consensus sequence of the hREV7-binding motif.

Very surprisingly, hMAD2 was also found to bind to the same hREV7-binding sequence of hREV3, while it did not interact with the hREV1, ADAM9 or ELK1. hREV7 did not interact with the hMAD2-binding sequence of MAD1 or CDC20, whose consensus sequence is often presented as (K/R)ϕϕxxxxP. While some previous papers suggested that hREV7 interacted with CDH1, a homologue of CDC20, we could not detect any interaction between hREV7 and CDH1 by our yeast two-hybrid assays. Furthermore, we found that mutations at some residues in the 1877-ILKPLMSPPS-1886 sequence of hREV3 differently affected the interactions with hREV7 and hMAD2. For example, I1877A and L1878A substitutions completely abolished the interaction with hMAD2, while they showed no or little affect on the interaction with hMAD2. On the other hand, P1880F substitution abolished the hREV7 interaction, while fully retaining the hMAD2 interaction. These results were confirmed by *in vitro* pull-down assays using fusion proteins (GST-hREV3 and hREV7-His or hMAD2-His) that were overproduced in *E. coli* cells. Thus, our results suggest a possibility that hREV3 may be involved in spindle assembly checkpoint via interaction with hMAD2. We are currently trying to determine the binding specificity of hREV7 and hMAD2 at the atomic level.

2) Testis specific 3'-mRNA processing in the *Polk* and *Ccdc89* genes: Y. S. CHAN, H. TANAKA[1], H. OHMORI ([1]Osaka University)

Polκ is one of DNA polymerases specialized for translesion DNA synthesis. While many genes involved in DNA repair, replication and recombination show higher

expressions in testis than in other organs, mRNAs for both human *POLK* and mouse *Polk* genes have a unique feature; their transcripts in testes are much shorter than those in other organs. The mRNAs coding for Polκ abundantly expressed in testes are about 2.8kb in length whereas those ubiquitously expressed in many other organs (including testis at lower level) are 4.2kb. The major and shorter transcripts found in testis have a polyA tail immediately downstream of the translation stop codon, whereas the longer transcripts have a much longer 3'-UTR with multiple copies of ARE (AU-rich elements) that is known to destabilize mRNA when present in 3'-UTR.

We are interested in examining whether transcripts of other genes are similarly regulated. *Ccdc89*, a mouse gene whose expression is dramatically up-regulated during spermiogenesis, was reported to generate shorter transcripts in testis than those found in other organs at embryonic stages. Our experiments with Northern blotting showed that *Ccdc89* is expressed specifically in testis of inborn and adult mice. The major transcripts found in testis are about 1.5kb in length while the transcripts expressed in embryo and the minor transcripts found in testis are 2.4kb. In addition, all of such transcripts have the same coding region, differing only in the length of 3'-UTR. By analyzing the 3'-end sequence of 3'-RACE products, we found that their polyA signals differ from each other. Furthermore, 5'-cap site hunting analysis showed that transcription of this intron-less gene starts from only one site. Thus, we concluded that *Ccdc89*-mRNAs with different lengths in 3'-UTR are generated due to alternative selection of polyA signal, not due to alternating splicing or initiation of the transcription at different sites. Because the longer transcripts of *Polk* and *Ccdc89* have long 3'-UTR containing multiple copies of AUUUA sequence (an essential and minimal units of ARE), it seems plausible that a similar mechanism operates to generate shorter transcript with shorter 3'-UTR that contain no or less copies of AUUUA, thereby increasing mRNA stability in testis.

CstF64 is a protein essential for polyadenylation of mRNA, involved in the cleavage process of nascent transcripts in the downstream of the consensus polyA signal AAUAAA. The gene coding for CstF64 is located on the X chromosome. Recently, it has been shown that a new form of CstF64 (named τCstF64) is highly expressed in testis during pachytene periods, while the *CstF64* gene expression is blocked due to X chromosome inactivation. Since our Northern blotting results showed that mRNAs of *Polk* and *Ccdc89* with different lengths in 3'-UTR are generated after the induction of

the *τCstF64* gene, we believe that τCstF64 plays a key role in causing the differences of polyadenylation site selections.

To verify whether τCstF64 alone is sufficient in causing alternative polyA signal selection, we plan to examine the change of size for *Polk*-transcripts under τCstF64 over-expressed condition in NIH3T3 cells. Next, we are also going to examine the change in length of *Ccdc89*-mRNA under τCstF64 over-expressed condition, by co-transfection of *Ccdc89* expression vector with *τCstF64* expression vector into NIH3T3 cells. On the other hand, since possibility that some factors other than τCstF64 may also take part in the selection of polyA signal exists, we plan to investigate the change of size for both *Polk*- and *Ccdc8*9-mRNA in mGS cells (male germ cells isolated from neonatal mouse testis) when the expression of τCstF64 is suppressed by siRNA.

3) DNA damage induced ubiquitylation of RFC2 subunit of RFC complex: J. Tomida, Y. Masuda[1], H. Ohmori, T. Todo[2] (**[1]Hiroshima University and [2]Osaka University**)

Many proteins involved in DNA replication and repair are known to undergo post-translational modifications such as phosphorylation and ubiquitination. PCNA (Proliferating Cell Nuclear Antigen, a homo-trimeric protein that encircles double-stranded DNA to function as a sliding clamp for DNA polymerases) is mono-ubiquitinated by the Rad6-Rad18 complex, and further poly-ubiquitinated by the Rad5-Mms2-Ubc13 complex, in response to various DNA-damaging agents. PCNA mono- and poly-ubiquitination activate an error-prone translesion synthesis pathway and an error-free pathway of damage avoidance, respectively. We showed that RFC (Replication Factor C, a hetero-pentameric protein complex that loads PCNA onto DNA) is also ubiquitinated in a RAD18-dependent manner in cells treated with alkylating agents or H_2O_2. A mutant form of RFC2 with a D228A substitution (corresponding to a yeast Rfc4 mutation that reduces an interaction with RPA, a ssDNA-binding protein) is heavily ubiquitinated in cells, even in the absence of DNA damage. Furthermore, RFC2 was ubiquitinated by the RAD6-RAD18 complex *in vitro* and its modification was inhibited in the presence of RPA. RPA-sensitive ubiquitination is unique to RFC2, not reported for PCNA. Our findings suggest that RPA plays a

regulatory role in DNA damage responses via RFC2 ubiquitination in human cells (Ref).

Ref. J. Tomida, Y. Masuda, H. Hiroaki, T. Ishikawa, I.Y. Song, T. Tsurimoto, S. Tateishi, T. Shiomi, Y. Kamei, J. Kim, K. Kamiya, C. Vaziri, H. Ohmori, T. Todo. JBC in press.

LIST OF PUBLICATIONS

Department of Genetics and Molecular Biology
Laboratory of Genetic Information Analysis

M. Kawanishi, K. Matsukawa, E. Ohashi, T. Takamura, Y. Totsuka, M. Watanabe, T. Sugimura, K. Wakabayashi, F. Hanaoka, H. Ohmori, T. Yagi.: Translesion DNA synthesis across mono ADP-ribosylated dG by Y-family DNA polymerases, *New Developments in Mutation Research*, pp 133-148, 2007

L. R. Barkley, H. Ohmori, C. Vaziri.: Integrating S-phase checkpoint signaling with trans-lesion synthesis of bulky DNA adducts. *Cell Biochem. Bioohys.,* 47, 393-408, 2007.

H. Ohmori：Protein-protein interactions during translesion DNA synthesis, 第３０回日本分子生物学会年会及び第８０回日本生化学会大会、横浜、１２月１１ー１５日、２００７

Y. S. CHAN、田中　宏光、大森　治夫：マウスPolκをコードする *Polk*-mRNAの3’末端プロセッシングの解析、第３０回日本分子生物学会年会及び第８０回日本生化学会大会、横浜、１２月１１ー１５日、２００７

花房　朋，大橋　英治，原　幸大，橋本　博，村雲　芳樹，大森　治夫：損傷乗り越え合成に関わるREV7タンパク質の結合特異性についての解析、第３０回日本分子生物学会年会及び第８０回日本生化学会大会、横浜、１２月１１ー１５日、２００７

冨田　純也、増田　雄司、廣明　秀一、 釣本　敏樹、立石　智、石川　智子、亀井　保博、金　鎭炯、大森　治夫、藤堂　剛：DNA損傷によって誘導される RFC 複合体のユビキチン化、日本放射線影響学会第５０回大会、千葉、１１月１４ー１７日、２００７

遺伝子動態調節研究部門　　Department of Genetic and Molecular Biology
遺伝子情報解析研究分野　　Laboratory of Gene Information Analysis

当グループでは２００７年に大きなメンバー交代があった。約１０年間に渡って当グループの重要メンバーであった大橋英治は２００６年８月１日から短期助手として採用されたが、２００７年２月末にその任期を終えて、米国国立衛生研究所に留学した。また技術補佐員として２００６年４月に加わった風間貴仁は２００７年３月末で退職し、４月から冨田純也が教務補佐員、佐々木克仁が技術補佐員として当グループに加わった。

冨田純也は京大放射線生物研究センターの藤堂剛教授の研究室で医学研究科の大学院生としてヒト培養細胞を用いてDNA損傷応答について実験していたが、藤堂教授が阪大医学研究科に転出されることになったので当グループに移って来た。彼はそれまでの研究課題について引き続き実験しながら、当グループが行ってきたHIVの宿主染色体への組み込みに関わる酵素Integrase (IN)とDNA損傷応答においてPCNAのUbiquitin (Ub)化に関わるE3酵素であるRAD18タンパク質との相互作用の実験にも関わることになった。INはそのN末端においてUb化されるとプロテアソームによる分解に向かうことが知られて来たが、RAD18との相互作用によってはむしろ安定化されることが米国のグループにより報告されていた。RAD18はE2酵素であるRAD6と複合体を形成して、DNA損傷を受けた細胞では多くのDNA polymerasesと結合してそのprocessivityを高めることが知られているPCNAの特定のアミノ酸（K164）をmono-Ub化することが知られていた。この場合もUb化されたPCNAは分解には向かわず、むしろ損傷バイパスに関わるPolηやPolκなどのDNA損傷箇所へのリクルートに関わると考えられている。冨田は、特にMMSなどのアルキル化剤によってDNAが損傷を受けたヒト細胞ではPCNAをDNA上にロードするRFC (Replication Factor Cの略で、1~5のサブユニットから構成される)のうち、RFC2及びRFC4がRAD18によるUb化を受けることを示した。RAD18はPolηとも直接結合することが報告されているが、HIV INタンパク質との相互作用の意義、メカニズムについては更なる研究が必要である。