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Molecular Mechanism of the Recruitment of XRCC4 and DNA Ligase IV to DNA Double-strand Breaks

Doctor Thesis

Sicheng LIU

Guided by

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Nov, 2013

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Abstract

DNA double-strand break (DSB) is the most severe type of DNA damages which can be induced by ionizing irradiation or chemotherapeutic drugs. Eukaryotic cells are able to repair DSB through Homologous-recombination (HR) or Non-homologous end-joining (NHEJ) pathways, where NHEJ is the major pathway that is fast, efficient and independent of cell cycle. Ligase IV and XRCC4 are two key molecules in NHEJ pathway, forming complex and ligating two broken DNA ends. We expressed Ligase IV gene in Ligase IV-deficient Nalm-6 cells, showing that the chromatin binding of XRCC4 requires Ligase IV. We focused on the role of Ligase IV C-terminal region and created two point mutations W725R and W893R. The mutated tryptophan site is highly conserved in Ligase IV protein across species and also in BRCT containing proteins involved in DNA repair.Both of the two mutations showed a significant decrease in survival fraction which is comparable to the reference mutation R278H, a mutation that was identified from a patient with Ligase IV syndrome. We analyzed the chromatin binding of Ligase IV and XRCC4 in the two mutants, and found thebinding in W725R was much reduced, but in W893R it was hardly seen. W893R is expressing at extremely low level, which might have protein instability.We have shown both BRCT domains are important for the function of Ligase IV, and suggested the two domains are involved in DSB recognition. We created a Ligase IV C-terminal fragmentnamed as LigIV-CT, which does not include the DNA binding domain on N-terminal region which is supposed to facilitatechromatin binding. We have found theLigIV-CT was capable of binding to chromatin with XRCC4. We then proposed a model that Ligase IV can bind to chromatin

with both N-terminal and C-terminal regions. C-terminal region especially binds to the DSB site where Ligase IV is recruited after IR. Ligase IV could possibly bridge DNA ends with its two binding domains on the two opposite terminiduring NHEJ repair. BRCT mutations disrupt the DSB induced C-terminal binding of Ligase IV, and result in high radiosensitivity. Our results also suggest two BRCT domains might be necessary for stable chromatin binding of XRCC4.

CHAPTER 1

Introduction

1-1 Ionizing Radiation

Radiation is a process that energy particle or energy wave passes through a certain space. Radio wave, visible light are examples of low energy radiation, ultra-violet light and α-ray are examples of high energy radiation.

High energy radiation composes particles individually with enough kinetic energy to liberate an electron from an atom or molecule(Satake*et al.,*1997). The atom or molecule will then become ionized, so the high energy radiation is also called ionizing radiation. As the ionization energy of a hydrogen atom is 13.6 eV, a kinetic energy of 10 eV is approximately considered as threshold for ionizing radiation(Nave, 2013).

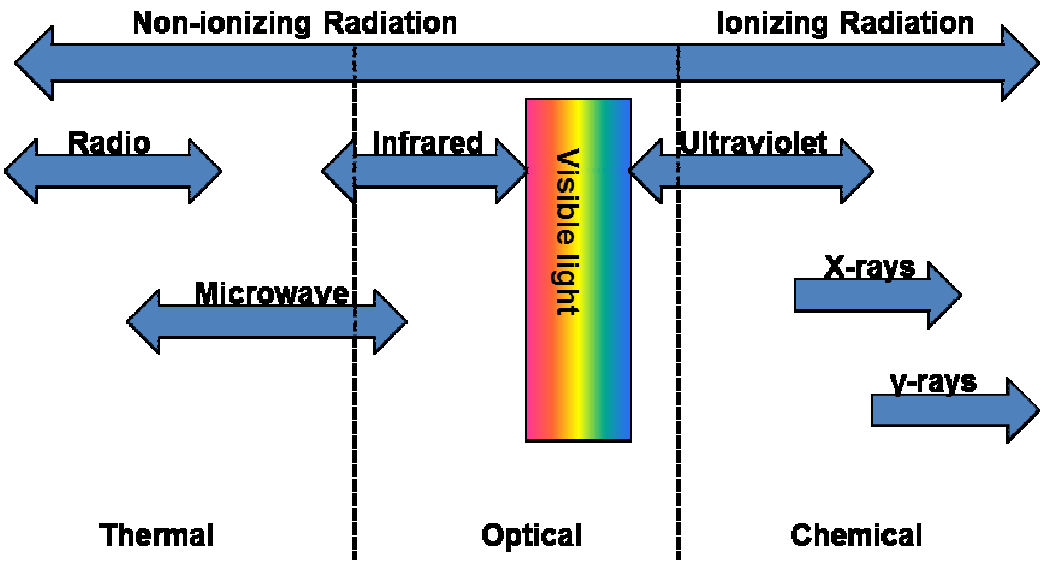

Fig.1 - 1*Types of radiation*

Ionizing radiation can be generated by nuclear reactions, either natural or artificial, in the action of fission or fusion. Emitted α-ray,β-ray or γ-ray all contains enough energy to

ionize a target atom or molecule. Ionizing radiation can also be generated by extremely high temperature or acceleration of charged particles, providing particles with enough energy.

When ionizing radiation is absorbed or emitted by an atom, it can liberate an atomic particle such as electron, photon, and neutron etc., resulting chemical bond change. Reactive ion-pairs are usually produced, leading to subsequent process. There are several types of ionizing irradiation categorized by the energy carrier. Electron, photon, heavy ion and neutron radiation have difference in energy deposition when penetrating a material. Different type or energy of ionizing radiation usually causes distinguishable radiation effects.

After the discovery of x-ray by Wilhelm Conrad Roentgen in 1985 and natural radioactive uranium compound by Henri Becquerel, scientists began to utilize the advantage of ionizing radiation and at the same time realized its harmfulness to human body. Exposure to ionizing radiation exceeding a certain limit can do severe damage to living tissues, resulting long-term biological effects known as mutation, radiation sickness and cancer, eventually leading todeath. In the 20^{th} century, nuclear bomb explosions and nuclear power plant disasters have caused severe aftereffects to survivors. The invisible and insensible nuclear ionizing radiation has become a public panic afterwards.Studies in radiobiology then become an urgent necessity to understand the cause of biology effects.

1-2 Mechanism of the action of ionizing radiation

As water is the major component of all living bodies, which capturing about 70% - 85% total weight of a cell, water plays a major role in radiobiology. When ionizing radiation

passes through a cell, it has high chances to interact withwater moleculeswhich are the most abundant. Water receives adequate energyfrom ionizing radiation and undergoes a breakdown sequence to hydrogen peroxide, hydrogen radicals and assorted oxygen compounds, such as ozone. These compounds are chemically active and subsequently produce indirect effects of ionizing radiation to the cell, named as water radiolysis.The indirect effect is a major cause for cell damage than direct effect. Water is extensively studied for radiobiology as the absorption property of water is very similar to human body. It is also widely used for clinical dosimetry(Plante, 2010).

The post radiation process can be divided into 4 more or less overlapping stagesby the time after radiation.

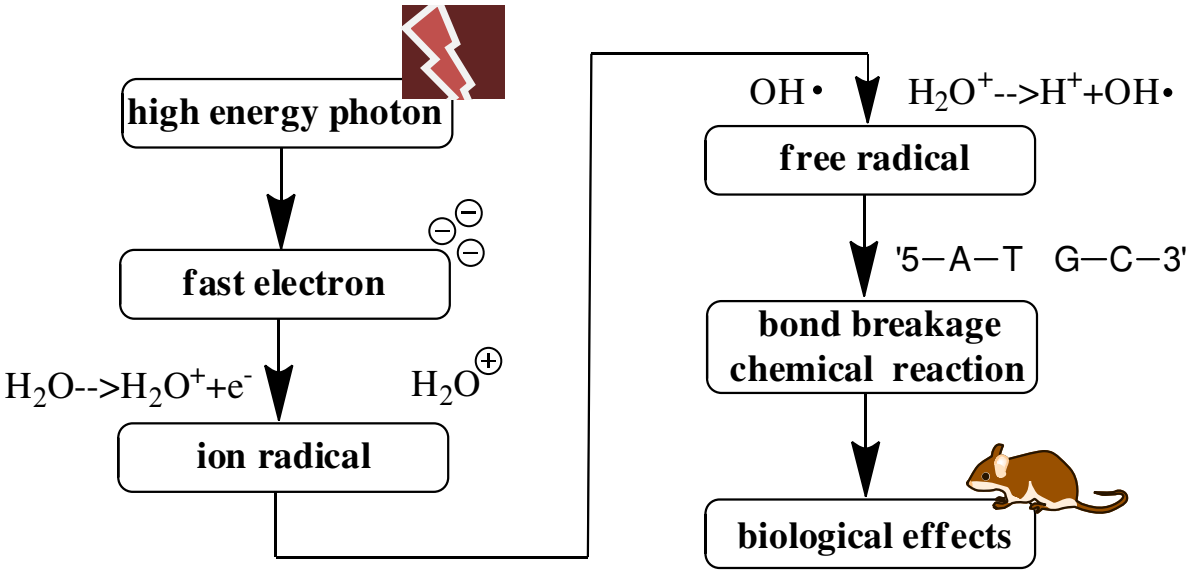

Fig.1 - 2 ***Mechanism of the action of ionizing radiation***

The physical stage is completed within 10^{-15} seconds. The kinetic energy is quickly transferred from ionizing radiation to target material, accompanying excitation and

ionization of the orbital electrons or nuclei. In this stage, if the energy is strong enough, an electron is will be removed from a water molecule, subsequently forming water ions, which is called ionization. If the energy is not enough for ionization, the electron will only change from fundamental state to an excited state, called excitation. The exited water molecule also has the potential to transfer its acquired energy.

The next stage is called physico-chemical stage, occurred within 10^{-15} and 10^{-12} seconds after ionizing radiation. The ionized and excited molecule in physical stage is highly unstable and will dissipate energy to neighboring molecules or cause bond rapture. ·OH radicals can be generated by proton transfer from an ionized water molecule to a neighboring water molecule. ·OH radicals are easy to interact with biological molecules in the cell as they are chemically reactive. Excited water molecules can dissipate energy by splitting into H· and ·OH, H_2 and O or 2H and O radicals. It may also dissipate energy in the form of heat, without causing any chemical bond breakage.

The third stage is calledchemical stage, which ranges from 10^{-12} to 10^{-6} seconds after ionizing radiation. It is the stage for free radical reaction. Chemically reactive products such as H·, ·OH, H_2 and H_2O_2 are created in high concentration. They will diffuse in the medium and induce chemical reactions. If H· luckily meets its counterpart ·OH, they will form back to water molecules and dissipate heat. But if the free radical meets a biological molecule, the molecule will be activated and undergoes unusual chemical reactions, leading to a loss of biological function.In this stage, some abnormal molecule structures and functions are likelyto appear, most of which is hazardous to cell survival.

The biological stage comes from seconds to years after radiation. It is the final stage that forms all level of injuries from cellular structure to organism and population. Either short or long, cells follow their own way to recognize and resolve the injury. Some of the

cellular misbehaviors can be detected and evaluated by experiments(Hall and Giaccia, 2011).

1-3 DNA

Deoxyribonucleic acid (DNA) is a molecule that encodes basic hereditary information used in development and functioning in all living cells and many viruses. DNA in the cell is organized ina long structure called chromosome. For eukaryotes, all genes are carried in the chromosomes of the nucleus, except small amounts of sequence information carried in mitochondria or chloroplasts (only for plants).

Most DNA molecules have a structure of double-stranded helix, which was first discovered by James Watson and Francis Crick in 1953. It consisting of two long biopolymers made of simpler units called nucleotides. Each nucleotide is composed of a nucleobase and a phosphate backbone. There are four types of nucleobases, named adenine (A), thymine (T), guanine (G) and cytosine (C) respectively. The nucleobases are attached to deoxyribose sugar and by the sequence arrangement on alternating phosphate and sugar residues it enables storageof biological information.

The two DNA strands are anti-parallel that runs in an opposite direction. Each stands has a 5’ end and a 3’ end, named from the position of carbon on the deoxyrobose sugar that is on the end of DNA strand. Nucleotides in vivo can only be synthesized from 5’ end to 3’ end, so sequence of DNA is conventionally recorded from 5’ end to 3’ end. The two stands are stabilized by hydrogen bonds between nucleotides and base-stacking interactions among aromatic nucleobases.

DNA along with protein and carbohydrate are three major macromolecules for the essential of life. Protein and carbohydrate have multiple copies in a single cell, but DNA may only present in a single copy. Damage to DNA can cause catastrophic results and it is

essential for cell to keep the integrity of its DNA molecule.

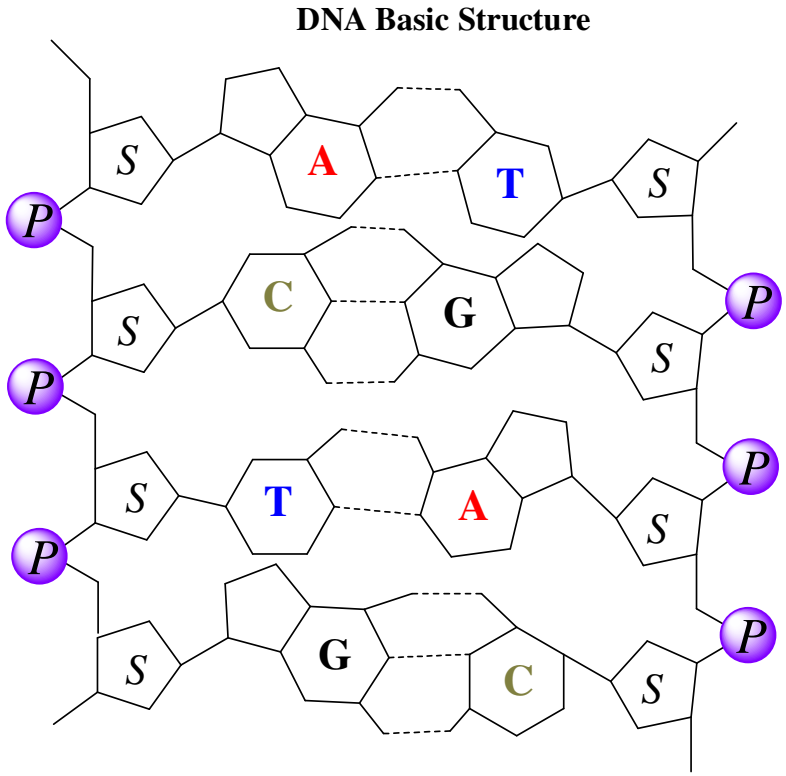

Fig.1 - 3 ***Basic unit of DNA***

There are around 6 billion base pairs distributed across 46 chromosomes in human cells. Cells can replicate the genetic information precisely to the progeny cells, with an error rate less than 1 nucleotide per cell.The cells also have error correction mechanism which further reduces the error during replication. Keeping a high fidelity in genetic information, cells in human body can maintain same DNA sequence and function properly(Watson *et al.,* 2007).

The error-prone property of DNA is contributed to the elegant regulation of the cell during long time evolution, as well as the DNA double-strand break repair we want to elaborate.

1-4 DNA damage

Use of focused micro-beam of α particle to selectively irradiate cell organelle has shown that the nucleus is the most sensitive part to radiation(Munro, 1970). As most space of cell nucleus is captured by chromatin, which is a complex arrangement of both DNA and nuclear proteins, the DNA is the most sensitive target of ionizing irradiation. For this reason, a lot of research in radiobiology is focused on DNA. It is important to know the mechanism of DNA damage and DNA repair.

Ionizing radiation to the cell can result DNA damage, which comes from direct effect and indirect effect to DNA molecule. The direct effect is the interaction of radiation directly with DNA molecule. DNA is either ionized or excited, acquired high chemical potential for reaction and eventually led to bond rupture. The indirect effect is the interaction between DNA and reactive species generated during water hydrolysis. The ·OH radical is the most important DNA-damaging agent. The indirect effect takes approximately 2/3 of total damage caused by ionizing radiation, but the proportion also varies from radiation type and energy.

After a cell exposed to ionizing radiation, various types of DNA damage are generated. The type of DNA injuries can be categorized to 7 groups, named single-strand break (SSB), double-strand break (DSB), abasic site, adduct, intra-strand crosslink, inter-strand crosslink and DNA-protein crosslink. DSB is likely caused by multiple DNA lesions within 10to 20 base pairs. DSB is considered to be the most severe type of damage as it can lead to acute and long-term radiation aftereffects(Hall and Giaccia, 2011).

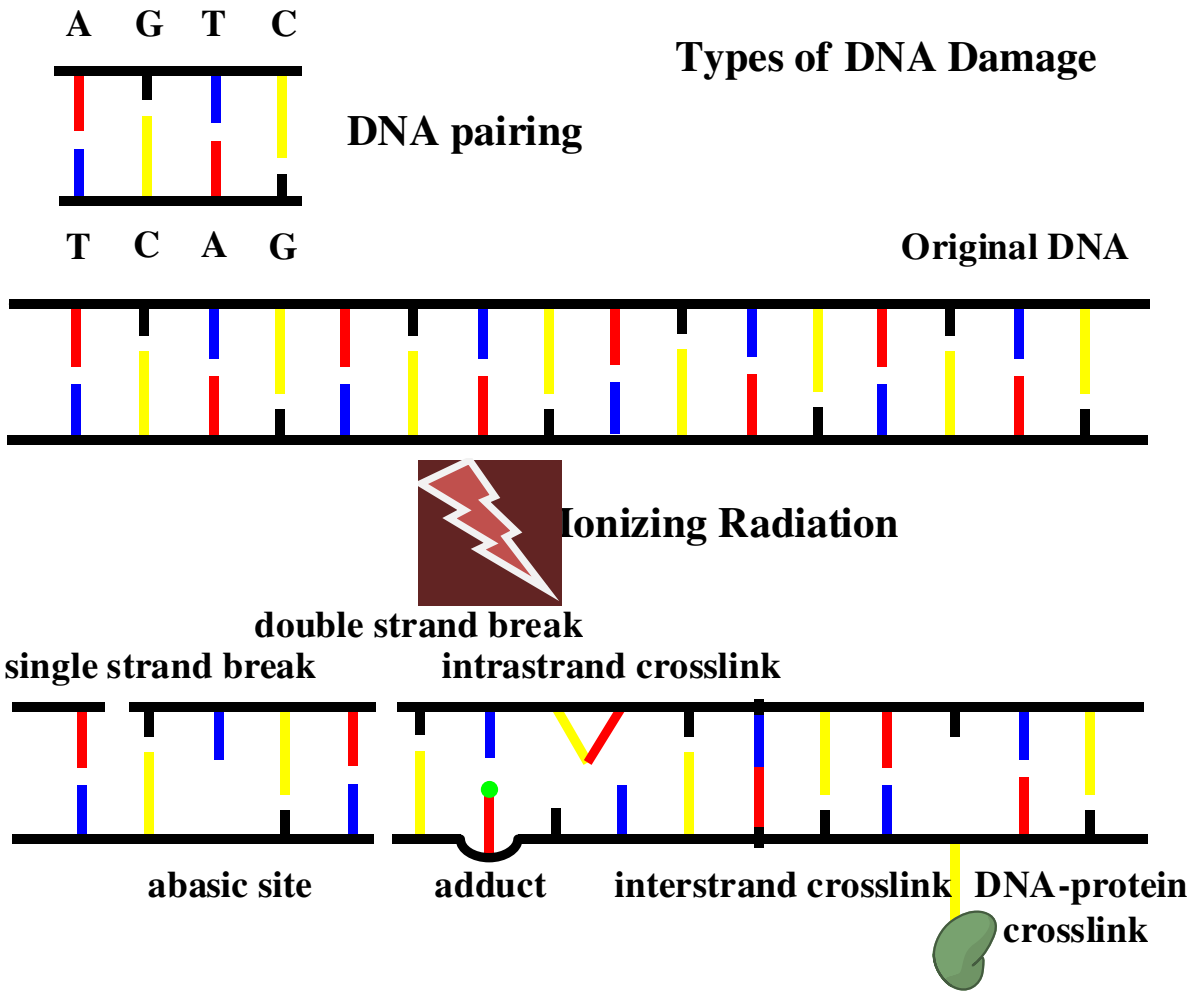

Fig.1 - 4*Types of DNA damage*

The gray (Gy) is the SI derived unit for absorbed dose. It is defined as the absorption of 1 joule of energy by 1 kilogram of matter. It is independent of any target material so that same absorbed dose may have different biological effects in different tissues or organisms. In radiobiology another unit sievert (Sv) is used for calculate the effective dose. It is a measure of the cancer risk to thewhole organism that the biological effects of ionizing radiation are unevenly delivered to the parts of the organism. For x-rays and γ rays the absorbed dose in gray is numerically the same value as the effective dose in sievert.

Normally for ionizing radiation with an intensity of 1Gy, it will induce 1000-2000 base damages, 500-1000 SSBs and 20-50 DSBs. SSBs do not lead to mutation or expression of injury. The remaining strands keep intact and can be simply rejoined by DNA ligase. Whilein the case of DSBs, the broken end of DNA will become unstable and separate away. If left unrepaired, the cell will lose chromosomal material during subsequent cell division or rejoined to a different chromosome, forming a chromosome aberration. Once the cell accumulated a large amount of DNA damage, it will eventually go into an irreversible state of dormancy, known as senescence, or it will suicide, known as apoptosis or programmed cell death. Also there is a catastrophic possibility for the cell that it will have unregulated cell division, which leads to carcinogenesis(Hall and Giaccia, 2011).

1-5 DNA repair

The human genome is under constant assault from both inside and outside of the cell. There will be tens of thousands DNA lesions in each human per day. If the genetic information remains corrupt, cells cannot function and proliferate properly. Cells must and they do have evolved the ability to identify and correct DNA damages. Depending on the type of damage on the DNA strands, the cells choose different repair strategies to restore the lost information. If possible, an error-free recovery is preferred. The unmodified complementary strand or the sister chromatid during DNA replication can act as a template to recover original information. If the DNA sequence is not restored, cells can still utilize an error-prone pathway as a last resort to rescue the cell.

If the DNA damage does not involve any breakage of the phosphodiester backbone, the DNA can be simply repaired by direct reversal. For example, pyrimidine dimers can

be reversed by an enzyme called photolyase, and methylation of guaine bases can be reversed by the enzyme called methyl guanine methyl transferase (MGMT). Cells can also have base excision repair (BER) to repair single base damage caused by oxidation, alkylation, hydrolysis, or deamination, nucleotide excision repair (NER) to eliminate bulky, helix-distorting lesions such as pyrimidine dimers and 6,4 photoproducts, and mismatch repair (MMR) to correct mispaired nucleotides errors from DNA replication and recombination.

In the case of backbone breakage, the cells need much more effort to recognize and repair the damage. DNA double-strand break (DSB) is the most deleterious lesion occurred on the DNA. Reactive oxygen species generated by cellular metabolism and replication or exogenous impact such as ionizing radiation and chemotherapeutic drugs are likely to induce hazardous DSBs to the cell. To rescue the nucleus, cells utilize twomajor pathways called homologous recombination (HR) and non-homologous end joining (NHEJ) to repair DSBs(Wang *and* Lees-Miller, 2013). HR is an error-free pathway which requires sister chromatid as a template to provide identical genetic information, so this pathway repair is strongly dependent on cell cycle and it is only available in late S and G2 phase after DNA replication. NHEJ, on the other side, can repair DNA DSB throughout the cell cycle. It is an error-prone pathway that does not require any sequence homology for repair. Damaged nucleotides are frequently lost after NHEJ repair and it sometimes introduces mutations. It has been reported that after the generation of DSBs, both HR and NHEJ pathways are activated in the cell and they compete with each other for the repair. The decision of the cell is thought to be the nature of broken DNA ends (Frank-Vaillant *et al.,* 2002). As NHEJ repair is much faster and more efficient than HR pathway, it is considered as the major and most important

pathway for the cell to repair DSBs.NHEJ is also required for joining hairpin-capped DSBs induced during V(D)J recombination, which is necessary to generate diverse immunoglobulin receptors in the vertebrate immune system. Besides the major HR and NHEJ pathways, DSBs in eukaryotic cells can also be repaired by microhomology-mediated joining (MMEJ) pathway, which is also named by alternative NHEJ pathway(Watson *et al.,* 2007).

1-6 DNA damage response

The DNA damage response (DDR) is a signal transduction pathway that senses DNA damage to activate repair process. The DDR is primarily mediated by proteins of the phosphatidylinositol 3-kinase-like protein kinases (PIKKs) family, which includes ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR) and DNA-dependent protein kinase (DNA-PK), and also mediated by proteins of the poly(ADP)ribose polymerase (PARP) family (Anderson,1993; Ciccia *et al.,* 2010).

ATM can be autophosphorylated and the chromatin binding event of MRN (MRE11/RAD50/NBS1) complex may promote ATM autophosphorylation.Activated ATM can phosphorylate hundreds of downstream substrates, among which H2AX is the best known one(Matsuoka et al., 2007). H2AX is a variant of H2A, which belongs to the histone family. Histones are highly alkaline proteins that bind tightly with DNA and form a packaged nucleosome structure, allowing higher order DNA conformation. γ-H2AX is the phosphorylated H2AX(Rogakou *et al.,* 1998), and it is considered as one of the earliest DSB markers in the cell. γ-H2AX rapidly accumulates at the DSB sites so that aγ-H2AX foci can be clearly seen with fluorescent staining. γ-H2AX is also frequently used to indicate the level of DNA damage (Vignard*et al.,* 2013).

DNA-PK however, only regulates a small group of proteins involved in DSB end joining, such as Ligase IV and XRCC4. ATR is mainly activated by SSB after the recruitment of RPA at stalled replication folk. Proteins PARP1 and PARP2 of PARP family are also found to be recruited to DNA end as a damage response to activateDNA polyADP-ribosylation(Harper *and* Elledge, 2007).

The DDR amplifies DNA damage signal and determines the future of the cell, facilitating events such as pathway choice and apoptosis. NHEJ and HR pathways both require the participation of ATM and ATR. ATM and ATR can phosphorylate checkpoint kinase 1 (CHK1), CHK2 and p53, which activate cell cycle arrest and halt cell cycle progression. The cells will be offered time that is necessary for DNA repair. Phosphorylate of p53, CHK1, CHK2, BRCA-1, RAD9, and RAD17 by ATM and ATR can also promote cell apoptosis(Zhu *et al.,* 2002; Helt *et al.,* 2005).

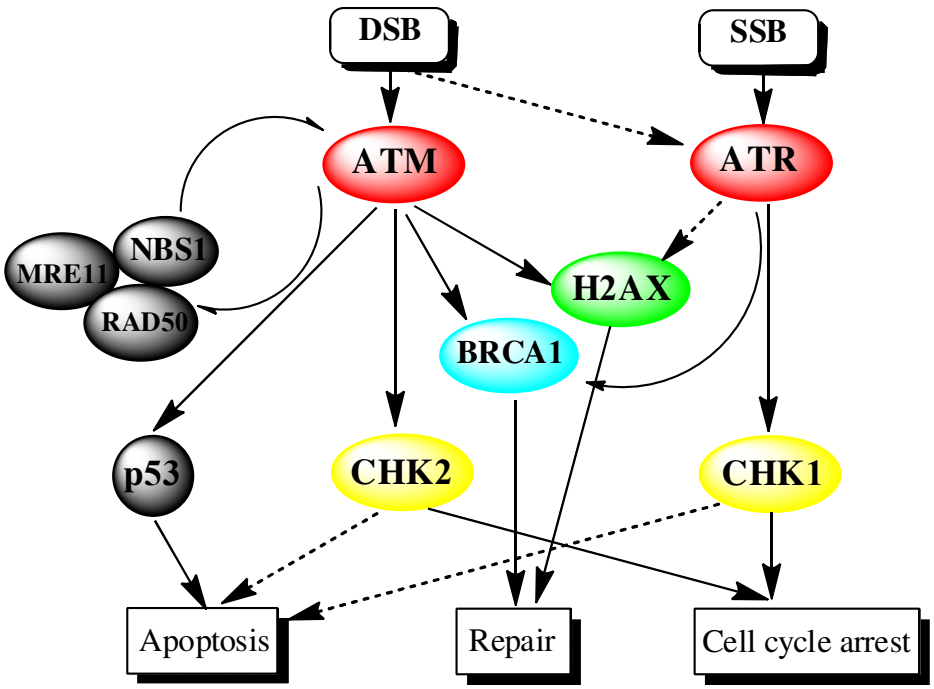

Fig.1 - 5 ***DNA damage response***

1-7 Cell cycle

The cell cycle is a series of events occurred in the cell, leading to its division and duplication. In eukaryotic cell, five cell phases can be divided. Any cell is in one of the five phases, and activation of each phase is dependent on the proper progression from the last phase.

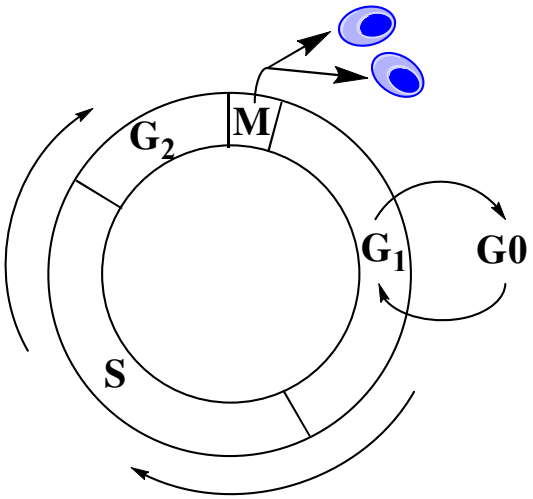

Fig.1 - 6*Cell cycle progression*

(a) G0 phase

It is a phase that the cell has stopped dividing and left the cell cycle progression.

(b) G1 phase

It is a gap phase that the cell is preparing for DNA synthesis. There is G1/S checkpoint that ensures the cell is ready for replication.

(c) S phase

The cell is replicating its DNA in this phase. Sister chromosome begins to appear.

(d) G2 phase

This is a phase that the cell finished synthesis but not yet divided. The cell is growing in this phase and the volume may become bigger. There is a G2/M checkpoint that ensures everything is ready for mitosis phase and divide.

(e) M phase

One cell will divide into two daughter cells in this phase. The chromosomes are being equally separated into two cells. There is an M phase checkpoint that ensures the cell is ready for final splitting and return to G1 phase.

1-8V(D)J recombination

V(D)J recombination is a mechanism of genetic recombination in the early stages of immunoglobulin and T cell receptors production of the immune system.In vertebrate lymphocytes, V(D)J recombination randomly combines variable(V), diverse(D), and joining(J) gene segments to generatea huge variety of antibody proteins with limited gene sequence to match antigens from bacteria, viruses, parasites, pollen and dysfunctional cells.

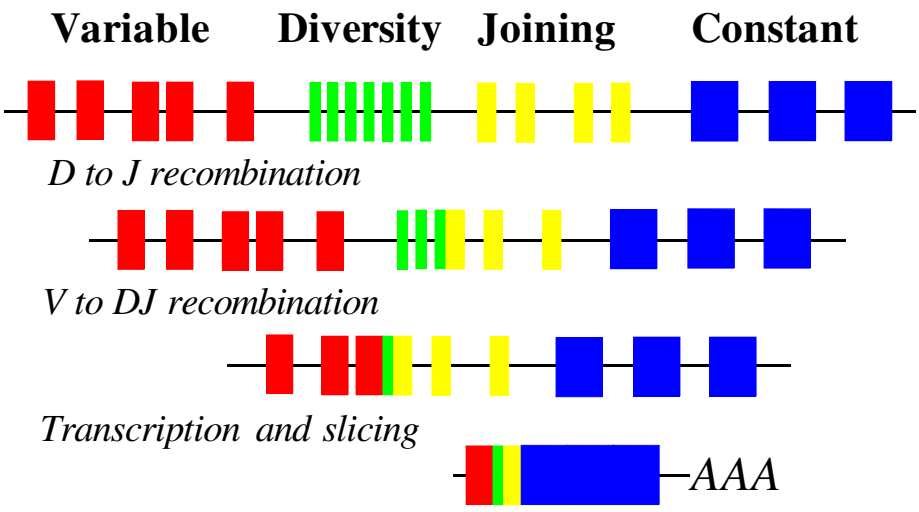

Fig.1 - 7*V(D)J recombination*

A V(D)J recombination initiates with D to J recombination, and subsequent V to DJ recombination. The gene is then transcript to RNA with necessary slicing, and instructs the genesis of immunoglobulin.

The NHEJ pathway is essential for V(D)J recombination as the DSBsare intentionally generated during the cleavage stage. Two hairpin-sealed coding ends and two blunt 5' phosphorylated signal endsare produced in the presence of RAG1 and RAG2 proteins. In the subsequent joining step, the signal ends are ligated directly to form precise signal joints by NHEJ pathway. The NHEJ repair proteins recognize the cleaved end and complete exon joint formation(Blunt *et al.,* 1995; Kirchgessner *et al.,* 1995). It is also reported that this type of joining are generally imprecise due to the addition or deletion of nucleotides, which contributes to further diversification of antibody.

Patients with deficiency in NHEJ pathway not only exhibit radiosensitive of body cells, but also manifest a severe immonodeficient phenotype, which is caused by the failure V(D)J recombination.(O'Driscoll *et al.,* 2001).

1-9 NHEJ repair pathway

The NHEJ pathway mediates direct ligation of broken DNA ends (Weterings *and* Chen, 2008). It has the potential to repair any kind of DNA ends that it does not reply on any homologous template and it is not restricted to any cell cycle (Davis*and* Chen, 2013). The NHEJ is considered as the major and most important pathway in DSB repair, which cannot be compensated HR pathway(Burma *et al.,*2006).

NHEJ pathway involves many supporting and functional proteins, among which Ku70/86, DNA-PKcs, DNA Ligase IV, XRCC4 and XLF play the major role in repair and

attract intensive study.

(a) Ku70/Ku86

Ku70 and Ku86, consisting of 609 amino acids and 732 amino acids, respectively, are two subunits of Ku heterodimer, which was first identified as an antigen of the autoantibodies from patients of polymiositis-scleroderma overlap syndrome (Mimori *et al.,* 1986). Though there is little sequence similarity between Ku70 and Ku86, they show similar structure in domain organization. Ku is an abundant protein, existing ~500,000 copies per cell (Ma *et al.,* 2005). Ku has a hollow ring-shaped structure which can accommodate a double-strand DNA helix and allows Ku sliding onto the DNA end(Walker *et al.,* 2001). Ku binds to DNA in its pore independent of DNA sequence. It has an extremely high DNA end binding affinity, with a binding constant of 2×10^{9} M^{-1}(Downs *and* Jackson, 2004). Ku has been shown to localize to laser generated DSBs within second after creation (Mari *et al.,* 2006). Once the Ku is recruited to DNA ends, it serves as a scaffold to recruit other NHEJ molecules to the damage site. DNA-PKcs, DNA Ligase IV, x-ray cross complementing protein 4 (XRCC4), XRCC4-like factor (XLF)and Aprataxin-and-PNK-like factor (APLF) are all directly or indirectly recruited by Ku(Nick McElhinny*et al.,* 2000;Davis *and* Chen,2013). It has been shown that Ku70 can physically interact with XRCC4 (Mari *et al.,* 2006), and the Ku complex can interact with Ligase IV through its BRCT1 domain(Costantini *et al.,* 2007). XLF also directly interacts with Ku complex through its C-terminal region (Yano et al.,2011). Loss of Ku results severeradiosensitivity and V(D)J recombination defects(Downs *and* Jackson, 2004).

Fig.1 - 8*Crystal structure of Ku binding to DNA*

(From RCSB Protein Data Bank, PDB ID: 1JEY, Ku70 in yellow, Ku86 in magenta and DNA in purple)(Walker *et al.,* 2001)

(b) DNA-PKcs

DNA-PKcs is a protein consisting of 4128 amino acids and is the catalytic subunit of DNA-dependent protein kinase (DNA-PK). DNA-PK complex including DNA-PKcs and Ku components was first purified from the nucleus of HeLa cell(Carter *et al.,* 1990)and it is capable of phosphorylating a number of DNA binding and regulatory proteins like p53 to control the cellular activity in transcription, replication, recombination and repair(Anderson *and* Lees-Miller,1992; Anderson1993). The structure study of DNA-PKcs reveals a central channel mainly formed by C-terminal mediating a binding ability with double-strand DNA (Sibanda *et al.,* 2010). Binding of DNA-PKcs to DNA-PK complex can translocate Ku heterodimer further into DNA end activate the kinase activity of DNA-PKcs,so that the complex stimulates the inter-molecularligation

in NHEJ pathway (Chen *et al.,* 2000). Mutation in DNA-PKcs is found in other radiosensitive or V(D)J recombination defective cell lines and animals, such as murine severe combined immunodeficiency *scid*(Biedermann *et al.,* 1991).

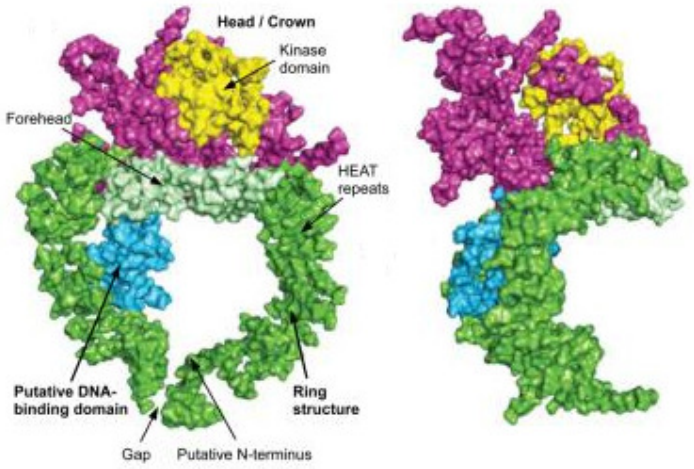

Fig.1 - 9*3D model of DNA-PKcs*(Sibanda *et al.,* 2010)

(c) Ligase IV

Ligase IV is an enzyme that is capable of direct annealing of two broken DNA ends (Grawunder *et al.,* 1997). Human DNA Ligase IV consists of 911 amino acids. In its N-terminal spanning ~600 amino acids, there are threecritical functional domains named DNA-binding domain (DBD), adenylation domain (AD) and oligo-binding domain(OBD)(Tomkinson *and* Mackey, 1998). Ligase IV bindsto DNA through its conserved DNA binding domain like other ligases(Jayaram*et al.,* 2008). In the C-terminal, Ligase IVhas two breast cancer associated 1 C-terminal (BRCT) domains, between which lies the XRCC4 interaction region (XIR)(Critchlow *et al.,* 1997). XRCC4 is an important partner that stabilizesLigase IV(Bryans *et al.,* 1999).

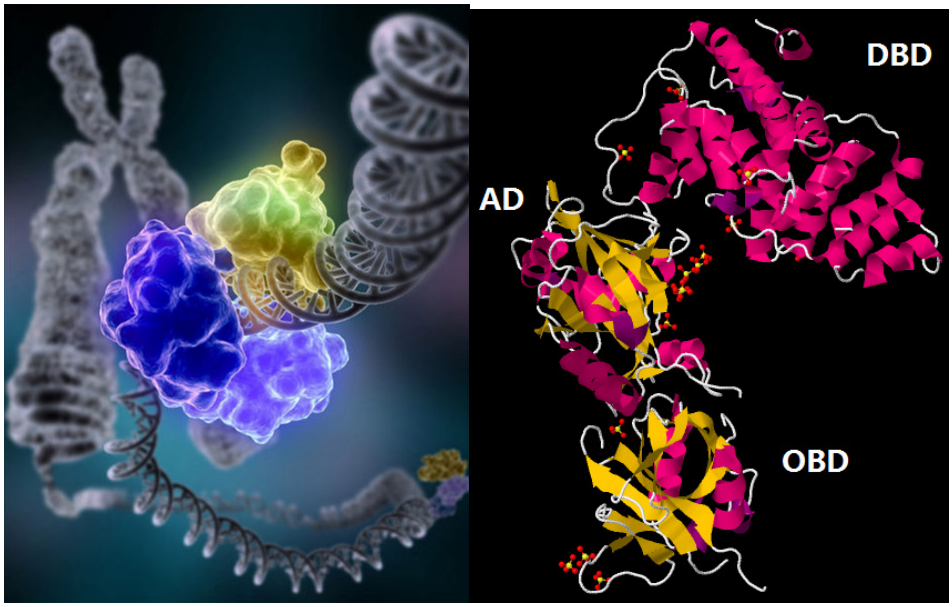

Fig.1 - 10*Left: 3D structureof DNA ligase binding to DNA (National Institute of General Medical Science), Right:N-terminal region of DNA Ligase IV (RSBC PDB ID: 3W1G)* (Ochi *et al.,* 2013)

Ligase IV belongs to the DNA ligase family, which has three members Ligase I, Ligase III and Ligase IV. Ligase IV is exclusively required for the Ku-dependent NHEJ pathway of DSB repair and that other DNA ligases (I and III) do not substitute for this function (Adachi *et al.,* 2001). All ligases share a similar structure in their functional N-terminal domain, and the ligation mechanism is also considered to be the same.

A standard ligation by DNA ligase usually contains 3 irreversible steps. First the adenylate group is transferred from ATP or NAD^+ to the active site of DNA ligase for adenylation. Later this adenylated DNA ligase transfers the adenylate group to 5' end of broken DNA. Finally the unadenylated DNA ligase catalyzes the nucleophilic attack by 3' end to 5' DNA adenylated, resulting a release of AMP (Ellenberger *and*Tomkinson, 2008).

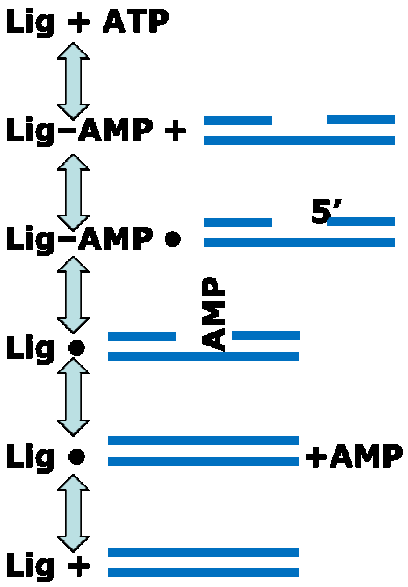

Fig.1 - 11*Mechanisms of DNA Ligation*

Ligase IV is the only ligase that participates in NHEJ repair. Compared to the other two ligases, Ligase IV has a unique C-terminal region which facilitates the binding with XRCC4. XRCC4, together with XLF formsrepair complex with Ligase IV.

Except for the role in DSB repair,Ligase IVhas also been found to be responsible for the early development of neural and immune system and participating in V(D)J recombination (Barnes *et al.,* 1998; Grawunder *et al.,* 1998)

(d) XRCC4

XRCC4 was initially found as the human cDNA, which could complement the defective V(D)J recombination and radiosensitivity of XR-1 cells, derived from Chinese hamster ovary cell (Li *et al.,* 1995). XRCC4 consists of 336 amino acids but has no known enzymatic function.XRCC4 homodimerizes and two dimers can make tetramers.The well-known feature of XRCC4 is its association with Ligase IV, and also it stabilizes and stimulates the ligation and adenylation activity of Ligase IV(Grawunder *et*

al., 1997; Critchlow *et al,* 1997). XRCC4 is likely to be a second NHEJ scaffold molecule and recruits other NHEJ factors to the DSB ends (Davis *and* Chen, 2013). Structural studies indicated that N-terminal which spanning200 amino acids, forms globular domain and coiled-coil domain. The latter domain mediates dimerization of XRCC4 and its interaction with Ligase IV(Junop *et al.,* 2000, Sibanda *et al.,* 2001).The XIR domain and BRCT2 domain on the C-terminal of Ligase IV are necessary and sufficient for the interaction(Grawunder *et al.,* 1998; Wu *et al.,* 2009). XRCC4 is known to interact with other repair enzymes like PNKP (Koch *et al.,* 2004), aprataxin(Clements *et al.,* 2004) and APLF (aprataxin- and PNK-like factor, also known as PALF, C2orf13 or XIP1) (Iles *et al.,* 2007; Kanno *et al.,* 2007). XRCC4 is also an important substrate of DNA-PKcs in DSB repair (Matsumoto *et al.,* 2000).

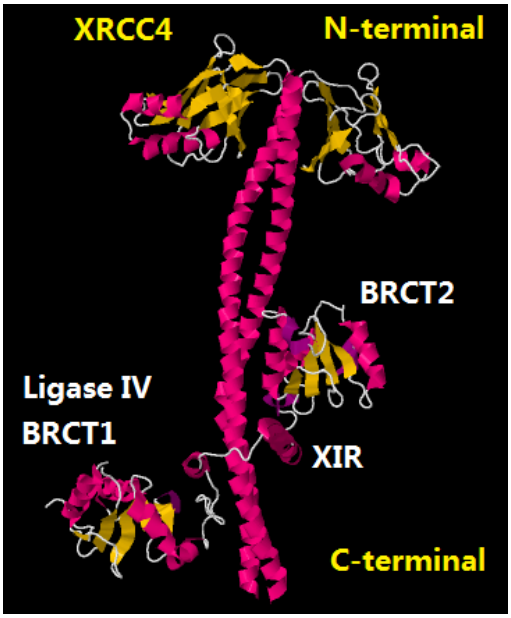

Fig.1 - 12 Structure of XRCC4 dimerization and its binding with Ligase IV C-terminal (RCSB PDB ID: 3II6) (Wu *et al.,* 2009)

(e) XLF

XRCC4-like factor(XLF), also known asCernunnos, consists of 299 amino acids and was identified as a mutated protein in patients with immunodeficiency and microcephaly (Ahnesorg *et al.,* 2006). XLF binds to and shares overall structural similarity with XRCC4(Li *et al.,* 2008). Although the precise role of XRCC4 is not well understood, it is reported that XLF is required for the joining of incompatible DNA ends by XRCC4-DNA Ligase IV complex(Wu *et al.,* 2007). The function of XLF cannot be compensated by XRCC4. Recent studies show that XLF and XRCC4 alternatively connect with each other through the globular head domain and form a filament structure (Malivert *et al.,* 2010; Hammel *et al.,* 2010). This filament can possibly help DNA bridging in DSB repair, which stabilizes the DNA ends(Andres *et al.,* 2012).

Fig.1 - 13*Filament structure of XLF and XRCC4 (RCSB PDB ID: 3RWR)* (Andres *et al.,* To be published)

In the traditional model of NHEJ repair, it is considered that immediately after a DSB formation,Ku heterodimer first recognizes the break site as a signal molecule and helps the recruitment of DNA-PKcs. DNA-PKcs is supposed to protect the DNA end and the

end is subsequently processed by nucleases and polymerases such as Artemis, PNKP, and DNA polymerase λ and μ. Later the Ligase IV/XRCC4/XLF complex is recruited to the DSB sites and ligates the broken DNA ends(Lieber*and* Michael, 2010).

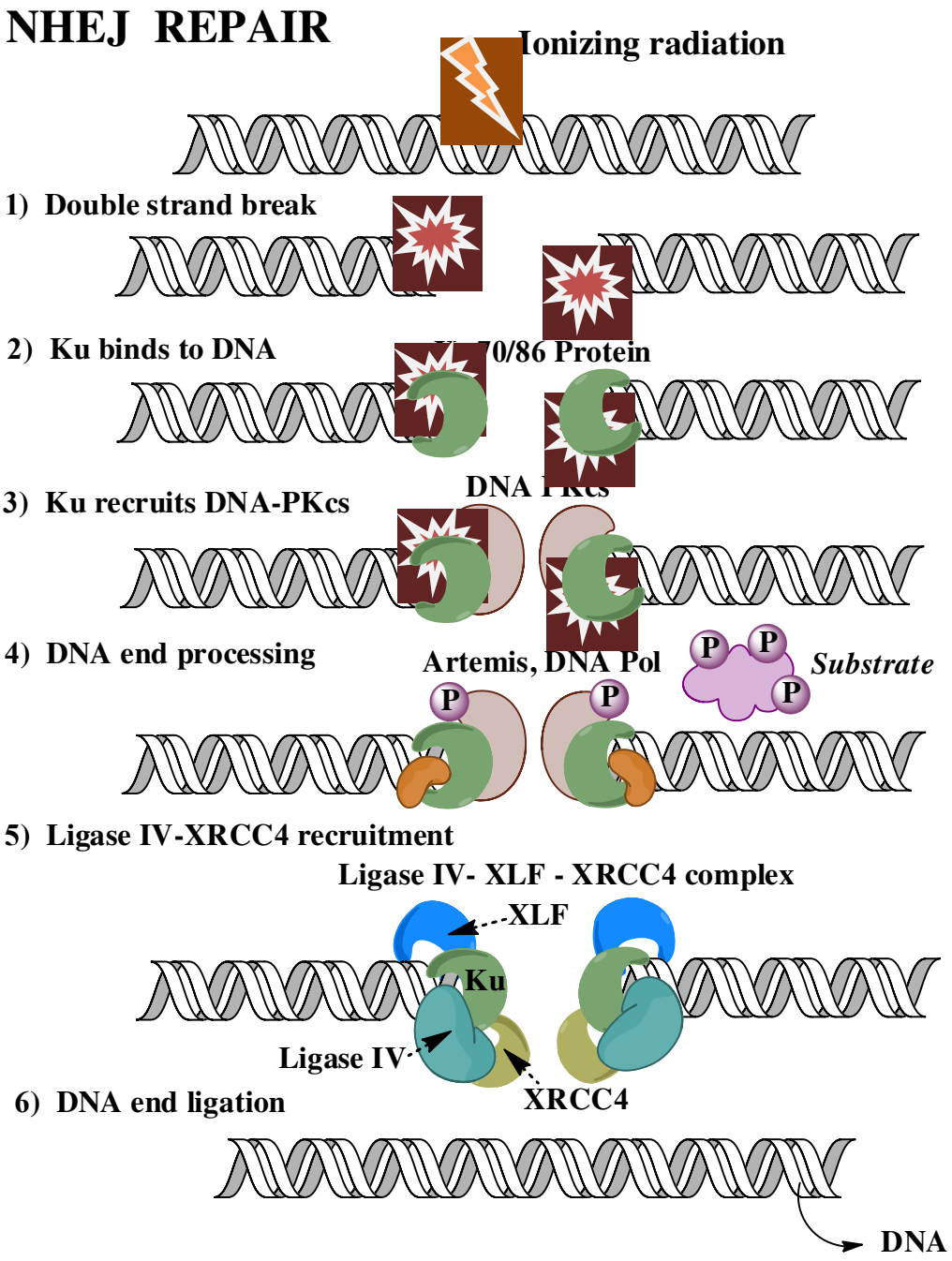

Fig.1 - 14*Traditional model of NHEJ repair*

However, recent studies show that the localization of NHEJ molecules Ligase IV, XRCC4 and XLF is not dependent on DNA-PKcs(Yano *and* Chen, 2008). Thus DNA-PKcs does not seem to be required to recruit these NHEJ molecules, and the recruitment of DNA-PKcs after Ku binding is not necessary. It has been suggested that the recruitment of DNA-PKcs can be flexible and probably depend on the complexity of the DNA damage (Reynolds *et al.,* 2012). If it is a simple damage, Ku can directly recruits Ligase IV and XRCC4, so that the complex can rapidly join the broken ends together without processing the DNA ends. The NHEJ model in this condition can be simplified.

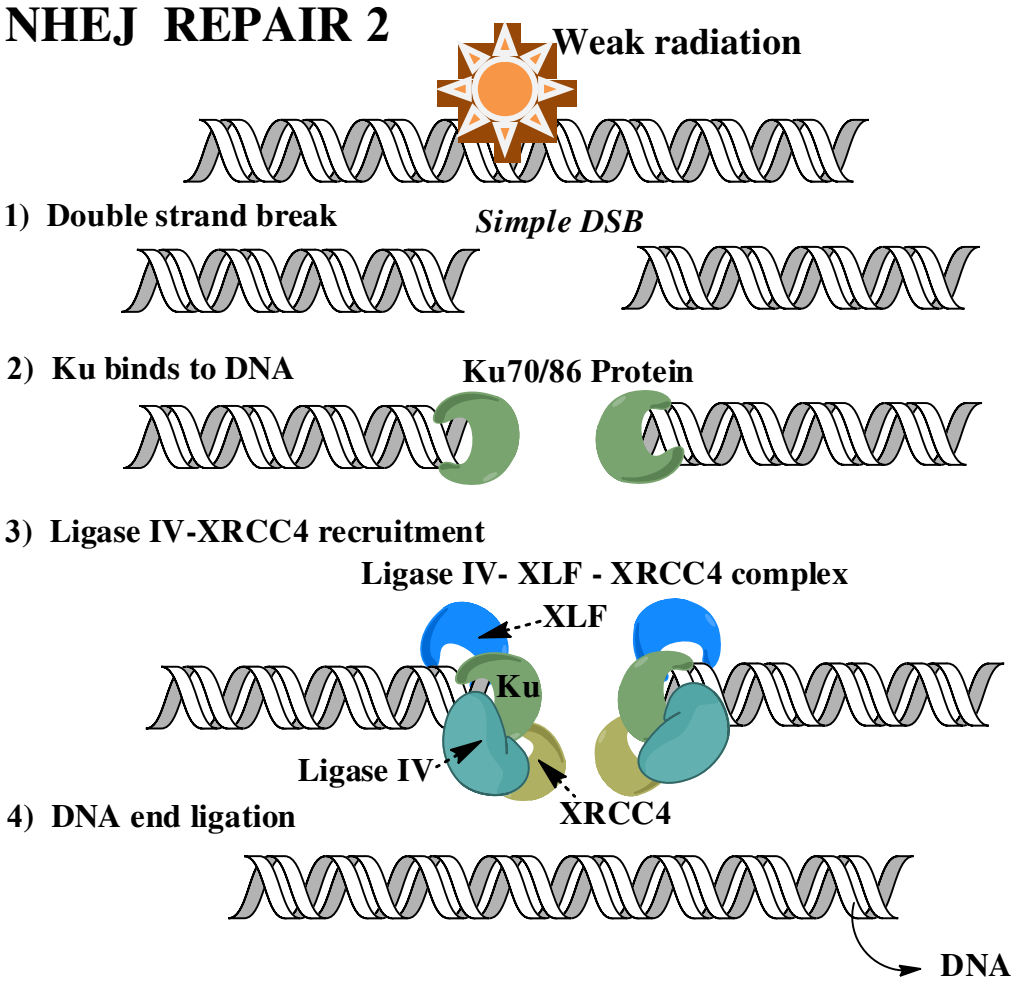

Fig.1 - 15*NHEJ pathway of simple DSB*

1-10 BRCT Domains

BRCT domains, named from Breast Cancer Associated 1 C-terminal, have been found present in many proteins involved in DNA damage response such as p53BP1, RAD9, XRCC1 and etc., participating in binding of phosphorylated proteins, non-phosphorylated proteins, and DNA in difference proteins(Bork *et al.,* 1997). A protein can have one BRCT domain, double BRCT domains or even multiple BRCT domains, giving the BRCT domains a diversity architecture and function (Leung *and* Glover,2011). BRCT sequences contain two highly conserved motifs, in which Motif-1

consists of a conserved Gly–Gly/Gly–Ala pair and Motif-2 consists a highly conserved Trp-X-X-X-Cys/Ser motif (Huyton *et al.,* 2000).

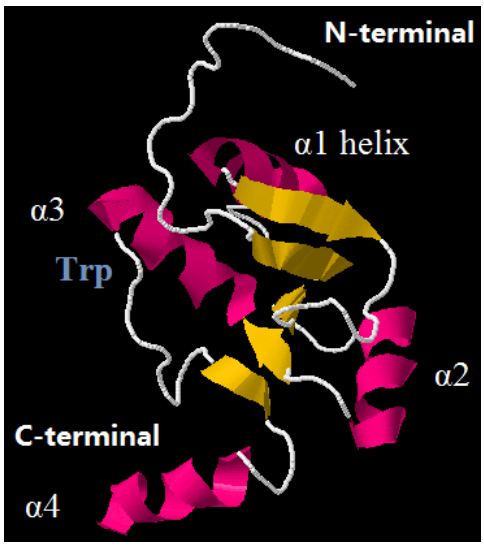

Fig.1 - 16*Structure of BRCT1 domain in DNA Ligase IV(RCSB PDB ID: 2E2W)*

(Nagashima*et al.,* To be published)

Ligase IV contains two BRCT domains in its C-terminal region.So far, there is poor understanding aboutthe role and functionof BRCT domains in Ligase IV. It is considered that BRCT domains in Ligase IVare not the domains themselves, but rather the linker between the domains that mediate the important interaction with XRCC4 (Doré *et al.,* 2006). Some recent researches pointed out thatthe BRCT1 domain of Ligase IV mediate the direct interaction with Ku (Costantini *et al.,* 2007), and the BRCT2 domain is necessary for stable binding to XRCC4 (Wu *et al.,* 2009). It is also reported that the α2 helix of Ligase IV BRCT1 domain is responsible for targeted degradation by adenovirus infection (Gilson *et al.,* 2012).

1-11Assembly of NHEJ repair complex

Although many key molecules in NHEJ pathway have been identified, the mechanisms how these molecules are recruited to DSB site and assembled into DNA repair complex remain to be elucidated. In the study of HR pathway, HR proteins have been tagged with fluorescent protein and introduced to the cells. After the irradiation induced DSB formation, ionizing radiation-induced foci (IRIF) at DSB can be easily detected by fluorescence microscope or with immunostaining techniques. By observing IRIF in HR gene deficient cells, including those established from patients of congenital disorders, and the effects of inhibitors or siRNA on IRIF, the understanding of the HR has been greatly advanced. But in the study of NHEJ proteins, IRIF formation wasonly observed forautophosphorylated form of DNA-PKcs where it co-localizes with both γ-H2AX and p53BP1 (Chan *et al.,* 2002). The failure or difficulty in the detection of IRIF for other NHEJ proteins in living cells may be attributable to several reasons which are not mutually exclusive.

a) Only a very small number of molecules might be recruited to each DSB site and thereby, do not appear as foci.
b) NHEJ proteins, especially Ku and DNA-PKcs are abundant. Therefore, even if some NHEJ molecules moved to the DSB site, their overall distribution would not change to a discernible extent.
c) NHEJ reaction is very rapid, healing most of the DSBs within minutes after irradiation. So, the association of repair enzymes with DSBs may be very transient and thus, difficult to be captured.

Recently, several studies using laser micro-irradiation technique have monitored the spatiotemporal dynamics of NHEJ molecules in irradiated areas but local high dose is

required (Kim *et al.,* 2005). Interestingly, a dynamic equilibrium between Ku heterodimer on DNA ends and in solution was detected, showing that NHEJ complex assembly is reversible. Recruitment of the XRCC4-DNA Ligase complex to DSBs occurs in the absence of DNA-PKcs, indicating a direct interaction of Ku70 – XRCC4 (Mari *et al.,* 2006). It was also shown that XLF is recruited to DSBs in a manner dependent on Ku, but not DNA-PKcs and XRCC4 (Yano *et al.,*2008, 2009).

Another approach to examine the association of DNA repair proteins with damaged DNA is sequential extraction with increasing concentration of detergent or salt. This approach has been used to demonstrate the recruitment of ATM and MRN complex to DSB sites (Andegeko *et al.,* 2001).

In our laboratory, Kamdar and Matsumoto have recently established a method to detect the binding of XRCC4 to chromatin after irradiation(Kamdar *and* Matsumoto, 2010). By using this method, they showed that DNA-PKcs was indispensable for the recruitment of XRCC4 to DSBs. Moreover, the results suggested that DNA Ligase IV is necessary for the chromatin binding of XRCC4.

1-12Research Purposes

As it is mentioned above, Ligase IV plays a criticalrole in the NHEJ pathway ofDSB repair,joining of two broken DNA ends in the final step. In addition, Ligase IV might also participate in the assembly of NHEJ repair complex. The purpose of this study is to elucidate how Ligase IV is activated in the repair of DSB and recruited itself as well as its partner XRCC4 to the necessary sites. It is known that all functional domains of Ligase IV lie in its N-terminal region. The C-terminal region, however, has not been solely crystalized and the function is not well understood. This region is uniquely present in

Ligase IV among all DNA ligases, possibly suggestingthe importance of C-terminal region in the NHEJ repair. I introduced several point mutations of Ligase IVin C-terminal region, as well as C-terminal fragments. BRCT domains frequently appeared in DNA repair related proteins.There are many mysteries in BRCT domains that need to be explored. Versatile interaction possibility, high sequential and structural conservation, and unique presence inLigase IV among all DNA ligasesmake BRCT domainsas interesting and potential research targets. With the study of BRCT domains in the C-terminal region, we can be able to know more about therole of Ligase IVin NHEJ repair.

CHAPTER 2

Experiment Procedures

2-1 Cell culture

2-1-1Nalm-6 cell culture

Nalm-6 cell is a human precursor-B acute lymphoblastic cell. It is highly proficient for gene disruption and the Ligase IV-deficient Nalm-6 cells were established by Grawunder and Adachi(So *et al.,* 2004). Both Wild-type Nalm-6 and Nalm-6 Ligase IV-deficient cellswere gifted by Dr. Noritaka Adachi from Yokohama City University.

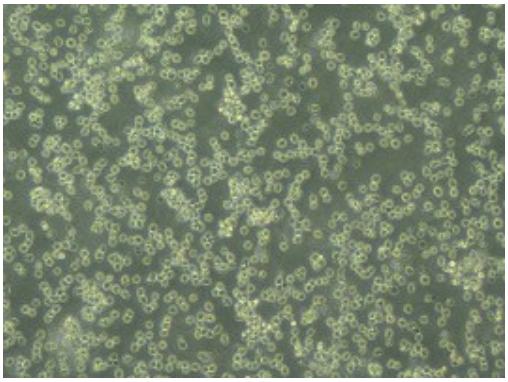

Fig.2 - 1 ***Nalm-6 cells (TKG 0413)***

Nalm-6 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 14 µM β-mercaptoethanol at 37 °C in humidified atmosphere containing 5% CO_2. Cell density was maintained between 10^5 and 10^6 cells/ml. FBS was purchased from HyClone and other agents were purchased from NacalaiTesque.For long time storage, 10^6 cells werestored in Cryo Tubes from Thermo Scientific Company, with 1ml Cell Reservoir One from Nacalai Company.The tubes were temporary storedin -85 °C deep freezer and then transferred to liquid nitrogen tank.

2-1-2JM109 cell culture

To select and propagate the plasmid DNA, JM109 strain of *E.coli* was used. The JM109 cells were purchased from SciTrove (Tokyo, Japan), available for convenient transformation in high efficiency. It harborsendA and recAmutations that prevent plasmid degradation during extraction and DNA recombination. They are suitable for plasmid purification and amplification.

Transformed JM109 cells were cultured in 37°C cell incubator with shaking.

The bacterial cell culture plate was prepared with 0.5g LB powder, 0.3g Agar powder and 20ml pure water supplemented with necessary antibiotics. The bacterial culture mediumfor plasmid propagation was prepared with 5g LB powder, 200ml pure water and necessary antibiotics.

Selection drug Kanamycin was used at aneffective concentration of 30μg/ml, and Ampicillin was used at 50μg/ml.

Transformed JM109 cells were stored in Cryo tubes, prepared with 900ml bacterial culture medium and 100ml 30% sterile glycerol. All stored samples were kept in deep freezer at -85°C.

2-1-3 Hela-Fucci cell culture

HeLa cell is a human cervical cancer cell named from the patient Henrietta Lacks. It is an adherent cell line that can be immobilized on the bottom of a culture dish. HeLa-Fucci cells were cultured in DMEM medium supplemented with 10% FBS, 1% Antibiootic/Antimycotic (100X, Gibco) at 37 °C with 5% CO_2. Cells were cultured in 25 cm^2 flask with 5 ml medium. During a cell passage, all medium was removed from the flash and washed with 5 ml of PBS. After removing all PBS, 1ml 0.25%

Trypsin-EDTA(1X, Gibco) was added into the flask, and kept in 37 °C for 3 min to make cells detach from the bottom of the flask. Finally the cells were suspended with 4 ml culture medium to make a total volume of 5 ml. The cell concentration was then measured and an aliquot of cell was transferred to new flask with preferred cell number.

2-2 DNA purification

2-2-1 Mini PREP

Mini PREP is used for small amount of DNA purification. In the case for insert check and sequencing, mini PREP is sufficient.

a) Sample preparation

2 ml *E.coli* cells are cultured in 15ml centrifuge tube overnight and transferred to 2 ml centrifuge tube.The cell pallets were collected after a centrifugation at 16000g for 1 minute. Supernatants are disposed.

b) *E.coli*lysis

The centrifuge tubesare vortexedto break all cell pellets, and the cells distributed equally with 175µl lysis buffer type 7.then add. 175µl lysis buffer type 8 are added later and flip the centrifuge tube to ensure complete reaction. At last350µl lysis buffer type 9 are added, and white sediments will appear in the tube. The sediments are to be removed by 5 minutes centrifugation.

c) Plasmid DNA binding

The column and collection tube are loaded with supernatants to the column and, centrifuged for 1 minute. All flow-through are disposed

d) Column wash and dry

Column is washed with 400µl wash buffer type 1 and centrifuged for 1 minute.

Remaining bufferare removed by a second time centrifugation.

e) Plasmid elution

New 1.5ml centrifuge tube is attached to the column and 50µl elution buffer type 4 are added to the column and incubated for 1 minute. The plasmid DNA is collected in the centrifuge tube after one minute centrifugation.

2-2-2 Midi/Maxi PREP

Midi PREP is used to purify large concentration of plasmid DNA. The plasmid suitable for gene transfection or DNA stock can be purified with this method.

Midi PREP kit utilizes the NucleoBondXtra Silica Resin to purify plasmid DNA. The negative charged DNA backbone phosphate group can bind with positive charged anion-exchanger group MAE in the resin.

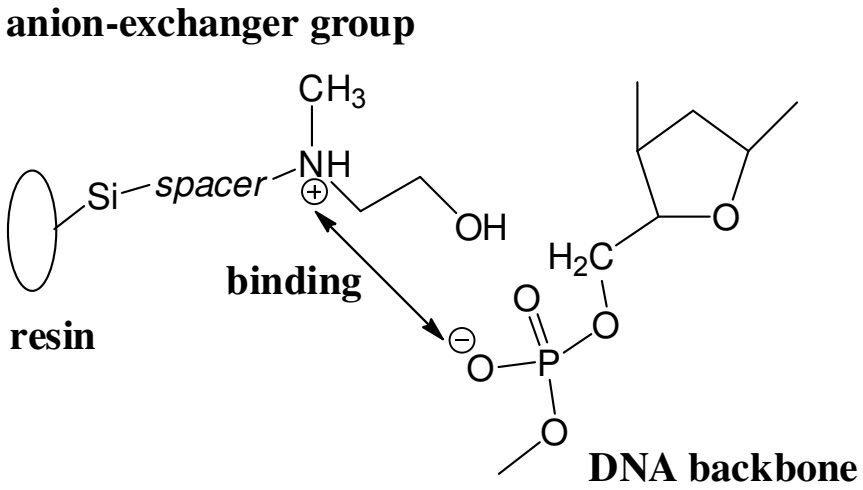

Fig.2 - 2 ***Mechanism of Midi PREP***

Cells for midi PREP are cultured in 200ml flask overnight and transferred to large

centrifuge bottles. High speed centrifuge machine is used for large volume centrifugation. The cell pallets are also vortexed repeatedly to disintegrate. RES LYS and NEU buffer are added in sequence to lyse the bacteria cells. The lysate is loaded to the NucleoBondXtra Column Filter equilibrated with EQU buffer. The plasmid DNA will be collected by the resin in column. The filter is discarded after wash with EQU buffer. The resin is washed with WASH buffer and the DNA is eluted from the resin with ELU buffer. The elutant is mixed with isopropanol and reconstituted using NucleoBondFinalizer, with TRIS buffer.

2-3 DNA techniques

2-3-1PCR

PCR is the abbreviation for polymerase chain reaction. It is a technique used to amplify DNA to several orders. Very small amount of DNA up to pictogram is needed as a template to run PCR. The PCR is based on thermal cycling. Repeated heating and cooling are used for DNA melting and enzymatic replication. Primers containing complementary sequences to the DNA template enable selective amplification and it can be elongated by DNA polymerase.

PrimeSTARmutagensis basal kit from Takara Companywas used for PCR. It provides an elongation speed as fast as 200 base pairs per second. The amount of required template in reaction is between 10pgto 100pg. In each PCR process, 25µl MAX Premix, 1 µl template DNA, 0.5µl forward primer, 0.5µl reverse primer and 23µl pure water are used for thermal cycling. The total volume is 50µl.

2-3-2Restriction enzyme digestion

Restriction enzymes which can recognize and cleave DNA double-strand at the specified

nucleotide sequences,enabling intended gene engineering. They are thought to be evolved from the cell's defense system against virus. Foreign DNA can be selectively cleaved as it lacks the modification of methylation.

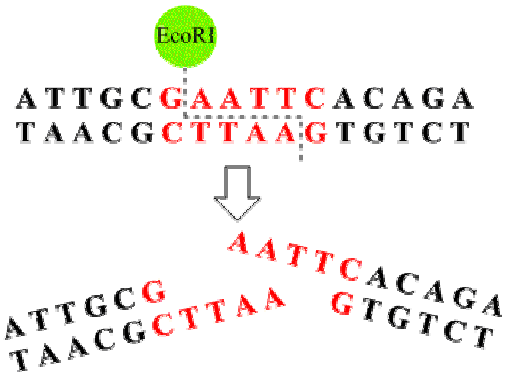

Fig.2 - 3 ***Restriction enzyme digestion***

Restriction enzymes can recognize 4 to 8 nucleotides, most of which are palindromic, meaning the reading forward or backward is the same. Restriction enzymes can produce both sticky ends and blunt ends. For example, EcoRI restriction enzyme recognizes the sequence GAATTC, and it will leave a sticky end of AATT. When there is a complement sticky end TTAA, it can be annealed with DNA ligase. SmaI restriction enzyme recognizes the sequence CCCGGG and it cleaves two strands in the middle, leaving no sticky end. In this case, ligation is not applicable. When DNA modification and recombination is needed, sticky ends are highly preferred.

When performing restriction enzyme digestion, suitable buffer should be carefully selected for combination of restriction enzymes. All enzymes are supposed to have a high reactivity in the selected buffer. The digestion is done in 37°C dry water bath for more than 2 hours.

2-3-3 DNA purification

Wizard SV Gel and PCR Clean-Up System (PROMEGA kit) is used for DNA purifications

(a) Gel Slice and PCR Product Preparation

A. Dissolving the Gel Slice

After the finish of electrophoresis, DNA band was excised from gel and placed inside 2 ml microcentrifuge tube. The weight of he gel fragment was measured to determine use of Membran binding solution. It was added1µl per 1mg of gel slice. The gel slice will be completely dissolved in 50–65°C water bath.

B. Processing PCR Amplifications

1. Equal volume of Membrane Binding Solution was to the PCR amplification.

(b) Binding of DNA

The dissolved DNA mixture or PCR products were first transferred the Minicolumnassembly and incubate at room temperature for 1 minute. The flowthrough was discarded after a centrifugation at 16,000 × g for 1 minute

(c) Washing

700µl Membrane Wash Solution with ethanol was added andcentrifuged at 16,000 × g for 1 minute, and flowthroughwas again discards. After it is cleaned, any residual ethanol should be removed by evaporation.

(d) Elution

Minicolumn should be put onmicrocentrifuge tube.50µl of Nuclease-Free Water releases DNA and it is collected byMinicolumn. Incubate at room temperature for 1 minute might be necessary. The DNA samples are centrifuged at 16,000 × g and kept in 4°C or –20°C

2-3-4 DNA electrophoresis

Agarose gel electrophoresis is a method of gel electrophoresis used to separate DNA in a matrix of agarose. An electric field is applied to move negatively charged DNA molecules through the agarose matrix, thus the DNA can be separated by length.

The gel for DNA electrophoresis is prepared with 1% agarose and 0.5x TBE buffer (Tris-Borate-EDTA, pH 8.3). A short gel is cast with total 25ml TBE buffer. It is then heated to 100°Cto allow fully dissolution of agarose, and solidified on the cast tool.

10x running buffer is added to DNA samples before injection to gel lane. No more than 30µl DNA can be loaded on a single lane. The electrophoresis is done with 100V, 45min for a typical separation.

To be visible with UV-light,the DNA gel needs to be stained withEthidiumBromide (EtBr). EtBr intercalates into DNA and allows the fluorescent emission after being excited by UV-light. The EtBr itself is a strong mispair inducer that will damage DNA, so the DNA to be purified should not be stained.

2-3-4DNA ligation

T4 DNA ligase joins two compatible sticky DNA ends. The ligation sample is commonly prepared at 10 µl, with 1µl T4 ligase, 1µl T4 ligase buffer and remaining 8µl DNA solution. The optimal molecular ratio of plasmid vector and insert gene is between 1:3 and 1:6. The ligation isdone in dry water bath at 16°C, with overnight reaction.

Fig.2 - 4*DNA ligation of compatible ends*

2-4 Transfection

Transfection is a process that deliberately introduce new gene into target eukaryotic cells. Transfection typically involves a transient opening of a hole on the cell membrane to allow the uptake of genetic material. Transfection can result unexpected morphologies and abnormalities in cells.

2-4-1 Magnetofection

Magnetofection exploits magnetic force exerted upon gene vectors associated with magnetic particles to drive the vectors into the target cells.

For a 6 well transfection, the cells were prepared at 4 x 10^5, DNA 6µg and 2 mL medium. 6 µL of PolyMag or PolyMag Neo is used.

2-4-2 NEON transfection

The NEON system from Invitrogen enables efficient and simple transfection with mammalian cell. Electronic pulses are applied to the mixture of mammalian cells with DNA to allow the gene entranceto cell nucleus and recombination.

Fig.2 - 5 ***NEON transfection system***

In the transfection of Nalm-6 cells, 6-well protocol is used, with 1×10^6 cells suspended in 100 µl T buffer, 4µg DNA, 100 µl tip and 2mL culture medium without any antibiotics.

2-4-3 Cell plating

After two days culture of transfected cells, it is plated on argarose containing medium to allow colony formation. The medium is first prepared with 0.2% agarose dissolved with 4ml water and sterilized at 121 °C for 20 min. Then 15% FBS containing culture medium plus 2µg/ml puromycin for colony selection is added after cooling down to 60 °C. Last, 8ml agarose medium is mixed with 2ml transfected cells and plated to 60mm^2 cell culture dishes. Good colonies can be visible after two to three weeks culture in cell incubator.

2-5 Irradiation

Ionizing irradiationof the cells wasdonewith Cobalt-60 irradiation facility of Tokyo Institute of Technology. The decay of Cobalt-60 emits characteristic γ-rays of 1.17MeV and 1.33MeV. The dose rate was measured with ionizing chamber-type explosure dosimeter C-110 (Oyo Giken, Tokyo, Japan)at 30cm and 90cm from the source for us every two months. For low dose irradiation samples were placed 90cm from the source, and higher dose was achieved at 30cm.

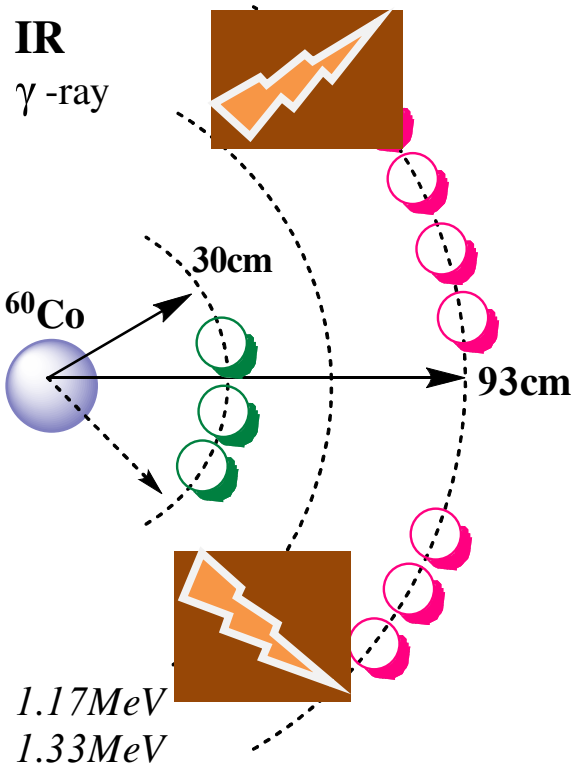

Fig.2 - 6*General irradiation with Co-60*

As a sample, the calibration data on 10th Apr.2013 is listed below

30cm, without stand: Room Temp. T = 12°C, Pressure p = 1002.3hPa

1st calibrationis measured after running into constant Co-60 irradiation with fixed dose:d_1 =1000 R, Time t_1= 86.34s

The transfer coefficient with correction from R to standard Gy unit k_1 = 0.01049

The temperature correction coefficient k_2 = T + 273 / 295 = 0.9661

The pressure correction coefficient k_3 = p / 1013 = 0.9900

Total received dose$D = d_1 * k_1 * k_2 * k_3$= 10.0333 Gy

Dose rate $\varphi = D / t_1$ = 0.1162 Gy/s

2nd calibration is measured with the startup of Co-60 IR operation with fixed time:

t_2 = 60 s, d2 = 536 R

The expected dose D_1 in constant operation will be $\varphi * t_2$ = 6.972 Gy

The actual dose $D_2 = d_2 * k_1 * k_2 * k_3$ = 5.378 Gy

The dose gap between D_1 and D_2 can be compensated by:

$\Delta t = (D_1 - D_2) / \varphi$ = 13.72 s

So the time needed for fixed dose from startup can be calculated as

$t_{ir} = D_{ir} / \varphi + \Delta t$

IR data sheet (30cm)

Dose	Time	Dose	Time
10Gy	99.8s	50Gy	440.0s
20Gy	185.8s	100Gy	874.3s

93cm without stand was calibrated

1st calibration t_1 = 74.93 s, D_1 = 100 R

2nd calibration t_2 = 120 s, D_2 = 146.1 R

After calculation, φ = 0.01331 Gy/s, Δt = 10.53 s

IR data sheet (93cm)

Dose	Time	Dose	Time
1Gy	87.5s	4Gy	311.1s
2Gy	160.8s	5Gy	386.3s
3Gy	236.0s	10Gy	762.0s

2-6Protein Techniques

2-6-1 General reagents

Running buffer is prepared with Glycine (192mM), Tris (25mM) and SDS (0.1%), Transfer buffer is prepared with Glycine (192mM), Tris (100 mM), Methanol (5%) and SDS (0.1%),TBS is prepared with 20mM Tris (PH=7.6) and 0.9% NaCl (w/v),

and TBS-T is prepared with 0.05% Tween-20 dissolved in TBS solution.

2-6-2 Antibodies

(a) Anti-Ligase IV rabbit polyclonal antibody from Abcam;

(b) Anti-XLF rabbit polyclonal antibody from BioVision;

(c) Anti-FLAG monoclonal antibody M2, conjugated with horseradish peroxidase, from Sigma–Aldrich;

(d) Anti-rabbit immunoglobulin swine antibody, conjugated with horseradish peroxidase, from DAKO.

(e) Anti-XRCC4 rabbit polyclonal antibody was generated in our laboratory as described

2-6-3 SDS-PAGE

a) Prepare gel plate

SDS gel plate is made of glass. It should be wiped with ethanol before use. Rubber strip is inserted between two plates.

b) Prepare gel solution

SDS separating gel solution is made up with 7.5ml pure water, 3.75ml 40% alkalyamid, 3.75ml separating gel buffer (1.5M Tris-HCl, 0.4% SDS buffer, pH8.8), 15µl TEMED and 45µl 10% APS.

SDS stacking get solution is made up with 5.2ml pure water, 0.8ml 40% alkalyamid, 2ml stacking gel buffer (0.5M Tris-HCl, 0.4% SDS buffer, pH6.8), 8µl TEMED, 60µl 10% APS.

The above volume is suitable for making two SDS gels. APS should be added

immediately before use.

c) Making SDS gel

First SDS separating gel solution is added to the gap of SDS plate, leaving a 2.5cm vacancy from the top of the plate. Add water to fill the plate and keep for 60 minutes in room temperature. Then remove all the pure water and fill with SDS stacking gel solution. Meanwhile, plastic comb is inserted into the plate from the top to make loading wells. Wait 15 minutes at room temperature for gel solidification. Gel is washed with pure water and water in wells is removed before loading samples. Gels are stored at 4°C.

d) Prepare samples

Sample buffer is made up with 500µl pure water, 500µl SDS-PAGE 2x sample buffer, 25µl BPB (Bromophenol blue), 5µl Crystal Violet and 50µl β-mercaptoethanol.

50~200µl sample buffer is added every 10^6 cells. After mixing with samples, heat at 100°C for 5 minutes. All proteins should be denatured.

e) Run SDS-PAGE

After setting up SDS-PAGE device, fill the tank with SDS buffer. Then load samples to the empty wells and fill all the wells with tank buffer. The current for electrophoresis is set to 20mA per gel and the voltage is fixed to 200V. For general SDS-PAGE, the time is around 75 minutes. Press the start button to run SDS-PAGE.

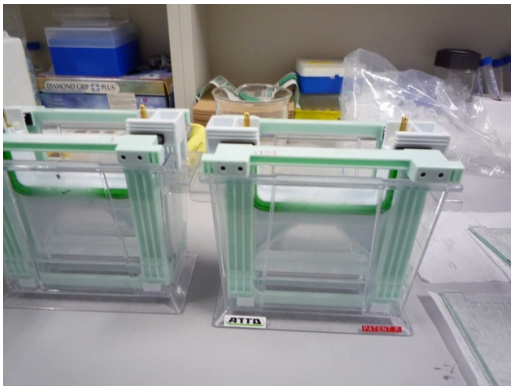

Fig.2 - 7*SDS gel loaded in SDS-PAGE tank*

2-6-4 Western blotting

a) Prepare for membrane transfer

One 6cm x 9cm PVDF membrane and six Chr paper pieces of the same should be prepared before membrane transfer. The PVDF membrane is treated with methanol for 1 minute and shaking with transfer buffer. The paper pieces are also absorbed with transfer buffer.

Put 3 Chr paper pieces on the center of the transfer device. Then put PVDF membrane above Chr paper. Carefully transfer the SDS gel to cover the PVDF membrane entirely, leaving no bubble or space. Cover the SDS gel with another 3 Chr paper.

b) Run membrane transfer

Connect DC cables to the transfer device and shut the lid firmly. The voltage for membrane transfer is set at 110V, and the current is set at 110mA per gel. The time for standard transfer is about 60 minutes. After the operation, the proteins in the SDS gel are transferred to the PVDF membrane for further examination.

c) Blocking

Immediately after the complete of membrane transfer, membrane should be blocked by blocking solution. The blocking solution is prepared with 1% milk in TBST solution right before blocking. More than 1 hour's shacking is needed for a complete blocking.

d) Primary antibody reaction

In some western blotting experiments they need two step antibody reactions. The membrane after blocking is put into the hybrid plastic bag. The corresponding antibody is added into 1ml blocking solution to make the antibody solution. After adding the first antibody solution, seal the hybribag completely. The reaction takes more than 1 hour.

e) Second antibody reaction

Second antibody reaction is similar to first time. After taking the membrane from hybrid plastic bag, the membrane needs to be washed to remove all unbinding antibody. It is done in 4 times' TBST wash, with a time of 2 minutes, 2 minutes, 5 minutes and 5 minutes respectively. Then prepare the 2^{nd} antibody solution and seal in the hybrid plastic bag in the same way. The 1^{st} antibody is the antibody that can recognize specific protein to examine, and the 2^{nd} antibody is something universal that can bind to the first antibody for fluorescent detection.

f) Fluorescent reaction

ECL plus kit are used to generate luminescent signals against second antibody. The solution A and solution B at a ratio of 40 are mixed before use. The membrane should also be washed four times after taking away from hybrid plastic bag. New bag is also made for fluorescent reactions. After 5 minutes' reaction, the abundant fluorescent mixture is removed from the bag and resealed tightly.

g) Fluorescent checking

The hybrid plastic bag is fixed in the cassette and a film is inserted into the cassette for exposure in the darkroom for a fixed time. Then develop the film to get the final results of western blotting.

The three groups of cells have different behavior upon irradiation because the mutated Nalm-6 cells cannot produce Ligase IV protein. This phenomenon can be detected by protein analysis with western blotting. The antibodies against NHEJ components such as Ligase IV, XRCC4, Ku70, Ku86 have already been purified in my lab. These antibodies were produced by the rabbit immune system with the injection of corresponding proteins as an antigen to the rabbit. These antibodies are collected and concentrated by column flow-through. Antibodies produced in the rabbit body contain the rabbit immunoglobulin, and anti-rabbit IgG is used as a second antibody for western blotting.

2-7 Colony formation assay

After ionizing irradiation, it is important to know how many cells can survive the DNA damage. Colony formation assay is such a method to distinguish healthy cells and dead cells. Cells either become physically dead or lose the proliferation ability are considered biologically dead.

Ligase IV deficiency will make cells radiosensitive to radiation, especially at a dose below 5Gy. So during a colony formation assay same numbers of cells were divided into Control, 1Gy, 2Gy, 3Gy and 5Gy group. The cells were maintained at a concentration around 3×10^5/ml before irradiation, and 1ml cells with medium were collected in 1.5ml centrifuge tube.

Cells were plated on 0.17% agarose culture tissue, with 15% FBS medium. For

Nalm-6 WT cells 200, 300, 500, 1000, 3000 cells were seeded onto the agarose medium, and for mutated cells 200, 400, 1000, 2000, 5000 cells were seeded according to the dose.

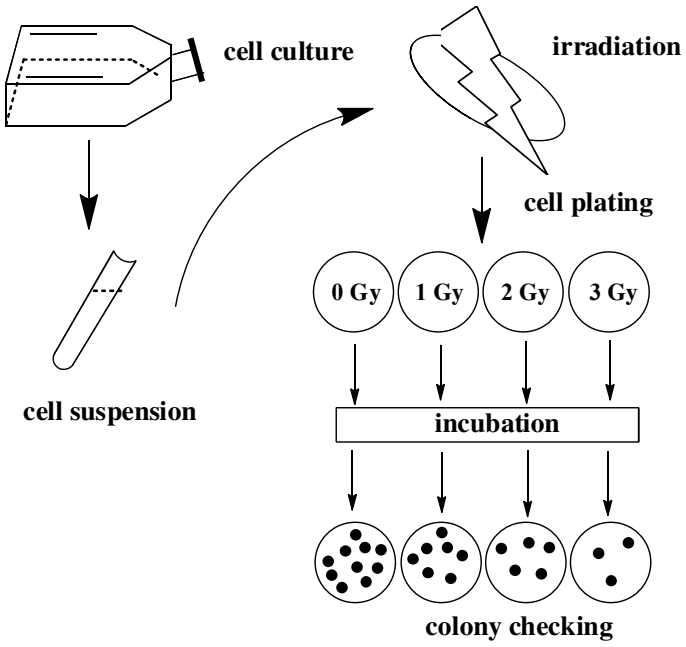

Fig.2 - 8*Demonstrationof colony formation assay*

Colony number was counted three weeks after plating. An average number of three plates were used for survival fraction calculation.

The plating efficiency (P.E) is defined by

P.E = Number of colonies formed / Number of cells seeded x 100%

The cell survive fraction (S.F) is defined by

S.F = P.E of irradiated cells / P.E of unirradiated cells x 100%

Experiments were repeated for three times to obtain reliable survival fraction.

2-8Silver staining of protein gel

Silver staining is used for protein detection after SDS-PAGE. Sil-best Stain One kit

were bought from NacalaiTesqueCompany.

Gel after SDS-PAGE is first treated with fixing solution I (5% acetic acid, 45% ethonol) for 20 mins and then fixing solution II (10% ethonol) for 10 mins to immobilize the protein on the gel. Next 1% pretreatment solution provided in the kit are used to treat the gel for 10 mins. Twice rinsing are necessary after pretreatment. 5% silver A solution and 5% silver B solution are used for silver staining. Again gel is rinsed with pure water for three times. 5% developing stock solution is used for developing the gel. After waiting for the stained signal to come to the promising intensity (usually 2~15 min), 5% acetic acid as the stopper solution is applied to the gel container.

2-9Chromatin binding assay by biochemical fractionation

Chromatin-binding status of XRCC4 andLigase IV proteins was examined by sequential extraction with increasing concentration of Nonidet P-40 to separate chromatin-binding and non-chromatin-binding proteins, as we described earlier.

Typically, 10^7 cells were suspended in 150 μl of buffer A (50 mM HEPES-NaOH (pH 7.5), 150 mMNaCl, 1 mM EDTA, 1/100 volume each of protease inhibitor cocktail for animal cells (NacalaiTesque), phosphatase inhibitor cocktail I and II (Sigma-Aldrich)) with 0.2 % Nonidet P-40. After standing on ice for 5 min, the suspension was centrifuged at 1,000 g for 5 min and the supernatant was recovered as F-I. The remaining cell pellet denoted as P-I, was resuspended in 150 μl of the same buffer and immediately centrifuged at 1,000 g for 5 min. The supernatant of this step was recovered as F-II.

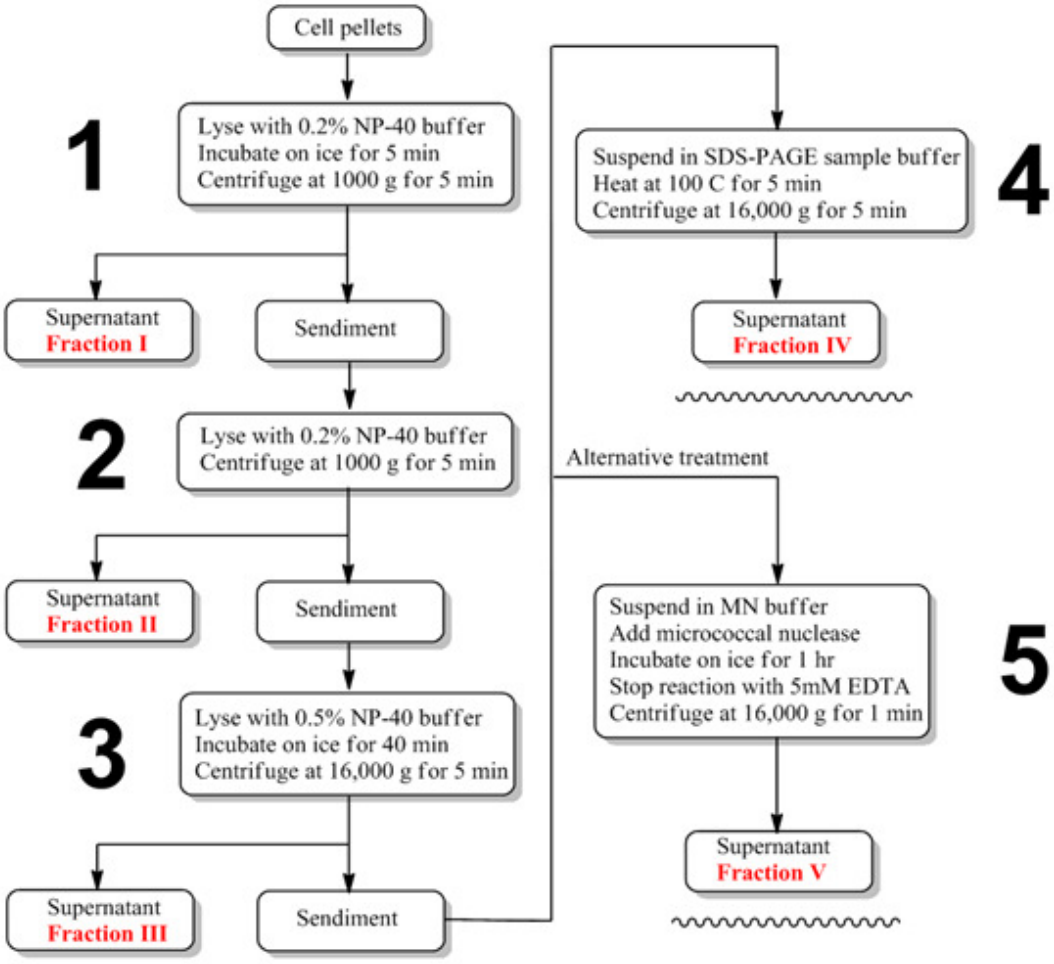

Fig.2 - 9*NP-40 Fractionation Protocol 4 fractions named F-I, F-II, F-III, F-IV can be collected after an NP-40 fractionation. F-V is collected from an alternative treatment with micrococcal nuclease treatment.*

The remaining cell pellet, denoted as P-II, was then resuspended in 150 µl of buffer A with 0.5 % Nonidet P-40. After standing on ice for 40 min, the suspension was centrifuged at 16,000 g for 5 min and the supernatant was recovered as F-III. The resultant pellet, denoted as P-III, was suspended in equivalent volume of 2x Sample buffer and heated in boiling water for 5 min. After centrifugation at 16,000 g for 5 min,

the supernatant was recovered as F-IV. F-IV could be regarded chromatin-binding fraction, as we have shown that XRCC4 protein found in P-III was liberated by micrococcal nuclease treatment, indicating that it had been tethered to chromatin DNA.

2-10Immunoprecipitation

Anti-FLAG M2 agarose(Sigma-Aldrich) is used for FLAG immunoprecipitation. 10^7 cells were harvested and washed twice with PBS. The pellet was lysed with Cell Lysis Buffer (50mMTris-HCl pH 7.6, 150mMNaCl, 0.5% TritonX-100) supplemented with 0.1% protease inhibitor cocktail for animal cells (NacalaiTesque) and phosphatase inhibitor cocktail I and II (Sigma-Aldrich). After 16,000g 5min centrifugation the supernatant was taken and mixed with 50 μl FLAG agarose. The tube was put on the rotator at 4 °Cfor 3hr reaction. After 2,000 g centrifugation the supernatant was disposed and the sentiment was washed with 1 ml Cell Lysis Buffer 5 times with centrifugation. The final sediment was mixed with 150 μl sample buffer for protein detection.

CHAPTER 3

The Chromatin Binding of XRCC4 Depends on the Presence of Ligase IV

3-1 Absence of chromatin binding of XRCC4 in Ligase IV-deficient cells

All the work started with Nalm-6 cells. Dr. Adachi gifted us the Nalm-6 Ligase IV-deficient cells. Our team has developed a biochemical analysis, named NP-40 fraction for the research in NHEJ molecules. Dr. Kamdar has successfully demonstrated the use of NP-40 fractionation in the study of XRCC4(Kamdar *and* Matsumoto, 2010). As a preliminary research, I followed the same protocol and examined the chromatin binding of Ligase IV.

Fig.3 - 1*Fractionation of Nalm-6 cells. 10^7 of Nalm-6 wild-type and Ligase IV-deficient cells collected for NP-40 fractionation. F-I, F-II, F-III, F-IV were mixed with sample buffer at an indicated ratio.*

Here are the results. Fraction I, II, III, IV were collected as described in the fractionation protocol, and immediately mixed with SDS sample buffer at a ratio of 20:1, 10:1, 2:1 and 1:1 respectively. As most proteins were thought to be collected in Fraction I, this operation was intended to equilibrate proteins in each portion to be blotted in a single gel. The western blotting results again confirmed that most of the Ligase IV were collected in

F-I, which is not chromatin binding. The chromatin binding Ligase IV was collected in F-IV, making only a small portion. From the approximate intensity of Ligase IV bands in different fraction, we can estimate that chromatin binding Ligase IV is about 5% of total Ligase IV in the cell, almost as same as the ratio of XRCC4. NHEJ molecules all seem to present with a larger number than necessary in the cell. This could possibly benefit the fast response toward a DSB action.

From the preliminary results, we have shown that this NP-40 fractionation is a powerful tool to separate chromatin binding Ligase IVwhich is thought to be active in DSB repair from those unbound form of Ligase IV. We established anexperiment foundationfor all following experiments. Every time we collect 10^7 cells for chromatin analysis, and the 5% chromatin binding proteins equal to a full cell lysate from 5×10^5 cells, which is considered enough and sufficient.

Learning from the Nalm-6 wild-type and Ligase IV-deficient cells, we could see that the XRCC4 chromatin binding was together missing with Ligase IV, suggesting Ligase IV is required for the chromatin binding of XRCC4. To verify this point, I have to re-introduce Ligase IV back to the deficient Nalm-6 cells.

3-2Creation of wild-type Ligase IVvector

To introduce Ligase IV back to Nalm-6 deficient cells, I have to create a Ligase IV vector which can transport the gene into cell nucleus. pCMV10 vector was chose as the initial vector for Ligase IV, as itwasavailable in my lab and was good for the study of XRCC4 in M10 cells.

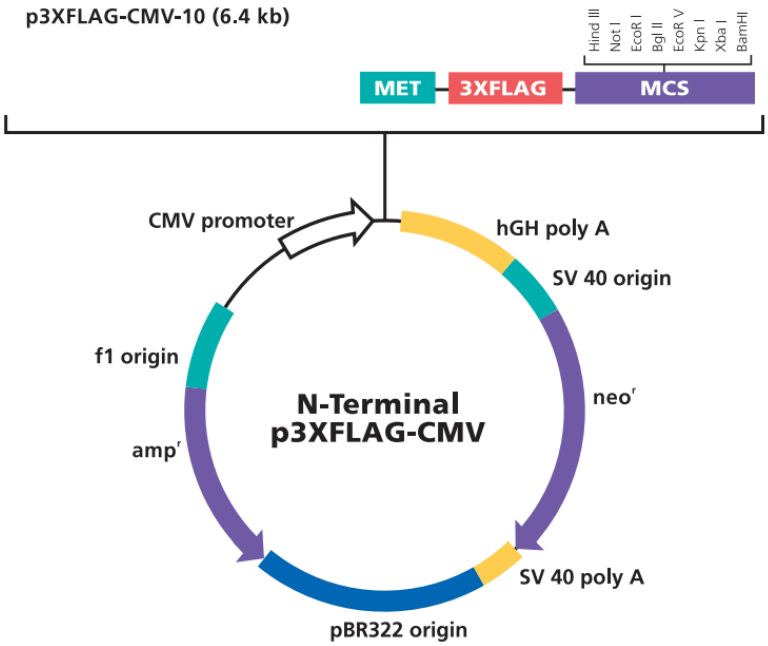

Fig.3 - 2*pCMV10 vector map*

The pCMV10 vector was originally purchased from Sigma-Aldrich Company. It is a shuttlevector forboth E. coliand mammalian cells. It contains an N-terminal 3xFLAG tag which allows detection of fusion protein with FLAG antibody.

On the other hand, Ligase IVcDNA was originally obtained from Open Biosystems(AL, USA),as cloneMGC:33819 IMAGE:5259632.The full length cDNA contains extensive 5'- and 3'- UTR (Untranslated Regions), which does not encode for protein. Only coding region of Ligase IV was intended to be inserted between EcoRI and KpnI site of pCMV10 MCS region. Necessary primers were carefully designed and ordered from FASMAC.

The primers are usually 20~30 long nucleotides served for DNA synthesis. A DNA replication can only be initiated from the 3' site of existing primers.Primers have complement sequences with the template DNA for recognition. In the polymerase chain reaction (PCR), the desired DNA replication initiates from the 3' primer, copy nucleotides

from the DNA template, and terminate until the end of 5' primer.

Primers were designed to acquire Ligase IV fragment with desirable restrictionenzyme sites.

NAME	R.E.SITES	PRIMER SEQUENCE
LigIV-F	EcoRI	ACGAATTCGATGGCTGCCTCACAAACTTCA
LigIV-R	KpnI	AACGGTACCTTAAATCAAATACTGGTTTTC

LigIV-F is the forward primer which starts from 3' ends, and it contains EcoRI site sequence (GAATTC) as the MCS of pCMV10. LigIV-R is the reverse primer which starts from 5' end, and it contains KpnI site sequence (GGTACC). The two primers together with Ligase IVcDNA were mixed and run for a PCR. Ligase IV was amplified after PCR and we obtained a DNA mixture.

The DNA mixture of PCR product contains large amount of amplified DNA Ligase IVwhich contains the two restriction enzyme sites. The mixture was purified with PROMEGA kit and subsequently digested with EcoRI and KpnI enzyme, as well as the original pCMV10 vector. After the digestion, both DNA left a sticky end. Now the digested pCMV10 vector and the digested Ligase IV were compatible for end ligation.

The digested solution contained fragment which was cut by restriction enzymes. In Ligase IV DNA solution there was also cDNA and primers. These products need to be separated before ligation.

It was done with DNA electrophoresis.Heavy DNA fragment moves slower than light fragment. The DNAs are separated mainly by molecule mass. After electrophoresis, DNA bands can be seen with UV-light by the treatment ofDNA intercalating agents.

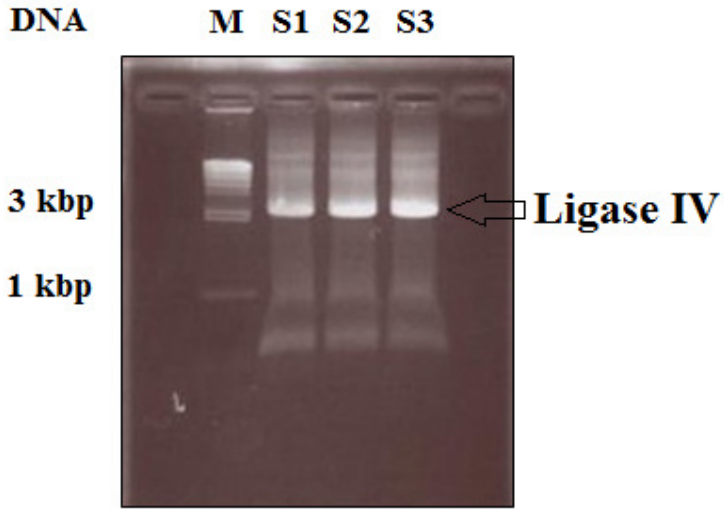

Fig.3 - 3*DNA gel checked by UV-illumination*

M: 1 kbp DNA Marker; S1,S2,S3: digested Ligase IV PCR products

The molecule mass of Ligase IV DNA is estimated 2.7kbp. With the reference to 1kbp marker, a band near 3kbp was clearly seen, indicating the amplification of Ligase IV was successful.It was carefully cut from the DNA gel and dissolved into solution. The digested pCMV10 vector and digested Ligase IV were to be ligated.

The optimal condition for DNA ligation requires a ratio of insert protein and vector molecule between 3 and 6. The DNA concentration was measured by Nucleic Acid Software. It gave the rough concentration data as:

pCMV10 (digested): 189 ng/µl

LigIV (digested): 41.2ng/µl

.Considering the mass of pCMV10 (6kb) and Ligase IV (2.7kb), the volume ratio of Ligase IV and pCMV10 was set to 7 versus 1. The mixture was then treated with T4 ligase and ligase buffer, and been kept in water bath at 16°C for 2 hours' ligation.

The ligation does not ensure 100% accuracy, so selection ofLigase IV vectors

isnecessary. It was done by a bacterial system called JM109. 5 µl ligation product was mixed with newly defreeze JM109 cells. The ligated DNA was transformed into JM109 cell and selected by ampicillin resistance.

The JM109 cells were plated on solid agarose medium. With correct plasmid in JM109, the bacterial cells can survive and form a colony. The next day 6 colonies were picked up and purified by MINI-PREP to extract plasmids. The plasmids were digested with EcoRI and KpnI and run a DNA electrophoresis.

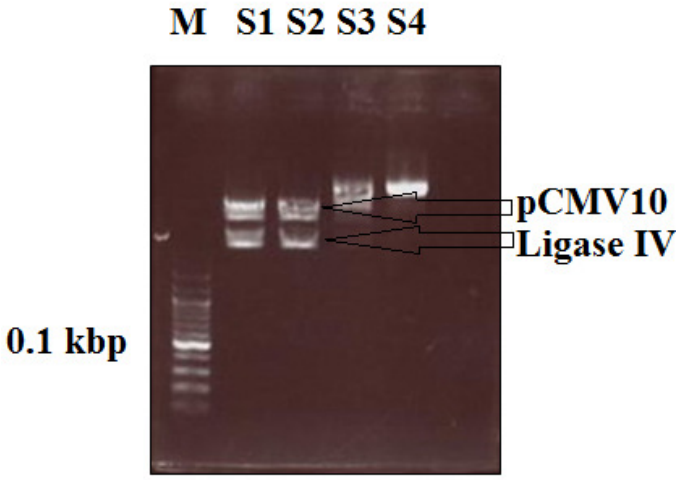

Fig.3 - 4 ***Insert check***

***** 0.1 kbp marker was used by mistake***

Correct plasmid contained a heavy DNA fragment, which is supposed to be pCMV10 vector at 6 kbp, and a light DNA fragment which is supposed to be Ligase IV at 2.7 kbp. S1 and S2 were selected as the correct vector and prepared for cell stock.

3-3 Validation of Ligase IVvector by DNA sequencing

Although insert check told the insert of Ligase IV gene into pCMV10 vector was successful, there is still possibilities that mutation was induced during the process of PCR.

The sequence of Ligase IV needs to be exactly the same as the database to ensure the production of wild-type Ligase IV protein. Such a small change in nucleotides will not be reflected by a noticeable change of molecular mass in insert check, so DNA sequencing is necessary to tell every nucleotide that was inserted in the pCMV10 vector.

Vector candidates were obtained after insert check. One colony in each mutant group was taken for sequencing. As each primer used for sequencing could give the credibility of about 800 amino acids, more than 4 primers were needed to cover the entire Ligase IV region which has 2888 amino acids to ensure the perfect fit.

3 old primers CMV30-F, CMV24-R, Lig4W725R-F were used to cover the edge region of Ligase IV and another 2 primers were design to sequence the center part. The two new primers were designed as

NAME	PRIMER SEQUENCE
LigIV703-F	AGTGGCTTATACGGATGATCATAAAGGATT
LigIV1377-F	CATACTAAGAATGAAGTAATTGATGCATTG

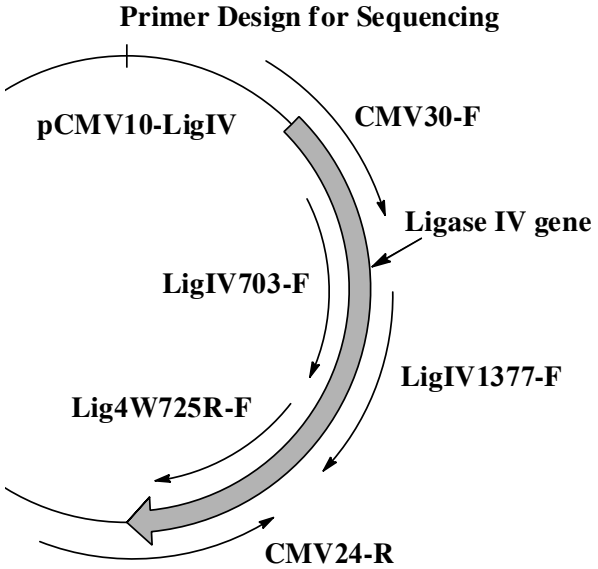

Fig.3 - 5*Demonstration for Ligase IV sequencing*

The sequencing samples were prepared with 1 primer mixed with plasmid DNA

according to the company protocol. They were later sent to FASMAC and thesequencing report was delivered by e-mail after one week's time. The sequencing report gave the most convincing information on the Ligase IV insert. Now the work of Ligase IV clone was finally over.

Full Human DNA Ligase IV sequence: 911 amino acids

1 MAASQTSQTV ASHVPFADLC STLERIQKSK GRAEKIRHFR

41 EFLDSWRKFH DALHKNHKDVTDSFYPAMRL ILPQLERERM

81 AYGIKETMLA KLYIELLNLP RDGKDALKLL NYRTPTGTHG

121 DAGDFAMIAY FVLKPRCLQK GSLTIQQVND LLDSIASNNS

161 AKRKDLIKKS LLQLITQSSALEQKWLIRMI IKDLKLGVSQ

200 QTIFSVFHND AAELHNVTTD LEKVCRQLHD PSVGLSDISI

241 TLFSAFKPML AAIADIEHIE KDMKHQSFYI ETKLDGERMQ

281 MHKDGDVYKY FSRNGYNYTDQFGASPTEGS LTPFIHNAFK

321 ADIQICILDG EMMAYNPNTQ TFMQKGTKFD IKRMVEDSDL

361 QTCYCVFDVL MVNNKKLGHE TLRKRYEILS SIFTPIPGRI

400 EIVQKTQAHT KNEVIDALNEAIDKREEGIM VKQPLSIYKP

441 DKRGEGWLKI KPEYVSGLMD ELDILIVGGY WGKGSRGGMM

481 SHFLCAVAEK PPPGEKPSVF HTLSRVGSGC TMKELYDLGL

521 KLAKYWKPFH RKAPPSSILCGTEKPEVYIE PCNSVIVQIK

561 AAEIVPSDMY KTGCTLRFPR IEKIRDDKEW HECMTLDDLE

601 QLRGKASGKL ASKHLYIGGD DEPQEKKRKA APKMKKVIGI

641 IEHLKAPNLT NVNKISNIFEDVEFCVMSGT DSQPKPDLEN

681 RIAEFGGYIV QNPGPDTYCV IAGSENIRVK NIILSNKHDV

721 VKPAWLLECF KTKSFVPWQP RFMIHMCPST KEHFAREYDC

761 YGDSYFIDTD LNQLKEVFSGIKNSNEQTPE EMASLIADLE

800 YRYSWDCSPL SMFRRHTVYL DSYAVINDLS TKNEGTRLAI

841 KALELRFHGA KVVSCLAEGV SHVIIGEDHS RVADFKAFRR

881 TFKRKFKILK ESWVTDSIDKCELQEENQYL I

(GENBANK: AAH37491.1) (Strausberg *et al.,* 2002)

3-4 Transfection ofLigase IV vector into Ligase IV deficient cells

New clone needed to be expressed in Nalm-6 deficient cells to rescue Ligase IV deficiency. It was a step looked pretty easy, but later proved as a nightmare in the whole study.

Magnetofection was used in early experiments to transfect Nalm-6 deficient cells with wild-type Ligase IV gene. Very few colonies appeared three weeks after plating on soft agarose medium, and of course these colonies were tested as incorrect colonies that did not produce any Ligase IV protein. This was the first time I noticed the low transfection efficiency, so I turned to use a more efficient transfection system, Neon transfection. GFP vector was tested on Nalm-6 cells and it worked well. I picked up the most optimized protocol #5, #8 and #18 according to the expressing level of GFP signal.

I had believed this new machine should work for me, and I just ran into a bigger trouble to get more incorrect colonies after plating. Many times I checking the new colonies with Ligase IV antibody, I got a band which was significantly lower from the expected position, indicating the newly expressed Ligase IV was not in full length. The Ligase IV could also be detected with FLAG antibody, and both of them shown at the same position. It is naturally think that the truncation was due to accidental termination during protein production. But afterI carefully checked the sequence data, I stillcould not find any single

mistake according to the Ligase IV sequencing report. The sequence of Ligase IV should be all right.

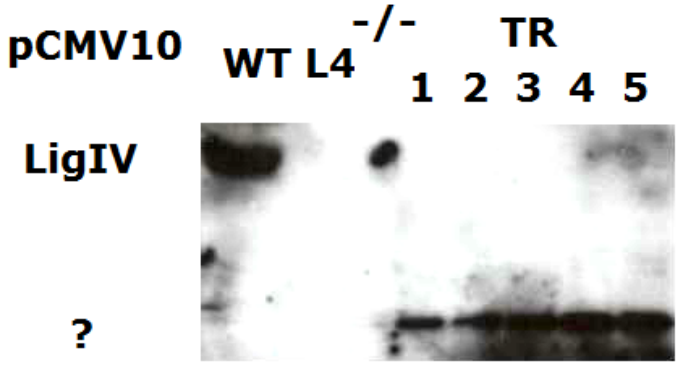

Fig.3 - 6*Failure of Ligase IV transfectionDetection with Ligase IV antibody. Original and Truncated Ligase IV was shown at a different position. TR is for transfected Ligase IV expressions. 5 colonies were examined in the same gel.*

Former lab member Dr. Kamdar gave me much support on the transfection work. Naturally there exists a possibility of bad gene recombination after successful transfection. So we togetherattempted many times with the same transfection of pCMV10-Ligase IV vector to increase the chance of a good Nalm-6 transfectant, but all ended with the same failure.

The pCMV10 was tagged with 3xFLAG, so all Ligase IV protein produced was tagged with FLAG protein. Ligase IV was found to be easily influencedby protein tags, so we modified the pCMV10 vector to exclude the 3xFLAG tag. Our Ligase IV gene contains its own start codon, so we prepared two pCMV10 vectors, with one kept start codon in 3xFLAG region, and one deleted.

We created the new vectors by PCR to short the unnecessary sequences. The primers are designed as:

NAME	PRIMER SEQUENCE
pCMV10-FLAG-F	TTA ACC ATG CTT GCG GCC GCG AAT TCA TCG
pCMV10-FLAG-R	GGC CGC AAG CAT GGT TAA TTC TGA CGG TTC
pCMV10-FLAG-NOMET-F	GAA TTA ACC CTT GCG GCC GCG AAT TCA TCG
pCMV10-FLAG-NOMET-R	GGC CGC AAG GGT TAA TTC TGA CGG TTC ACT

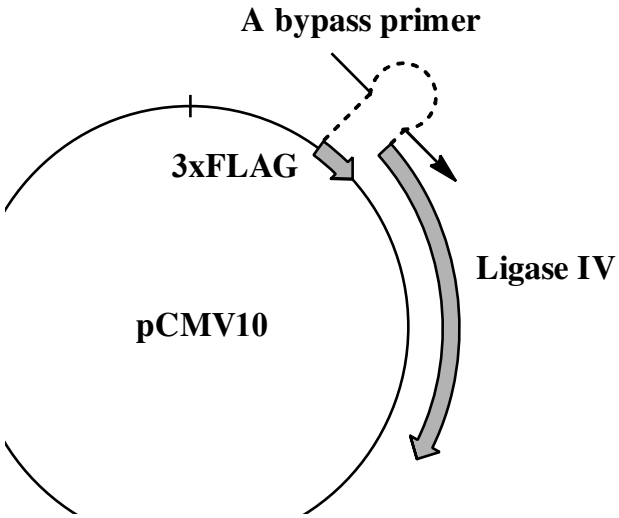

Fig.3 - 7*Mechanism of primer design for 3xFLAG tag deletion*

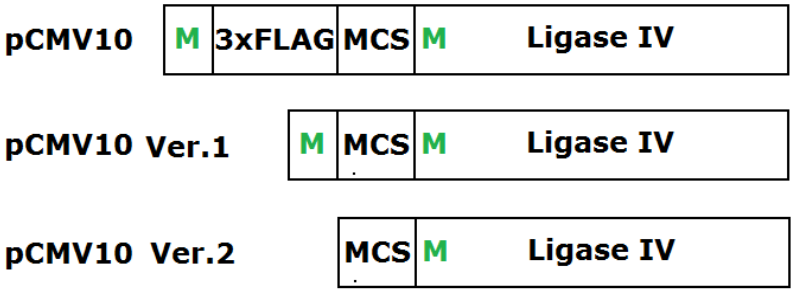

Fig.3 - 8*Modified pCMV10 vector with WT Ligase IV 3xFLAG was removed from original pCMV10 vector. M stands for the start codon as it is also coded for amino acid Methionine. MCS is the multiple cloning site, where there are several restriction enzyme site for gene operation.*

We later examined the new vectors by sequencing and ensured that there was no

mutation induced to the Ligase IV insert. We transfected to Nalm-6 cells by both magnetofection and NEON transfection protocols, and the transient expression was examined after 3 days cell culture by western-blotting against Ligase IV antibody. As itwas anothersad story, no normalLigase IVexpressing cells could be obtained.

From the fact that full length Ligase IV gene couldnot be integrated into Nalm-6 cells, it suggested that gene recombinationpossibly requires the participation of NHEJ pathway to join transfected DNA fragments to the chromosome. IfLigase IV was absent, the cell might meet difficulty for gene integration. But except for NHEJ there also existed some other DNA joining pathways which might not be efficient.

As the pCMV10 vector could not work properly, we turned our sight to other vectors. There werepcDNA3 and pcDNA4 vector in my lab. So together with Dr. Kamdar we copied the Ligase IV sequence from pCMV10 to pcDNA3 and pcDNA4 vectors. We transfected them into Nalm-6 deficient cells, but results again disappointed us.

So far, I noticed that all the vectors we used have the same CMV promoter. Could this be the cause for the Ligase IV truncation? It might be a good attempt to switch to a new promoter other than CMV. EF1α is a stronger promoter that yields higher transfection efficiency in many cells compared to CMV (Qin *et al.,* 2010). So we purchased the EF1α vector from TaKaRa.

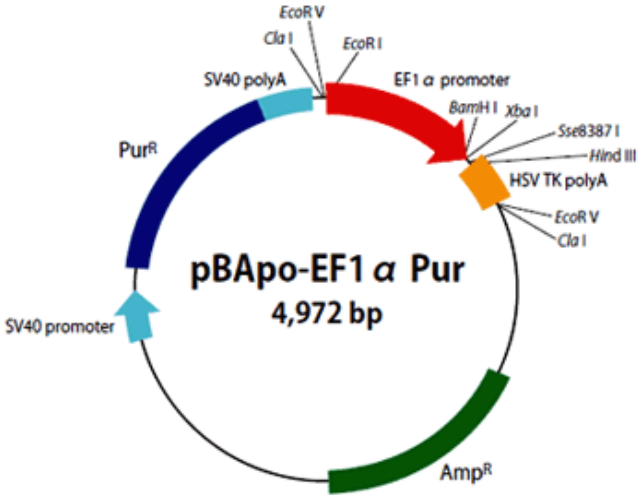

Fig.3 - 9*Vector map of EF1α*

The pBApo-EF1α vector contains a human polypeptide chain elongation factor (EF1 alpha) promoter and a polyA signal site from the herpes simplex virus thymidine kinase gene, which enables a high infection efficiency and wide target cell spectrum, suitable for in vitro and in vivo gene transductions.

The original MCS region of EF1α vectorcontains very few restriction enzymes sites. To copy Ligase IV from pCMV10 vector, we modifiedthe MCS region to allow gene operations.

New MCS was designed for EF1α vector to accept Ligase IV insertion. The newly introduced restriction enzyme sites were not contained in the original vector, which was verified by NEBCutter 2.0.

The new primer was designed to contain as many available sites as possible to allow future use. And the primer should not be too long for the economical concern. It was inserted between BamHI and HindIII site on the original EF1α vector.

New MCS:

GGATCCGCTAGCAGATCTTCCGGA --

-- CCACGTGCGGCCGCTCGACGGTACCAAGCTT

BamHINheIBglIIBspEI

PmlINotIKpnIHindIII

NAME	SEQUENCE
EF1a-BamHI-HindIII-New-Fwd	GATCCGCTAGCAGATCTTCCGGACCACGTGCGGCCGCTCGACGGTACCA
EF1a-BamHI-HindIII-New-Rev	AGCTTGGTACCGTCGAGCGGCCGCACGTGGTCCGGAAGATCTGCTAGCG

The modified EF1α vector was produced by PCR with the above two primers and purified by PROMEGA kit. The modified vector has also been sequenced by newly designed primers outside the MCS region

NAME	SEQUENCE
EF1a-Seq-Forward	AGCCTCAGACAGTGGTTCAAAGTT
EF1a-Seq-Reverse	CGCGGGTTCCTTCCGGTATTGTCT

Ligase IV gene was later inserted into NheI andKpnI sites. The new Ligase IVvector was transfected into Nalm-6 deficient cells by Neon transfection.

After been stuck on transfection for almost two years,I finally get Ligase IV expressed in Nalm-6 cells. Thanks to EF1α, I could be able to continue my research on Ligase IV, though the transfection efficiency still remains low.

3-5Restoration of the chromatin binding of XRCC4 by introduction of Ligase IV

It is already know that the chromatin binding of XRCC4 is missing in Nalm-6 Ligase IV-deficient cells. Now wild-type Ligase IV has been stably tranfected into deficient cells, and a recovery of XRCC4 chromatin binding could be clearly seen in F-IV fraction, where chromatin binding proteins present. This observation indicated that the chromatin binding of XRCC4 is dependent on the presence of Ligase IV.

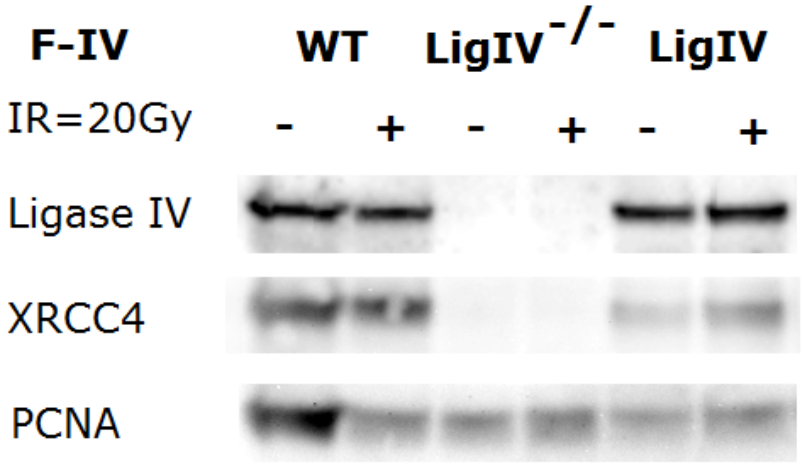

Fig.3 - 10*LigIV restored XRCC4 chromatin binding.Ligase IV c-DNA was introduced to deficient Nalm-6 cells and named asLigIV.*

As Ligase IV and XRCC4 are molecules involved in NHEJ repair, we irradiated the cells by 20Gy to generate considerable DSBs, and observe the changes after irradiation. Here the expected increase after IR was not observed in chromatin binding of either XRCC4 or Ligase IV, which is a contrast to our earlier study with murine leukemia M10 cells. In M10 cell, IR induced increase was observed even at 2Gy (Kamdar *and* Matsumoto, 2010), but in human cells others have reported same results as us (Drouet e*t*

*al.,*2005).

We also checked the chromatin binding of XLF in Nalm-6 cells. XLF is another key player in NHEJ repair, and it shares much structural similarity with XRCC4.Results showed that the chromatin binding of XLF was also present in Ligase IV-deficient cells as well as in wild-type cells.

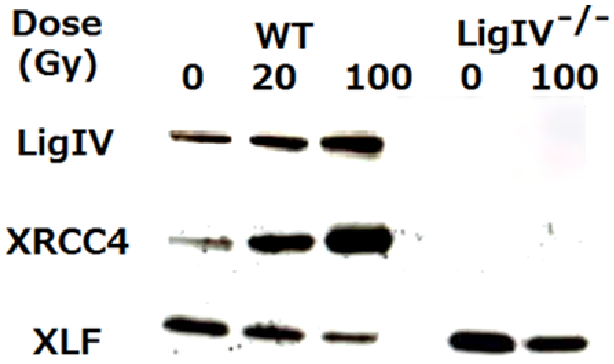

Fig.3 - 11*Chromatin-binding of XLF is independent of Ligase IV. Wild-type Nalm-6 were irradiated at a dose of 0, 20, 100 Gy, and Ligase IV-deficient cells were irradiated at a dose of 0, 100 Gy. All samples were fractionationed and F-IV was western-blotted against LigIV, XRCC4 and XLF.*

It was unlike XRCC4 that the chromatin binding of XLF was not affected by the absence of Ligase IV. The chromatin binding of XLF does not require Ligase IV, which fits the observation that Ku independently and sufficiently recruits XLF to chromatin (Yano*et al.,* 2008), but contradicts to a previous report that loss of Ligase IV prevents DSB recognition of both XRCC4 and XLF(Jayaram *et al.,* 2008). The difference behavior between XRCC4 and XLF indicates a different recruitment mechanism. Although Ku can directly interact with XRCC4 (Mari *et al.,* 2006), it is not likely that Ku can

independently recruits XRCC4 to chromatin. It is also reported that XLF and XRCC4 forms a filament structure that possibly facilitate the bridge of DNA during DNA repair (Andres *et al.,* 2012). There is an N-terminal head to head interaction between XLF and XRCC4 and they connected each other alternatively to form a filament structure(Hammel*et al.,* 2010). We don't know if this filament can be formed without Ligase IV. If so, why only chromatin binding of XLF can be observed, but not XRCC4? If not, what is the role of Ligase IV in filament formation? It still remains to be answered.

It is known that NHEJ repair is activated after a DSB event, and Ligase IV and XRCC4 are considered to be recruited to chromatin. An increase in chromatin binding proteins could be clear seen at high IR dose such as 100Gy. We noticed that even in non-irradiated cells there were alreadylarge amount of chromatin binding proteins.

At first I suspect that the chromatin binding might be caused by endogeneous DSBs induced by cell metabolism. I tested this idea by treating cells with Actinomycin D, a potential inhibitor for transcription that was supposed to stop cellular activity which may lead to endogenous DSBs.

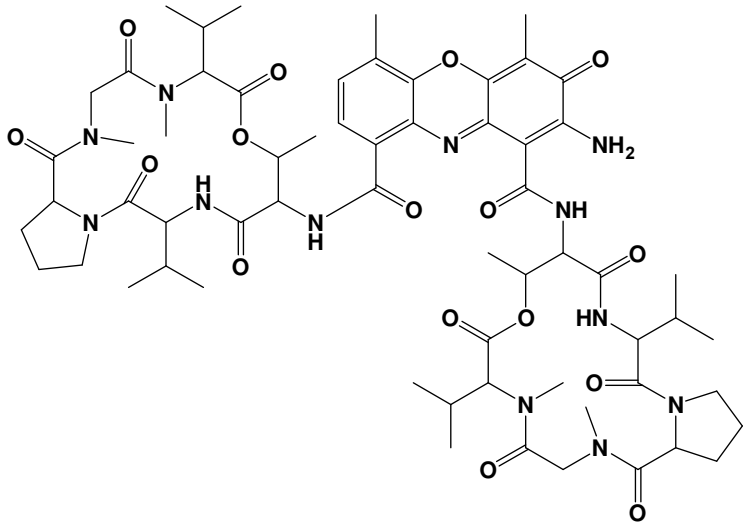

Fig.3 - 12*Structure of Actinomycin D, a transcription inhibitor*

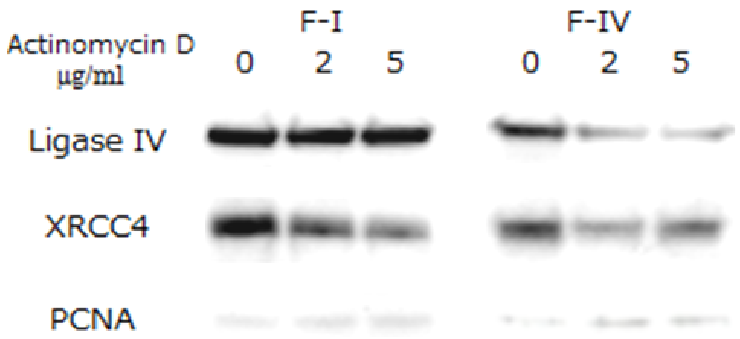

Fig.3 - 13 Actinomycin D reduced chromatin binding of Ligase IV and XRCC4**. A:** ***0 (control), 2, 5 µg/ml Actinomycin D was added in wild-type Nalm-6 cells 2h before fractionation. F-I and F-IV were western-blotted against Ligase IV, XRCC4 and PCNA (as a loading control).***

Both chromatin binding of Ligase IV and XRCC4 in wild-type cells were reduced after 2 h treatment with Actinomycin D, while the Ligase IV in F-I remained unchanged. From this observation I had once assumed chromatin binding of Ligase IV was due to endogenesously generated DSBs.

However, this assumption could not explain why the increase of chromatin binding Ligase IV was so insignificant from 0 to 20Gy. 20Gy is much higher than the half lethal dose, so cells are not supposed to survive after an irradiation of 20Gy. Surely there would be much more DSBs as well as other types of DNA damages compared to non-irradiated cells. If it is possible to observe chromatin binding proteins in non-irradiated cells, it should be greatly increased after dose that is beyond the repair capacity of the cell.

I have noticed that some reports support that Actinomycin D induces DNA damage rather than eliminate endogenous DSBs, with elevated γ-H2AX foci formation which is commonly considered as a direct DNA damage response (Mischo *et al.,* 2005; Haffner *et al.,* 2011).

The mechanism of Actinomycin D in transcription inhibition is that it forms complex with transcription initiation complex and prevents elongation of RNA chain by RNA polymerase (Sobell, 1985). When the complex accumulates on DNA, it might capture most of the space around DNA that makeunable to bind.

Now we suppose the naturally occurred chromatin binding of Ligase IV could be a dynamic process which is independent of DSBs. Ligase IV contains the DNA binding domain so that it has intrinsic binding affinity with DNA like other DNA ligases.

CHAPTER 4

Role of C-terminal Region of Ligase IV in Chromatin Binding

4-1Ligase IV point mutagenesis

Now it is the time to start the research on the function of Ligase IV molecule. Ligase IV is a protein with a catalytic N-terminal and a supporting C-terminal. Ligase IV has not been crystalized yet so that there are less information on the protein structure. The N-terminal has been extensively studied, while the C-terminal is still remained as a myth. All we know now it that it contains two BRCT domains, and a XIR domain in between, which is specifically mediating the interaction with XRCC4. XRCC4 is critical for the stability of Ligase IV.

BRCT domains of Ligase IV have not been found to have significant function, except for possible roles in XRCC4 interaction, but the sequence is so conserved within the eukaryotic species.

I made my decision to step into the research on Ligase IV C-terminal, especially the two BRCT domains. BRCT domain in various proteins share a similar structure and most of the BRCT domains have a strong conservation in the amino acid tryptophan of helix α3. Here I compare the BRCT helix α3 sequence in human DNA Ligase IV to other eukaryotic cells. This tryptophan site is highly conserved from human to frog, fruit fly, yeast, and even to some fungi and plants, as well as from the BRCT domain of Ligase IV, BRCA1, BARD1, p53BP1 and XRCC1. From the comparison between species it seems W725 region is more conserved than W893, so that the function might be more important.

Fig.4 - 1*Ligase IV gene conservation in BRCT domain.The gene sequence was obtained from INSDC (GenBank), aligned with Clustal Omega and arranged with Color Align Conservation. Upper: Comparison between species; Lower: Comparison between human proteins which contain BRCT domain and participate in DNA repair.*

To find the potential function of BRCT domains, a commonly used study method is point mutation. Point mutation changes the characteristic of single amino acid, usually accompanied by a change of electric charge. The chemical reaction and protein interaction are usually charge sensitive. By doing point mutation it can effectively reduce the binding affinity with a possible partner, which disrupts the protein's normal function. Tryptophan in α3 helix is highly conserved in BRCT domains. By mutating this site, it will possibly disrupt the entire function of BRCT domain. By comparison between BRCT

mutated Ligase IV with wild-type, we can get the information on the role of BRCT domains.

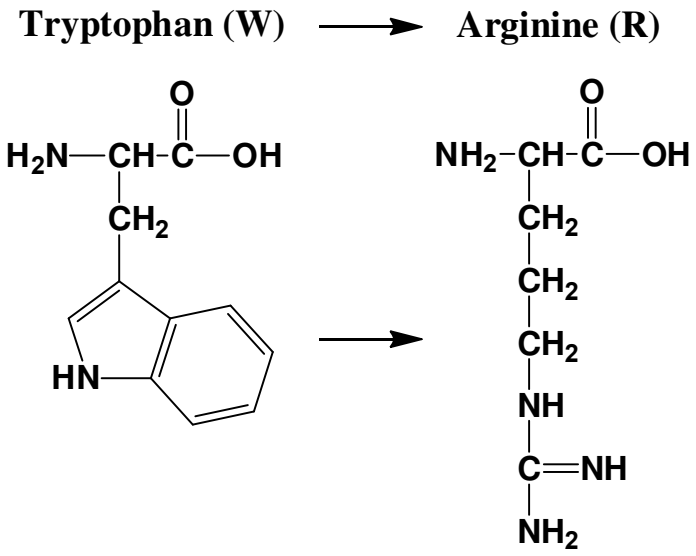

Fig.4 - 2*Molecule structure of mutated amino acid in BRCT domains*

Changing hydrophilic amino acid tryptophan (W) to hydrophobic amino acidarginine (R) in BRCT domains in BARD1 and 53BP1 was shown to compromise the binding to phosphorylated peptide (Yu *et al.,* 2003).In the case W to R mutation, the distance from N-terminal to C-terminal is not changed, so it has a merit to keep the stacking structure of mutated protein unchanged, with only change in its side chain. So I created two Ligase IV mutants W725R and W893R to compromise the function of BRCT domains.

Forthe evaluation of defective Ligase IV activity, I also created another Ligase IV point mutation R278H. This mutation was first discovered from a patient with Ligase IV syndrome(Riballo *et al.,* 2001). The mutation was not critical but reduced Ligase IV activity to 10% of wild-type. If the BRCT mutations show some radiosensitivity, this extensively studied mutation can be a good reference.

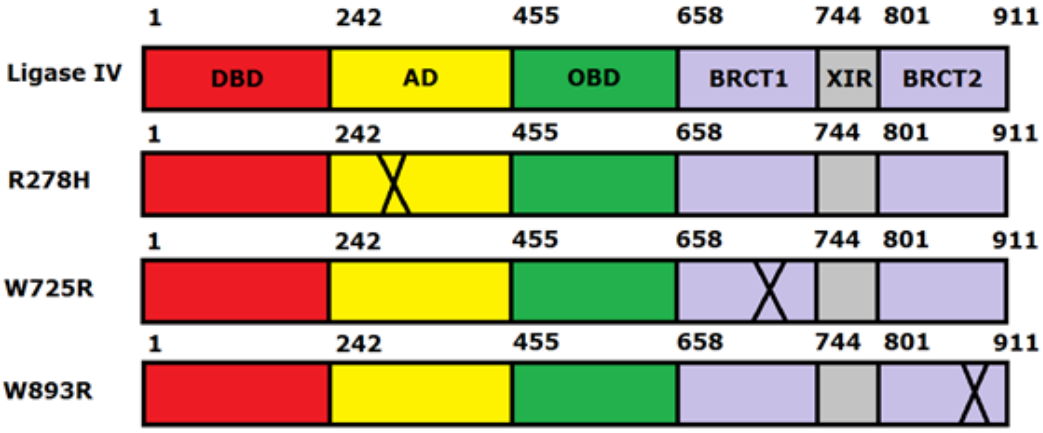

Fig.4 - 3*Ligase IV point mutations. Human Ligase IV contains 911 amino acids, with functional domains of DNA-binding domain (DBD), Adenylation domain (AD) and oligo-binding domain (OBD). In the C-terminal it contains BRCT1 domain, XRCC4 interacting domain and BRCT2 domain. The position of point mutations are marked with "X".*

The primers for point mutagenesis were created as below.

NAME	PRIMER SEQUENCE	ORIGINAL
LIG4-K273M-F	GAAACCATGCTAGATGGTGAACGTATGCAA	AAG
LIG4-K273M-R	ATCTAGCATGGTTTCTATGTAGAAACTCTG	
LIG4-R278H-F	GGTGAACATATGCAAATGCACAAAGATGGA	CGT
LIG4-R278H-R	TTGCATATGTTCACCATCTAGCTTGGTTTC	
LIG4-W725R-F	CCTGCACGGCTTTTAGAATGTTTTAAGACC	TGG
LIG4-W725R-R	TAAAAGCCGTGCAGGCTTGACAACATCATG	
LIG4-W893R-F	GAAAGTCGGGTAACTGATTCAATAGACAAG	TGG
LIG4-W893R-R	AGTTACCCGACTTTCTTTTAGGATTTTAAA	

All four mutants were created by PCR with PrimSTAR mutagenesis kit, and sequencing by FASMAC Co Ltd. And incorrect vectors were abandoned and repeated with new mutagenesis to ensure there is no single mutation expect for the desired site. K273M was

initially created but unfortunately not used in later experiment.

After getting the stable clones, we examined the expression level of Ligase IV in Ligase IV mutations. R278H and W725R had relatively same expression level as Ligase IV transfected cell, while the expression level of W893R was greatly reduced. I have repeated the transfection W893R, and found the low expression level was common in all W893 transfectants. I think the mutation in BRCT2 might lead to the instability of Ligase IV, as it has been shown that BRCT2 domain is necessary for Ligase IV and XRCC4 interactions (Wu *et al.,* 2009). Patients with mutation in BRCT2 also have low expression level of Ligase IV (Girard *et al.,* 2004).

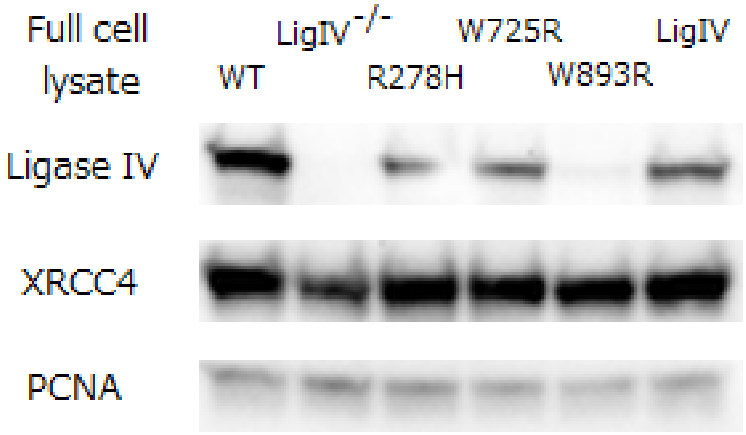

Fig.4 - 4*Protein expression in Ligase IV mutants. 10^6 Nalm-6 wild-type, Ligase IV-deficient, LigIV-R278H, LigIV-W725R, LigIV-W893R, Ligase IV transfected cells were collected and lysed with 100μl sample buffer, western-blotted against Ligase IV, XRCC4 and PCNA.*

4-2Both BRCT mutations of Ligase IV sensitized cell and reduced chromatin binding of Ligase IV

We examined theradiosensitivity ofLigase IVBRCT mutants by colony formation assay,

and multiple experiments were repeated to reduce the variance. We counted number of survival colonies and calculated the survival fraction for each mutant at each IR dose. We plotted the curve of survival fraction.

Wild-type Nalm-6 and Ligase IVtransfectant cells showed exactly same survival fraction, indicating the Ligase IV deficiency was completely compensated by introduction of Ligase IV gene. Although the Ligase IV expression in Ligase IVtransfectant was noticeably lower than wild-type, the Ligase IV did not seem to be affected, suggesting the Ligase IV in normal cells exist more than necessary.

The point mutation R278H, as expected, showed a survival fraction between wild-type and deficient cells. It is quite understandable thatR278H hasdefect in Ligase IVadenylation so that it could not effectively repair the DSBs induced by IR.It was not as radiosensitive as deficient cells that R278H still remains certain DSB repair capability.

The BRCT mutants W725R and W893R, however, both showed reduced survival fraction that is comparable to R278H. This result strongly suggested the important role of both BRCT domains in the function of Ligase IV which has never been studied before.

From the similarity curve between BRCT mutants and R278H, I questioned myself if they have the any common reason for its radiosensitivy. As it is clearly known R278H was mutated near catalytic center for the adenylation that lies in the N-terminal. It is not likely to have a direct influence on the C-terminal BRCT domains. We have not examined the adenylation efficiency of BRCT mutants so far. It can be possible that the similar defect in Ligase IV is just a coincidence. To eventually get the answer to this question, double point mutation of R278H and W725R or W893R will be necessary, which has not yet be done in our lab.

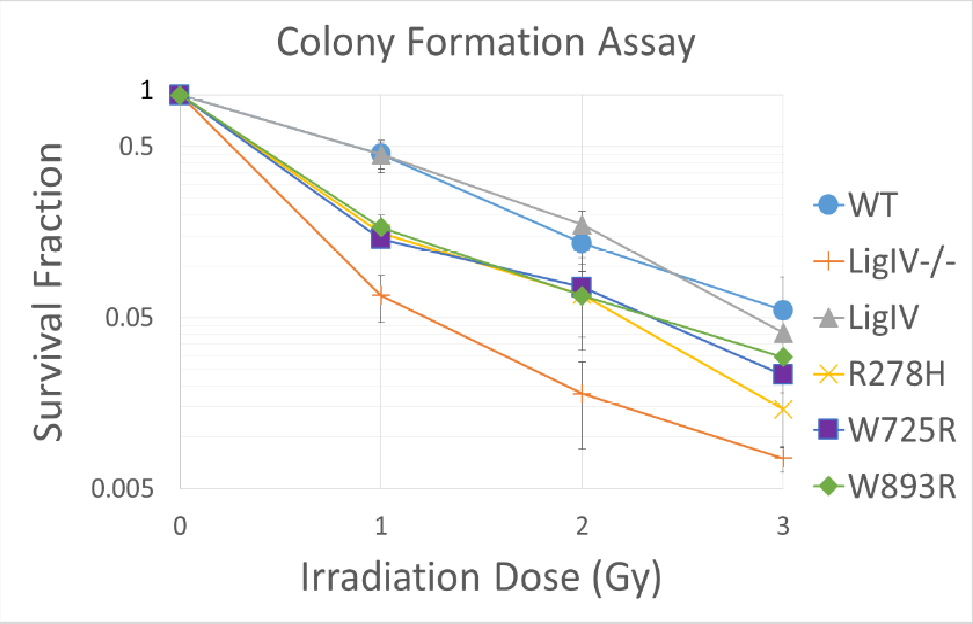

Fig.4 - 5*Colony formation assay. LigIV is transfected with wild-type Ligase IV. R278H, W725R, W893R are three point mutations of Ligase IV. Cells were irradiated at 0, 1, ,2, 3 Gy.*

Again I followed the NP-40 fractionation protocol to examine the chromatin binding molecules. F-I and F-IV have been western-blotted. A phosphorylation of XRCC4 can be clearly seen after 20Gy irradiation. It is still not clear about the meaning of XRCC4 phosphorylation, at least it is not required for cell survival after irradiation or V(D)J recombination (Yu*et al.,* 2003). A considerable decrease of chromatin binding Ligase IV and XRCC4 can be seen in W725R compared to non-mutated LigIV, indicating the BRCT1 domain is important for chromatin binding of Ligase IV and XRCC4 complex.

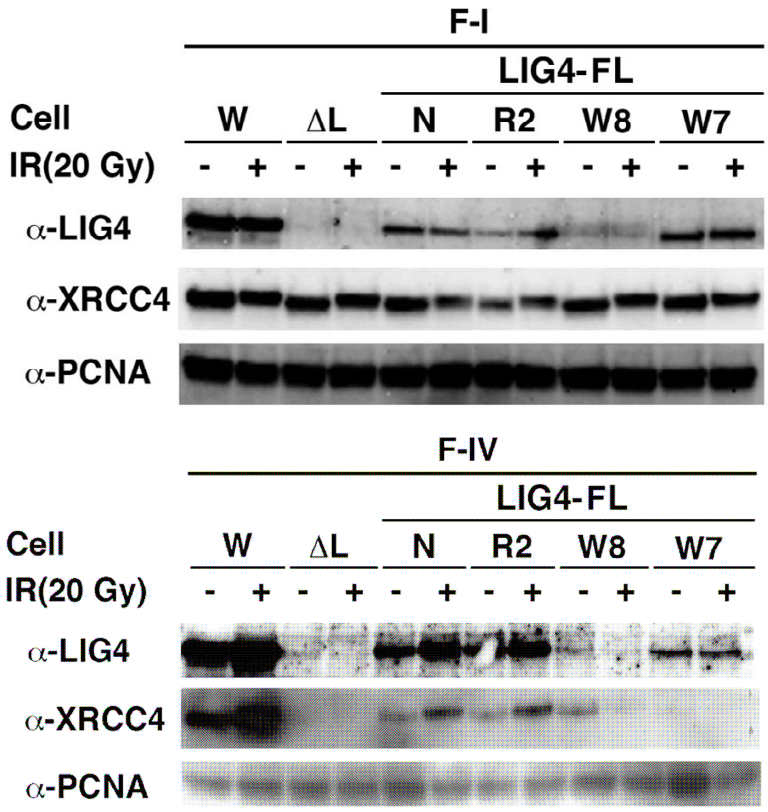

Fig.4 - 6*Fractionation analysis. R278H, W725R, W893R are abbreviated as R2, W7 and W8. N is the wild-type Ligase IV transfectant served as a control. All cells were fractionationed with NP-40 buffer and F-IV was western-blotted against Ligase IV, XRCC4 and PCNA.*

In W893R mutant, the Ligase IV but not XRCC4 was hardly detectable in F-I, suggesting the role of BRCT2 in maintaining Ligase IV stability. The Ligase IV snd XRCC4 were marginally detectable in F-IV, possibly due to the diminished level of Ligase IV in the whole cell.

It has been reported that R278H only have 10% Ligase IV activity in clinical study (Girard *et al.,* 2004). As W725R and W893R had similar survival fraction curves as R278H, I believe the activity of Ligase IV in BRCT domains have also be reduced to

nearly 10%, which means a great loss for Ligase IV function. The significant decrease of Ligase IV further indicated the importance of both BRCT domains.

I also noticed there was difference in chromatin binding of Ligase IV and XRCC4 between non-mutated Ligase IV and wild-type cells. However, the difference in chromatin binding did not affect the cell survival after IR, indicating the chromatin binding Ligase IV and XRCC4 were in excess amount. Some of the Ligase IV and in chromatin was not binding to DSB sites, but some other positions in chromosome. Only a small part of Ligase IV moved to DSB site after the occurrence of DSB. This part of Ligase IV could hardly be seen from 0 to 20Gy IR, but pretty easier from 0 to 100Gy or even higher dose. Base on this observation we later proposed two binding model for Ligase IV.

Another important observation is that Ligase IV and XRCC4 in chromatin binding kept a certain ratio. Simultaneously increase or decrease could be seen between Ligase IV mutants, indicating that chromatin binding of XRCC4 was pretty much relied on the number of Ligase IV that had been binding to chromatin. Considering from the fact that Ligase IV and XRCC4 forms a complex with high dissociation constant, it is not strange to believe that XRCC4 binds to chromatin as part of the Ligase IV / XRCC4 complex.

4-3 Chromatin binding of NHEJ molecules at extreme high dose behaves differently

As the chromatin binding molecules have only slight change when we use 20Gy irradiation, recently I started to use extreme high dose to observe clear change in recruitment of NHEJ molecules after IR.

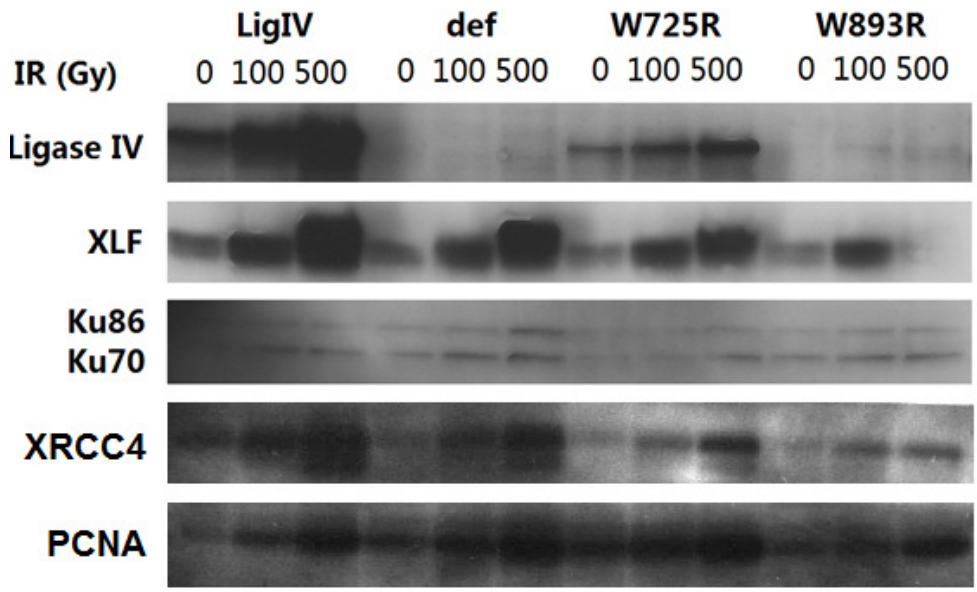

Fig.4 - 7*Chromatin bindingof NHEJ molecules at extreme high dose. Chromatin binding of NHEJ molecule Ligase IV, XLF, Ku70/86 and XRCC4 was examined; PCNA served as a loading control.*

I found that XLF appeared to be most active player to respond to induced DSB, and it was not dependent with Ligase IV at all. Ku70/86, in contrast, did not have any change with IR dose, which contradicts to our prediction. Ku is considered as the first sensor complex that is surely to be recruited to DSB. Now I don't knowany answerto this.

I also observed XRCC4 signal in deficient cells, as well as in other mutants. I think over 100Gy is extreme high dose that is far beyond the DNA repair capacity. It would possibly caused damage more than DSBs. It indicated a possibility that XRCC4 might be recruited by molecules other than Ligase IV, among which XLF is a good candidate. But at dose lower than 20Gy, the interaction with Ligase IV is still dominant.

In the case of Ligase IV, we could see the BRCT1 mutation of W725R did not eliminate the DSB induced response, butthe chromatin binding of Ligase IV in BRCT2

mutation was completely diminished. BRCT2 domain is more likely to regulate the damage response than the BRCT1 domain.

The extreme high dose response may give different results to low dose, which possibly contradicts current understanding. As dose above 100Gy has not been well studied in DNA damage repair, we have no idea if the chromatin binding molecules were really participating DNA repair or not.

4-4Creation of truncated Ligase IV mutants

The creation of Ligase IV was initially aimed to find C-terminal binding proteins. The fragment protein, LigIV-CT contains only the two BRCT domains as well as a linking region known as XIR. It does not contain N-terminal functional domains, so at first it was considered that this region was incapable of DNA binding. LigIV-CT is good for focused study on BRCT domains. Also the two BRCT mutations were introduced for comparison.

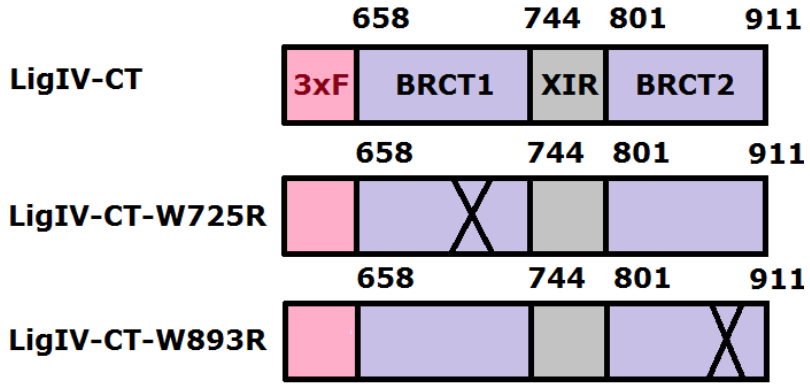

Fig.4 - 8*Ligase IV C-terminal mutations. LigIV CT mutants contain BRCT1, XIR and BRCT2 domains. All proteins are tagged with 3xFLAG in the N-terminal.*

The mutagenesis took same procedure as copying the entire Ligase IV gene. But as the truncated Ligase IV lacks some basic domains that it will not be detected by Ligase IV antibody we had used. So a tag was necessary for the detection of new protein. The EF1α

vector must be modified to incorporate some protein label. The 3xFLAG tag from pCMV10 vector is a good choice for sensitive and selective detection.

A 3xFLAG nucleotide sequence was directly obtainedfrom the map of p3xFLAG-CMV-10 vector.

ATG GAC TAC AAA GAC CAT GAC GGT GAT TAT AAA GAT CAT GAC ATC GAT TAC AAG GAT GAC GAT GAC AAG

Primers were designed to copy 3xFLAG gene from pCMV10 vector

NAME	SEQUENCE
3xFLAG-XbaI-Forward	GAATCTAGAATGGACTACAAAGACCATGAC
3xFLAG-NotI-KpnI-Hind III-Reverse	ATCAAGCTTGGTACCCGCGCGGCCGCGCTTGTCAT CGTCATCCTTGTA

The 3xFLAG fragment was inserted between XbaI and HindIII site of original EF1α vector. NotI and KpnI sites included on the primer were prepared for insert of Ligase IV C-terminal.

NAME	SEQUENCE
LigIV-BRCT-NotI-Forward	AGCGGCCGCATTGGAATTATTGAGCACTTA
LigIV-BRCT-KpnI-Reverse	ACTGGTACCTTAAATCAAATACTGGTTTTC

With this system, I have successfully created a LigIV-CT vector that expressed the C-terminal fragment of Ligase IV. The LigIV-CT expression can be detected with FLAG antibody.

W725R and W893R reduced the chromatin binding of Ligase IV and XRCC4, leading to a decreased capability for DSB repair. What would happen if same mutation

occurred in LigIV-CT? Would the IR induced response be diminished? Or the XRCC4 binding would disappear? In seek of answer to above questions, I also introduced point mutation W725R and W893R on C-terminal fragment, denotes as W7CT and W8CT.

I continued touse this system in expressing several other fragments of Ligase IV. We met some difficulty to get stable cloned with LigIV-NT. We could get very few colonies and we were unable to detect any FLAG tagged proteins. I have no idea whether LigIV can be normally expressed. If not, there must be some relation with XRCC4. Some of the workis still going on.

NAME	SEQUENCE
L4BRCT2-NotI-F	GGCGGCCGCGCATGGCTTTTAGAATGTTTT
BRCT1-KpnI-R	AGAGGTACCTTATCCTGAGAATACTTCCTTCAGTTG
LigIVW/OBRCT_R	AATGGTACCTTAAACTTTCTTCATCTTTGG
LigIVW/BRCT1_R	AAGGGTACCTTAAGGCTTGACAACATCATG
LigIVPuBRCT2_F	GTATGCGGCCGCATTAAAAATTCTAACGAG
LigIVNeBRCT1_F	CACCGCGGCCGCGGTGGTGATGATGAACCA

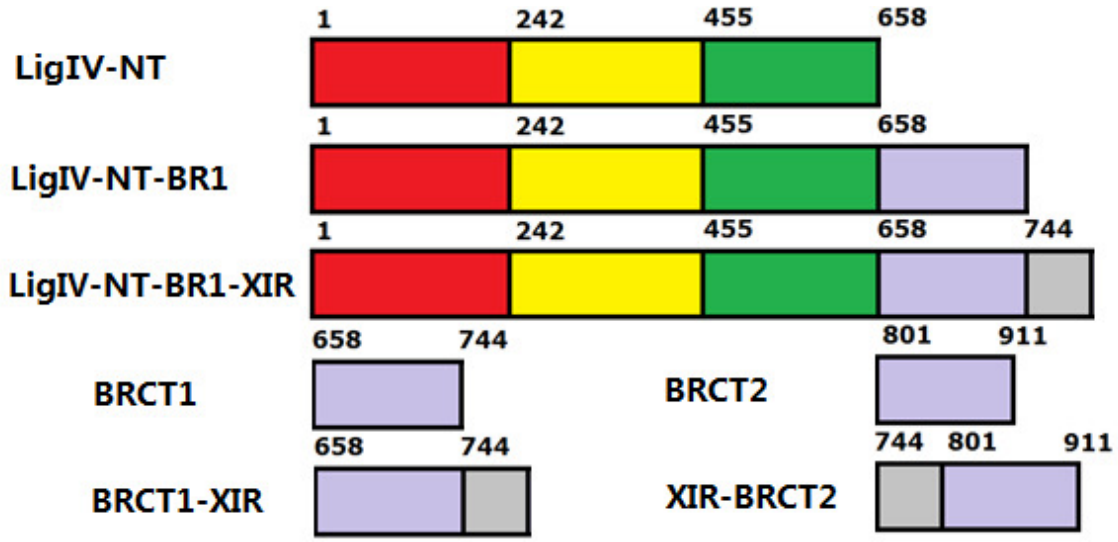

Fig.4 - 9*Mutagenesis for Ligase IV fragments. A stop codon was added to the end of*

each Ligase IV fragment by PCR. All proteins were tagged with 3xFLAG

4-5Ligase IV C-terminal region is capable of binding to chromatin with XRCC4

I examined whether or not LigIV-CT alone can recruit itself as well as XRCC4 to chromatin. F-IV after fractionation was western-blotted against LigIV-CT (FLAG)and XRCC4.

A clearly band of FLAG signal was been in the blot, showing the C-terminal can be self-recruited, and furthermore, it increased with IR dose, which is a characteristic of NHEJ molecules. The LigIV-CT was recruited the DSB sites which were increased after IR. Also the chromatin binding of XRCC4 was rescued, but rather partial in contrast to wild-type. I believe this result is in correspondence to the hypothesis that Ligase IV / XRCC4 can bind to chromatin, but only a very small part is binding to DSB sites where the repair takes place. This DSB induced binding could possibly be mediated by C-terminal of Ligase IV, which are not present in other DNA ligases. Together with the finding with W725R and W893R, it is clear that C-terminal of Ligase IV recruits Ligase IV / XRCC4 to DSB sites and plays a key role in NHEJ repair.

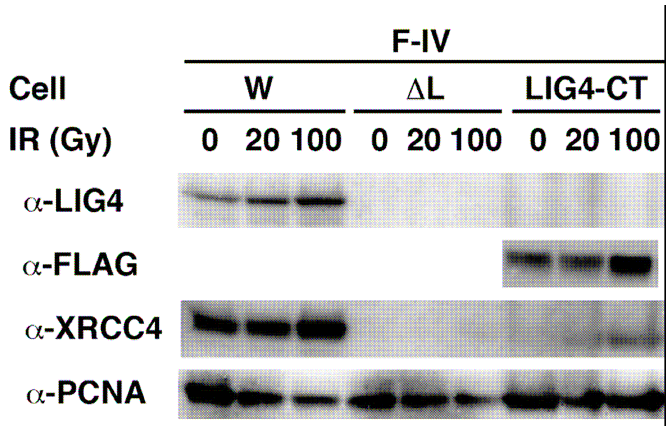

Fig.4 - 10*Chromatin binding of Ligase IV C-terminal fragment. The wild-type Ligase IV was detected by Ligase IV antibody, and the C-terminal fragment LigIV-CT was detected by FLAG antibody. The cells were irradiated with 0, 20 and 100 Gy dose.*

4-6BRCT domains possibly mediate repair response to DSB

I examined the chromatin binding of LigIV-CT in two BRCT mutations W7CT and W8CT. W8CT was expressing in a much reduced level, corresponding to W893R in Ligase IV expression. This fact indicated that BRCT2 stabilizes the Ligase IV C-terminal, as well as full length Ligase IV. Ligase IV might be targeted degradation through C-terminal, or BRCT2 mutation has changed the solubility of C-terminal.

Nevertheless, W7CT was expressing in a level comparable to LigIV-CT. Chromatin binding LigIV-CT presented in both W7CT and W8CT, though it was merely seen in W8CT. It is noticeable that the LigIV-CT did not increase after IR in both mutants, suggesting the response to DSB was diminished. Here we again could see chromatin binding signals in un-irradiated cells, but from the intensity of corresponding XRCC4 we think it was a small portion. The chromatin binding of LigIV-CT in un-irradiated cell could be inflicted by simultaneous DSBs generated in the cell, or it is possible that LigIV-CT can interact with other nuclear proteins which are also chromatin binding.

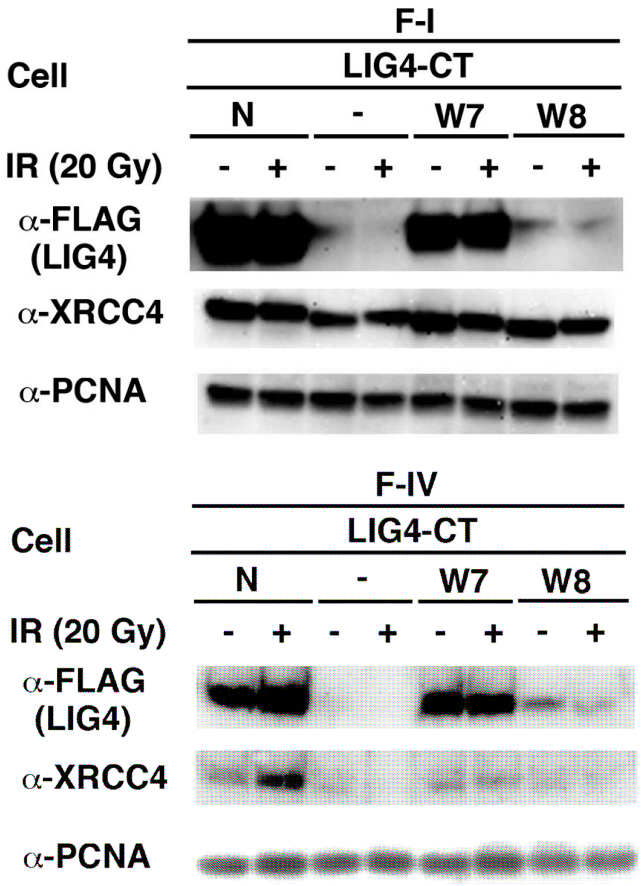

Fig.4 - 11*Chromatin binding of LigIV-CT mutants. W7CT and W8CT are two point mutated version of LigIV-CT, which only contains the C-terminal part of Ligase IV. The chromatin binding of XRCC4 was greatly reduced in these mutants.*

An immune-precipitation with anti-FLAG agarose was performed to evaluate the interaction between LigIV-CT mutants and XRCC4 partner. We could see that the interaction between LigIV-CT and XRCC4 was not disrupted by point mutation in BRCT domains with or without irradiation. LigIV-CT has high binding affinity with either unphosphorylated or phosphorylated XRCC4.

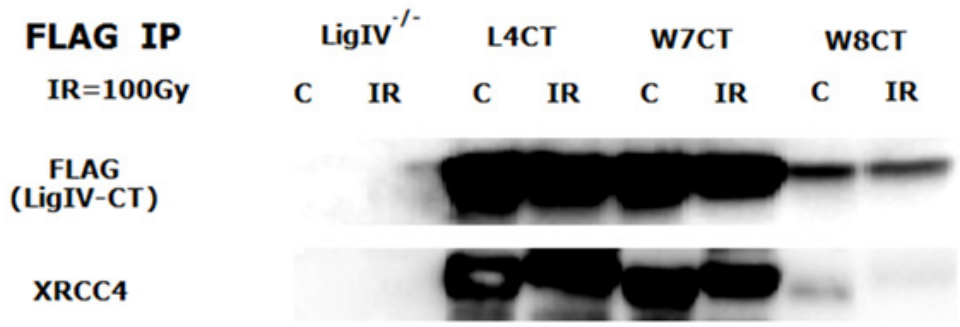

Fig.4 - 12*FLAG immunoprecipitation. Nalm-6 LigIV-CT cells were collected for irradiation and immunoprecipitation with FLAG-agarose. We FLAG-argarose was washed and treated with sample buffer. FLAG tagged LigIV-CT and XRCC4 was successfully immunoprecipitated.*

Based on these observations we suggest that the BRCT domains, more likely BRCT1, mediate repair response in Ligase IV. Disruption of BRCT domain possibly deactivatesthe DSB recognition of Ligase IV. The interaction is mediated by direct BRCT / DNA interaction or intermediate proteins are still unknown.

We have tried the silver staining with LigIV-CT after fractionation, but did not get promising results. XRCC4 band was overwhelmed by other binding protein in the chromosome. We have also tried FLAG immuno-precipitation, but either Ku70/86 or XLF was not co-precipitated with LigIV and XRCC4, both of which do have physical interaction with XRCC4. It suggests that immuno-precipitation condition was not suitable to study molecules interacting with LigIV-CT.

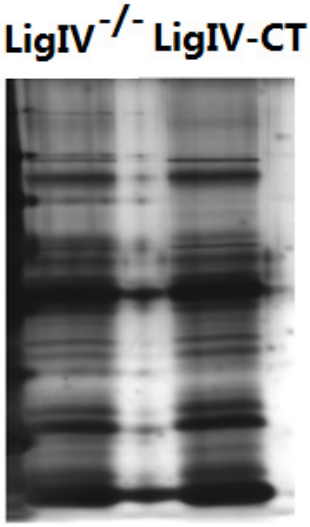

Fig.4 - 13*Silver staining 10^7 cells of LigIV$^{-/-}$ and LigIV-CT were first collected for NP-40 fractionation. The F-IV fraction after SDS-PAGE was silver stained.*

4-7Ligase IVhas two domains mediating the chromatin binding

Based on the chromatin binding characteristic of Ligase IV in non-mutated and mutated clones, we suppose Ligase IV has two distinct regions mediating the chromatin binding events.

One region lies in the N-terminal, which is widely known as DNA-binding domain. According to the structural similarity to other DNA ligases, the binding to DNA is sequence independent. This non-specific binding gives the explanation that though WT Nalm-6 cell has higher chromatin binding Ligase IV/ XRCC4 signals than LigIV transfected cells, they do not differ in DSB repair capability, or radiosensitivity examined by colony formation assay after irradiation. The non-specific binding amount relies on the total amount of proteins in the cell. When Ligase IV is up-regulated, it will be reflected also on the chromatin binding molecules. This binding model explains why there are considerable amount of chromatin binding Ligase IV even without irradiation. The

chromatin binding Ligase IV does not solely depend on the number of DSBs.

Another region lies in the C-terminal which has been explored in this chapter. An increase of chromatin binding LigIVsignal as well as XRCC4 was seen in non-mutated LigIV-CT cells after IR. LigIV-CT is thought to bind to DSB sites after DSB formation, and either BRCT domains, or at least BRCT1,wouldbe necessary for the recognition and recruitment of Ligase IV to DSBs. We still have no idea whether it is a direct interaction as Ku binding to DNA ends or indirect interaction as XLF recruited to DSBs by the help of Ku.

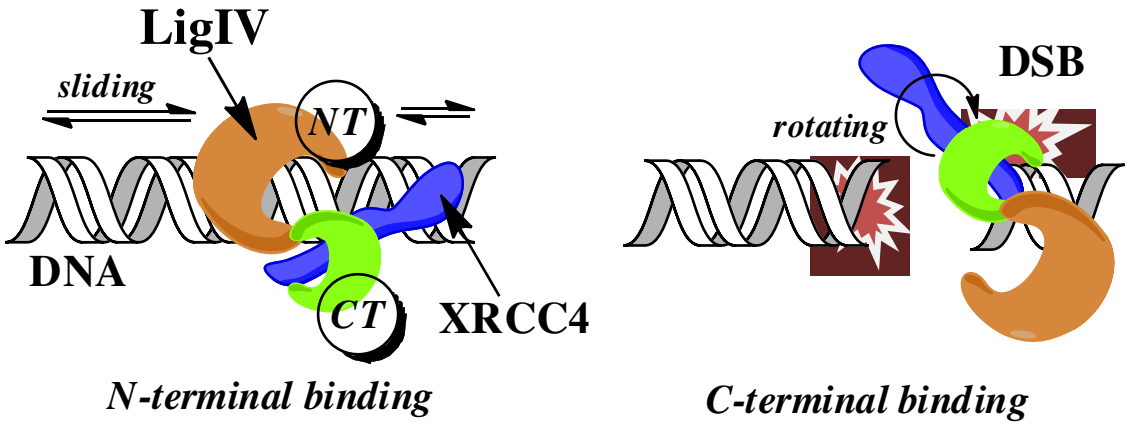

Fig.4 - 14*Two binding domains of Ligase IV*

According to theLigase IVbinding model, the N-terminal region which non-specifically binds to DNA with DBD domain, possibly allow sliding of Ligase IV to nearby by. The C-terminal region, on the other hand, specifically recognizes the DSB sites that need repair, and it will not allow horizontal moving on the DNA strand.

Presence of dual DNA binding domain would enable "hooking" another DNA end. It might be noted that a recent electron microscopic study showed the end bridging by

human or yeast Ligase IV / XRCC4 complex, engaging two Ku-bound DNA ends (Grob *et al.,* 2012). Alternatively, Ligase IV may move along DNA with DNA-binding domain and then anchor at DSB via C-terminal region.

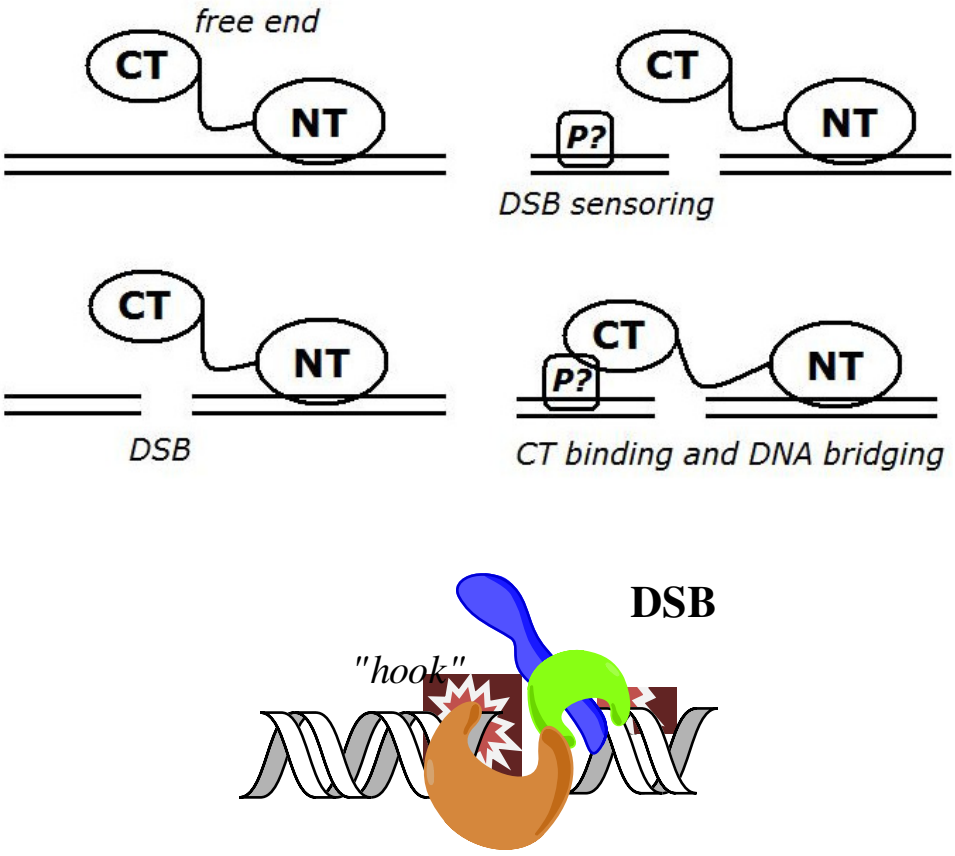

Fig.4 - 15*Ligase IV bridgestwo DNA ends*

C-terminal region seems to be more important for Ligase IVfunction. In case of W725R mutation, the function of N-terminal region isnot thought to be affected by disruption of BRCT1 domain. The N-terminal region can bind to DNA so that considerable chromatin binding Ligase IV was detected, in an amount much higher than W893R. The Ligase IV activity, however, was not rescued by the chromatin binding molecules, and both W725R

and W893R show similar radio-sensitivity.

The chromatin binding Ligase IV in W725R are considered as inactive Ligase IV, which cannot, or not efficiently carry on end joining task. We suppose that W725R is capable of N-terminal binding, but not C-terminal binding. While in W893R, due to a low expression level, both binding have been reduced.

Ligase IV gene	N-terminal binding activity	C-terminal binding activity	Overall activity
WT	++	+	+
def	X	X	X
LigIV	+	+	+
R278H	+	+	-
W725R	+	-	-
W893R	-	-	-
LigIV-CT	X	+	X

The Ligase IV detected in F-IV is a mixture of both N-terminal binding and C-terminal binding molecules that cannot be separated. An increase of binding Ligase IV contributes to an increase of C-terminal binding. The N-terminal binding is rather served as “background”. In LigIV-CT mutants, we deleted the N-terminal, so what we see is the C-terminal binding molecules. It can better reflect the number of DSBs in the cell.

4-8Interaction between Ligase IV C-terminal fragment and DSB sites

Known that Ligase IV C-terminal is activated by DSBs and recruits itself to DSB site, the next addressed question is that how this event happen. There are three domains in LigIV-CT, BRCT1, XIR and BRCT2 respectively. As mutation in either BRCT domain

greatly reduces Ligase IV activity, both BRCT domains seems to be involved in this event.

BRCT domain is shown to be a phosphopeptide binding module (Manke *et al.,* 2003)(Yu *et al.,* 2003). ATM and DNA-PKcs are thought to phosphorylate a number of proteins,

including histone H2AX, in response to DNA damage, phosphorylated H2AX or other protein might be a bait of BRCT domains in Ligase IV. If this is the case, it is expected that depletion of ATM and/or DNA-PKcs would reduce chromatin binding of Ligase IV / XRCC4/ complex. However, we and others demonstrated that the chromatin binding of XRCC4 was not affected by wortmannin, a potent inhibitor of both of ATM and DNA-PK (Drouet *et al.,* 2005; Kamdar *and* Matsumoto, 2010). As another possibility, it was reported that Ligase IV interacts with Ku via BRCT1 (Costantini *et al.,* 2007). In the present study, however, W725R mutation in BRCT1 did not affect the chromatin binding of LigIV-CT but the chromatin binding of XRCC4 was much reduced. It might be expected that the interaction between LigIV-CT and XRCC4 is weakened by W725R mutation. However, the interaction between XRCC4 with W725R mutant and non-mutated LigIV-CT was similar in co-immunoprecipitation experiments. Considering this, BRCT1 might have a role in stabilizing XRCC4 on chromatin. XRCC4 can then interact with XLF. Nevertheless, as we introduced mutation in putative phosphopeptide binding pocket, it remains possible that another interface of BRCT1 mediates the interaction between Ligase IV and Ku. It may appear contradictory that the chromatin binding of Ligase IV was considerably reduced by W725R mutation in the full-length context, but not in the C-terminal context. In this regard, we would note that an electron microscopy study suggested the juxtaposition of LigIV-NT to XRCC4 (Recuero-Checa *et*

al., 2009). Although it is currently unclear whether there is a direct, physical interaction between LigIV-NT and XRCC4, possible tertiary interaction among LigIV-CT, XRCC4 and LigIV-NT, which would be lost in W725R mutant, might firm the complex.

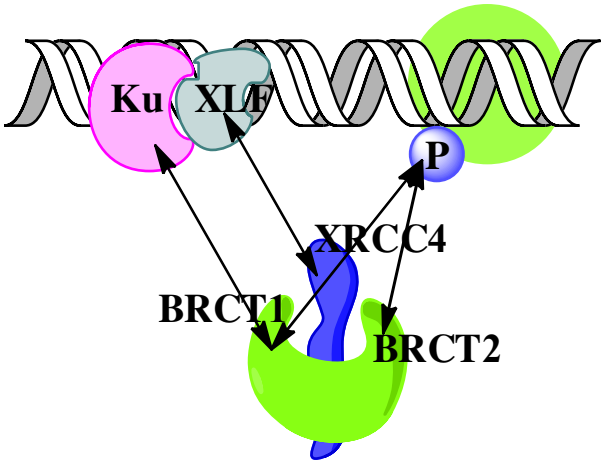

Fig.4 - 16*Possible binding model ofLigIV-CT*

Together with the work of LigIV-CT, we also created other Ligase IV fragment to verify the DNA binding model, especially the LigIV-NT we mentioned above. The vectors were successfully created. However, we failed to get stable colonies by transfection. The deletion of BRCT2 domain as well as disruption of Ligase IV and XRCC4 interaction greatly destabilized Ligase IV, which might be a reason for the failure.

CHAPTER 5

CONTENTS PROTECTED

CHAPTER 6

Discussion and Conclusions

6-1 Conclusions

In the Ligase IV research, I have successfully created and examined two novel BRCT mutations W725R and W893R in cell radiosensitivity and chromatin binding of NHEJ molecules. The results fit my prediction that the highly conserved sites are critical for the function of Ligase IV. It explains why during long time of evolution the BRCT domains are highly conserved. I proposed a two domain binding model for Ligase IV and I have shown the C-terminal domain is involved in DSB responseby the fact that C-terminal of Ligase IV successfully recruits itself and XRCC4 to chromatin after IR induced DSB, and Ligase IV C-terminal fragment successfully sensitized HeLa-Fucci cell after IR. From the result that either BRCT mutation cause similar level of radiosensitivity, I think they might together participate in DSB repair as an interacting domain to some repair molecules, which have not been identified yet.

6-2 Reduced expression level in Ligase IV BRCT2 mutation

In the study of Ligase IV and Ligase IV C-terminal, I found both LigIV-W893R and LigIV-CT-W893R were expressing at a low level. I have attempted many times to get a higher expressing colony but ended with failure, so I think this is not just a coincidence.It has been reported that patient with R814X mutation also has a low Ligase IV expression level(Girard *et al.,* 2004). According to this research, R814X is less than 10% expressed in nucleus and has a Ligase IV activity as low as 10%. My results showed that W893R exhibited exactly similar phenomenon as this R814X mutation, both low expression level and low repair ability. The Ligase IV defect caused by R814X is similar to the changes in highly conserved W893 site of BRCT2. The very C-terminal region is possibly mediating

some interaction to stabilize Ligase IV, which is not known yet.

In the study of LigIV-CT mutation with FLAG immunoprecipitation, low binding affinity between W8CT and XRCC4 has been confirmed, but not W7CT.The entire BRCT2 domain is possibly mediating the interaction with XRCC4, not only a small region which has been reported before.

6-3 Overview to the future

I have successfully explored the role of DNA Ligase IV in the recruitment of NHEJ repair molecules through my work. I have proved that Ligase IV but not XLF is necessary for the chromatin binding of XRCC4, but XRCC4 can also be recruited at the extreme high dose. This kind of binding appears trivial at low lose, but it is amplified with the extreme high dose. So far almost all the NHEJ repair work are done with a dose not exceeding 20Gy. The high dose response seems an interesting new field for future research.

Next, by FLAG immunoprecipitation I have shown that W725R mutation did not alter the interaction with XRCC4, which is correspond with previous data that only XIR and BRCT2 domain are necessary for XRCC4 interaction. It is still not clear why chromatin binding of Ligase IV was reduced in W725R mutant. Somehow, the chromatin binding of XRCC4 seems to be more reduced than Ligase IV and it was also suggested by the C-terminal fragment mutation W7CT. We have not explored the mechanism how NHEJ molecules leave DSB sites after repair. Before that, maybe we need to know XRCC4 and Ligase IV, which one leaves chromatin first, and how?

Third, I found Ligase IV C-terminal is capable to drive Ligase IV as well as XRCC4 to chromatin. The dose dependent LigIV-CT chromatin binding shows a DSB activation

of the C-terminal. The BRCT domains are evolved from DNA binding motif to phosphorylation protein binding motif (Leung *and* Glover, 2011). I suspect the Ligase IV BRCT domains participate in protein-protein interaction, and phosphorylated histone γ-H2A, γ-H2AX or even phosphorylated XRCC4 could be a target.

The highly conserved W725 and W893 sites are critical for normal function of Ligase IV. R278H, W725R, W893R all give a similar defect of Ligase IV, leading to elevated radio sensitivity. So far we have not yet established any connection between these three mutants. We know R278H mutation leads to impaired ability to form an adenylate complex as well as to rejoin DNA (Riballo *et al.,* 2001), reducing Ligase IV activity to 10%. Also R814X mutation significantly impairs adenylate complex formation (Girard *et al.,* 2004). Is it possible that W725R and W893R also have reduced Ligase IV adenylation activity?

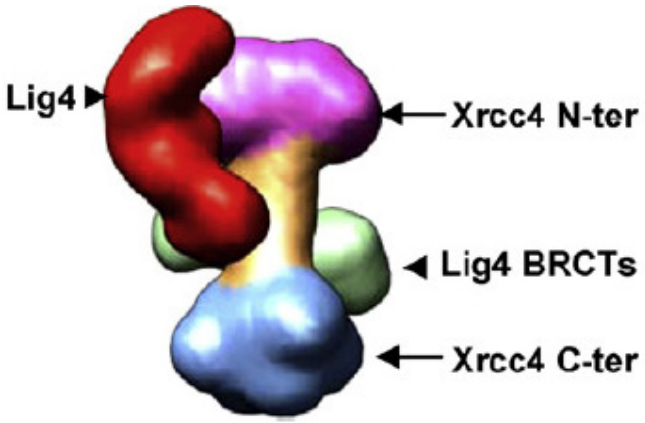

Fig.6 - 1*Model of Ligase IV / XRCC4 complex* (Recuero-Checa *et al.,* 2009)

There is an interesting model of NHEJ repair complex. The XRCC4 N-terminal is very close to the Ligase IV N-terminal. R278H is mutated in the adenylation pocket which lies

in the N-terminal, and resulting 10% adenylation activity to normal Ligase IV(Girard *et al.,* 2004). Is it possible that XRCC4 has some relation with Ligase IV adenylation? Mutation in either BRCT domain could possibly affect the orientation of XRCC4 and thus make the N-terminal head of XRCC4 may collide into the functional N-terminal region of Ligase IV.

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