SYNOPSIS

To counteract the potentially calamitous effects of genomic instability in the form of double-strand breaks (DSBs), cells have evolved with two major mechanisms. First, DNA non-homologous end joining (NHEJ) which requires no significant homology, and second, homologous recombination (HR) that uses intact sequences on the sister chromatid or homologous chromosome as a template to repair the broken DNA. Although NHEJ repairs DSBs in all stages of cell cycle; it is generally error-prone due to insertions or deletions of few nucleotides at the breakpoint. In contrast, DSBs that are generated during S and G2 phase of the cell are preferentially repaired by HR that utilizes neighboring sister chromatid as a template. A central role in the HR reaction is promoted by the RAD51 recombinase which polymerizes onto single-stranded DNA (ssDNA), catalyzes pairing and strand invasion with homologous DNA molecule. Assembly of RAD51 monomers onto ssDNA is a relatively slow process and is facilitated by several mediator proteins. The tumor suppressor protein BRCA2 is the best-characterized RAD51 mediator in DSB repair by HR. Many reports in the past two decades have established that RAD51 recruitment at break sites also depends on the RAD51 paralogs.

Mammalian cells encode five RAD51 paralogs; RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3 which share 20–30% identity at amino acid level with RAD51 and with each other. In addition to their role in HR, RAD51 paralogs have been identified to be involved in DNA damage signaling and replication fork maintenance. In addition, mouse knockout of RAD51 paralogs causes early embryonic lethality. Recent studies show that germline mutations in all five *RAD51* paralogs cause various types of cancer including breast and ovarian cancers. Pedigree analyses revealed that similar to *BRCA1* and *BRCA2*, pathological missense mutants of *RAD51C* were of high penetrance. Historically, defects in the DNA repair pathways have been exploited for cancer chemo- and radiotherapy. In an attempt to develop better cancer therapeutic approaches, the concept of synthetic lethality for cancer therapy has been recently proposed. One such example is the use of PARP1 inhibitors to treat tumors carrying mutations in HR genes, such as *BRCA1* and *BRCA2*. Inhibition of PARP1 compromises single-strand break repair

(SSBR) pathway. Upon replication fork collision, the accumulated SSBs are converted to one-ended DSBs, which are efficiently repaired by the HR for cell survival. As a result, HR-deficient tumors with BRCA1- or BRCA2- deficiency exhibit extreme sensitivity to PARP-1 inhibition resulting in cell death. This approach was highly successful in targeting tumors with severe defects in Fanconi anemia (FA)-BRCA proteins which led to PARP inhibitors being tested in clinical trials. However, targeting cancer cells that express hypomorphic mutants of HR proteins is highly challenging since such partially functional mutants require a high dosage of PARP inhibitors for effective sensitization which renders normal cells toxic and can also lead to tumor resistance.

The pathological RAD51C mutants that were identified in breast and ovarian cancer patients are hypomorphic with partial repair function. The first part of my Ph.D. thesis is aimed at developing an effective strategy to target cells that express hypomorphic RAD51C mutants. To this end, we used RAD51C deficient CL-V4B hamster cells and expressed the pathological RAD51C mutants associated with breast and ovarian cancers. Cells expressing RAD51C mutants that were severely defective for HR function exhibited high sensitivity to low doses of PARP1 inhibitor (4-ANI). These cells also accumulated in G2/M and displayed chromosomal aberrations. However, RAD51C mutants that were hypomorphic were partially sensitized even at higher concentrations of PARP inhibitor. RAD51C$^{-/-}$ CL-V4B cells displayed higher PARP activity compared WT V79B cells. Notably, PARP activity was directly proportional to the sensitivity of RAD51C mutants towards 4-ANI where highly sensitive RAD51C mutants showed higher PARP activity and vice versa. Increased PARP activity was associated with replication stress as confirmed by an increase of PARP activity in cells treated with replication stress inducer, hydroxyurea (HU). Notably, treatment of CL-V4B cells with PARP1 inhibitor (4-ANI) resulted in the accumulation of PARP1 onto the chromatin which eventually led to the formation of DSBs which suggests that PARP1 entrapment triggers replication fork collapse leading to one-ended DSBs in S-phase.

To further understand the molecular mechanism of PARP inhibitor-induced toxicity of RAD51C deficient cells, we carried out chromatin fractionation from V79B and CL-V4B cells at

varying time points of 4-ANI treatment. Surprisingly, there was an enhanced loading of NHEJ proteins on chromatin in CL-V4B compared to V79B cells. Consistently, an increased error-prone NHEJ was observed in CL-V4B cells which resulted in increased chromosomal aberrations and cell death. Furthermore, inhibition of DNA-PKcs or depletion of KU70 or Ligase IV restored this phenotype. Thus, error-prone NHEJ in collaboration with PARP inhibition sensitizes RAD51C deficient cells. Since ionizing radiation (IR) is known to stimulate NHEJ activity, we hypothesized that irradiation in combination with PARP inhibitor would further sensitize the RAD51C deficient tumors. Strikingly, stimulation of NHEJ by a low dose of IR in the PARP inhibitor-treated RAD51C deficient cells and cells expressing pathological RAD51C mutants induced enhanced toxicity 'synergistically'. These results demonstrate that cancer cells arising due to hypomorphic mutations in RAD51C can be specifically targeted by a 'synergistic approach' and imply that this strategy can be potentially applied to cancers with hypomorphic mutations in other HR pathway genes.

In addition to nuclear functions, RAD51 paralogs RAD51C and XRCC3 have been shown to localize to mitochondria and contribute to mitochondrial genome stability. However, the molecular mechanism by which RAD51 and RAD51 paralogs carry out this function is unclear. The second part of my thesis was dedicated to studying whether RAD51C/XRCC3 facilitates mitochondrial DNA replication and the underlying mechanism by which RAD51C/XRCC3 participate in mitochondrial genome maintenance during unperturbed conditions. Using mitochondrial subfractionation we show that RAD51 and RAD51 paralogs (RAD51C and XRCC3) are an integral part of mitochondrial nucleoid and absence of RAD51C/XRCC3 and RAD51 prevents the restoration of mtDNA upon depletion of mtDNA. This suggests that RAD51 and RAD5C/XRCC3 participate in mtDNA replication. To determine whether this function of RAD51C is exclusive to mitochondria we expressed NLS mutant of RAD51C which was defective for nuclear functions. Interestingly, cells expressing RAD51C R366Q were able to efficiently repopulate the depleted mtDNA after EtBr stress similar to that of WT RAD51C expressing cells, suggesting a nuclear independent function of RAD51C in mitochondrial genome maintenance. mtDNA-IP analysis revealed that RAD51 and RAD51C/XRCC3 are recruited to the mtDNA control regions spontaneously along with mitochondrial polymerase POLG. Moreover, RAD51 was

found to associate with TWINKLE helicase and this association was required for the recruitment of RAD51 and RAD51C/XRCC3 at the D-loop.

As in nucleus, mtDNA replisome also encounters replication stresses like altered dNTP pools, a collision between replication and transcription machinery, rNTP incorporation, oxidative stress which hampers replication fork progression. Using Dideoxycytidine (ddC) as replication stress inducer in mitochondria, we observed nearly 3-4 fold enrichment of RAD51, RAD51C, XRCC3 and POLG at the mtDNA mutation hotspot region D310. Notably, RAD51C/XRCC3 deficient cells exhibited increased lesions in the mitochondrial genome spontaneously, pointing towards the importance of RAD51C/XRCC3 in the prevention of mtDNA lesions. Moreover, RAD51C/XRCC3 deficiency prevented the repair of ddC induced mtDNA lesions. Given that RAD51C/XRCC3 and RAD51 are localized to mtDNA control regions along with POLG and their deficiency affects mtDNA replication we were curious to learn the effect of RAD51C/XRCC3 deficiency on the recruitment of POLG in mtDNA. To test this we performed a mtDNA-IP assay of POLG in RAD51C deficient cells which revealed that deficiency of RAD51C/XRCC3 and RAD51 affected the recruitment of POLG on mtDNA control regions. As a consequence RAD51C/XRCC3 deficient cells exhibit aberrant mitochondrial functions. These findings propose a mechanism for a direct role of RAD51C/XRCC3 in maintaining the mtDNA integrity under replication stress conditions.