DNA REPAIR PATHWAYS INVOLVED IN THE FORMATION OF ANAPHASE BRIDGES

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Chromosomal alterations can arise from numerous events, including errors during cell division or repair of damaged DNA. Of these errors, segregational defects such as anaphase bridges and multipolar spindles play a major role in chromosomal instability, leading to tumorigenesis.

Bridges can theoretically be produced by several mechanisms including telomere-telomere fusion, persistence of chromatid cohesion into anaphase or repair of broken DNA ends. DNA damage can induce anaphase bridges following exposure to agents such as hydrogen peroxide or ionizing radiation (IR). Our hypothesis is that while the majority of double strand breaks (DSBs) are repaired, to restore the original chromosome structure, incorrect fusion events also occur leading to bridging and that bridge formation allows cells to bypass the apoptotic pathways that are activated in response to DNA damage. To test this, we set out to determine what pathways the cells use to heal the damage and form bridges. Our data suggest that neither of the two major pathways used by the cell for repair of double strand breaks, homologous recombination (HR) and non-homologous end joining (NHEJ), is required for bridge formation. In fact, the NHEJ pathway seems to play a role in the prevention of bridges. When NHEJ is compromised, the cell appears to use HR to repair the break, resulting in increased anaphase bridge formation. Moreover, intrinsic NHEJ activity of different cell lines appears to be

correlated with induction of bridges from DNA damage. Our preliminary data also suggest that cell lines with high levels of bridging are capable of apoptosis, yet further experiments are required to test if blocking bridging can enhance cell death.

Multipolar spindles are aberrant mitotic figures that occur when a cell divides with two or more poles, which can lead to uneven segregation of the chromosomes. In our studies, we found that IR treatment can lead to an increase in multipolarity shortly after treatment and changes the distribution of spindle pole components. Initial observations on the splitting of centrosomal proteins following IR treatment are presented.

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PREFACE

FOR MY DEAR FAMILY

Melek Açılan

Muharrem Açılan

Burak Açılan

First and foremost, I would like to thank my thesis advisor, Dr. William Saunders, for accepting me in his lab and giving me such a wonderful project to work on. He has guided me through tough times with many helpful suggestions and endless discussions. I appreciate all the insights he has given me in science and his encouragements in my personal life.

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1.0 CHAPTER I: INTRODUCTION

1.1 CONCEPTS IN GENOMIC INSTABILITY

Cancer is a result of accumulation of mutations that control cell division or cell death. It is widely accepted that a cell has to undergo multiple mutations before it becomes tumorogenic (Lengauer et al., 1997). Hence, events speeding up the mutation rate or which interfere with the stability of the genome are likely to give the some cells a growth advantage, which could potentially proceed to cancer development. Some of these events that may contribute to destabilization of the genome are described below:

Changes in nucleotide sequence: These are alterations in the DNA sequence by substitution, addition or deletion of nucleotides. Examples include mutations in c-K-Ras, which are found in most pancreatic cancers (Almoguera et al., 1988), BRCA1/2 genes, which are associated with 30-50% of the heritable breast cancer cases (Rahman and Stratton 1998; Nathanson et al., 2001), or the well-studied p53 gene, which is defective in 50% of all human cancers (Hollstein et al., 1991; Hainaut and Hollstein 2000; Soussi and Beroud 2001).

Changes in chromosome number: This can be described as the gain or loss of an entire chromosome, which leads to the state of aneuploidy. Aneuploidy is known to be associated with almost all cancer types (Mitelman 1983) and its severity is correlated with the stage of tumorogenesis (Cavalli et al., 1989).

Translocations: A translocation is defined as the exchange of DNA material between non homologous chromosomes. Translocations may create fusion of genes with a change in activity of hybrid sequences. One famous example includes the Philadelphia chromosome in chronic myeloid leukemia, where the nuclear protein Bcr is translocationally juxtaposed to the Abl kinase, producing a hybrid Bcr-Abl protein with increased kinase activity/localization (Ben-Neriah et al., 1986; Jeffs et al., 1998). Likewise, in Burkitt's lymphoma, the myc oncogene is translocated to the immunoglobin promoter leading to enhanced expression of Myc in lymphocytes (Dean et al., 1983).

Amplifications: Gene amplifications involve an increase in the copy number of a proto-oncogene, which may lead to over production of the associated gene product and drive the cell to an abnormal growth stage. For instance, amplification of the N-Myc oncogene has been reported in 30% of neuroblastomas (Seeger et al., 1985).

In normal cells, the biological processes preventing these changes are intact, thus they occur rarely, if ever. On the other hand, all of these events are readily observed in different cancer types, and are tightly linked to genomic instability. For example, failure in mismatch repair system (MMR) has been shown to elevate the rate of DNA mutation by ~1000-fold

(Christmann et al., 2003) and results in the destabilization of simple repeat sequences. This phenomenon, which is known as microsattelite instability (MIN), is found in most hereditary non-polyposis colon cancers (HNPCC) (Aaltonen et al., 1993), and sporadic colon cancers (Narayan and Roy 2003). Phenotypically, MIN cannot be detected by karyotypic analysis and does not involve the large scale genomic changes discussed above.

The other major form of instability involves continuous gross alterations in chromosomal structure and number, which is defined as chromosomal instability (CIN). While experimental data support a direct link for MIN and carcinogenesis, the link between CIN and cancer is ambiguous. Whilst it is clear that many tumor types exhibit the CIN phenotype (Lengauer et al., 1997; Lengauer et al., 1998), there is a chicken-and-egg dilemma as to whether CIN is an early event leading to cancer or an outcome of malignant transformation. Despite the modeling studies, which indicate that CIN alone is sufficient to drive the cell into tumorogenesis (Nowak et al., 2002), other studies challenge this hypothesis with the evidence of adenomas without any obvious changes in CIN (Haigis et al., 2002). Regardless, CIN is a hallmark of cancer and studying the molecular events leading to chromosomal instability will enlighten our understanding of it.

1.2 SOURCES OF CHROMOSOMAL INSTABILITY

Although there are numerous events that may lead to CIN, the main reasons can be listed as defects in chromosomal segregation, repair of DNA damage and cell-cycle checkpoint regulation. Seemingly diverse at first, these events are actually very closely intertwined. The

different defects that lead to CIN usually coexist in tumor cell lines perhaps cooperatively contributing to the multistep tumorogenesis process (Saunders et al., 2000; Jallepalli and Lengauer 2001; Gisselsson 2003; Deng 2006). For instance, incompetency to repair a checkpoint gene might allow mitosis despite incorrect chromosome congression. Experimental evidence from BRCA2 deficient mice show that these mice which harbor mutations in spindle checkpoint proteins (Bub1, BubR1) exhibit higher incidences of lymphomas (Lee et al., 1999). BRCA2 is also reported to interact with the mitotic kinase PLK1 (Lee et al., 2004) and the checkpoint kinase BubR1 (Futamura et al., 2000) indicating a tie between DNA repair and checkpoint proteins.

While the links between DNA repair/checkpoint regulation and chromosomal segregation/checkpoint regulation are well studied, it is less clear how errors in the repair of DNA damage can lead to segregation defects. In this dissertation, evidence for the molecular mechanisms regarding this connection will be presented. Below, I will start by discussing the commonly observed segregation defects in cancer cells.

1.2.1 Chromosome Segregation Defects

The major segregation defects visible in cancer cells can be summarized as lagging chromosomes, micronuclei, anaphase bridges and multipolar mitoses.

1.2.1.1 Lagging Chromosomes

Lagging chromosomes can be defined as chromosomes or fragments of chromosomes, which fail to align properly at the metaphase plate or those that lag behind the separating

chromosomal masses during anaphase (Figure 1). Lagging chromosomes have been shown to emerge as a consequence of failure in attachment of the spindle microtubules to the kinetochore proteins (Dulout and Olivero 1984) or by attachment to a single spindle pole (merotelic attachment) (Cimini et al., 2001; Cimini et al., 2002). It has also been uncovered in the Saunders lab that resolution of anaphase bridges may also result in lagging chromosomes as pieces of chromosomal material (Hoffelder et al., 2004). In any case, the daughter cells are under the risk of losing a chromosome or having both copies of the sister chromatids, which contribute to CIN.

1.2.1.2 Micronuclei

Micronuclei are derived from whole chromosomes or chromosomal fragments, which have a nuclear envelope and are anywhere from 1/16 to 1/3 of the size of the main nucleus (Fenech 1993). Micronuclei are distinct from the nucleus, yet remain in the same cell (Figure 2). While the major reason for micronuclei formation appears to be due to lagging chromosomes, breakage of an anaphase bridge has also been shown to result in micronuclei in 50% of divisions with a bridge (Hoffelder et al., 2004). Micronuclei occur frequently upon treatment with DNA damaging reagents and have been proposed as a diagnostic tool to test for chemotherapy damage (Driessens et al., 2003).

Once a micronucleus forms, many scenarios are possible for its fate. It may be excluded from the cell, reincorporated into the main nucleus or function separately in the cell's cytoplasm (Leach and Jackson-Cook 2004). In the first situation, the cell would face the threat of losing chromosomal material, resulting in aneuploidy. In the second scenario, the micronuclear mass might potentially continue normal biological activity. In the third case, micronuclei do not

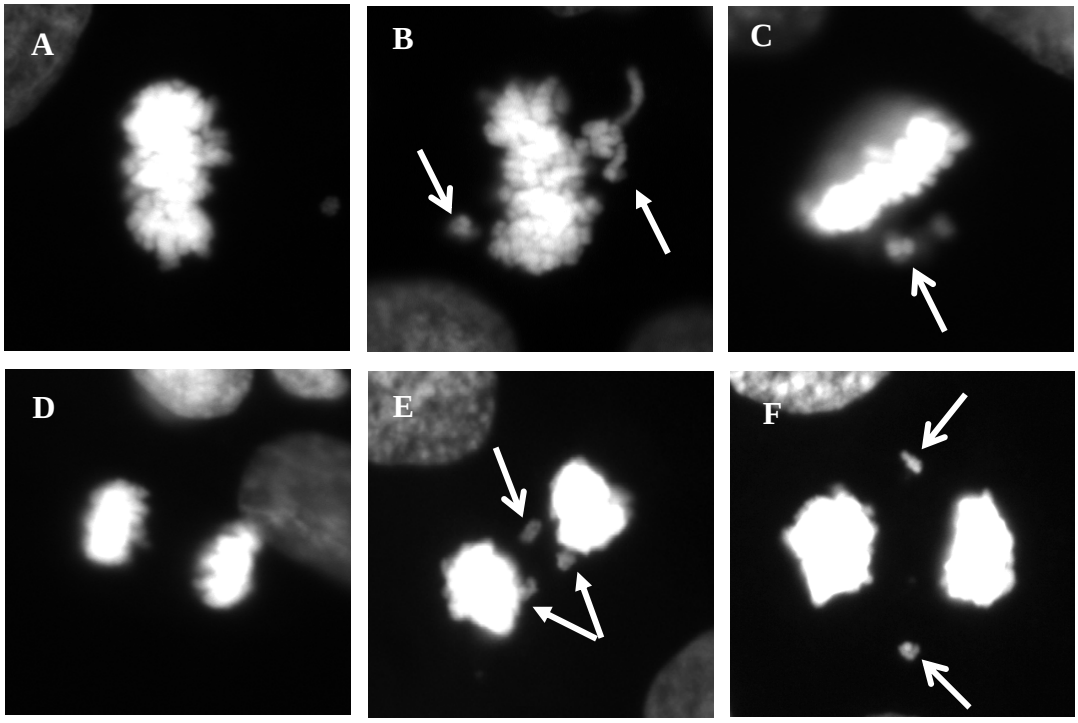


Figure 1 UPCI:SCC103 cells dividing normally in metaphase (A) or anaphase (D). Examples of cells in corresponding stages with lagging chromosomes are indicated by arrows (B, C, E, and F). DAPI is used for DNA staining and images are taken with an Olympus camera with 100X magnification lens.

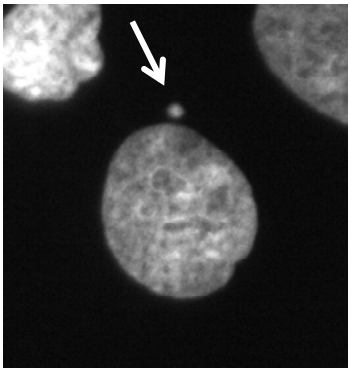


Figure 2 Example of a cell with a micronucleus (shown by arrow) in UPCI:SCC103 cell line. DAPI is used for DNA staining and images are taken with an Olympus camera with 100X magnification lens. (Image: Courtesy of Qian Wu.)

appear to be fully transcriptionally active as they are incapable of nucleotide incorporation and contain reduced numbers of nuclear pore complexes, which could potentially limit regular transport (Hoffelder et al., 2004).

As described above, both lagging chromosomes and micronuclei could lead to genomic instability if not corrected. Fortunately, these defects can trigger checkpoint proteins to pause mitosis until all chromosomes are properly attached to both poles. There is evidence that merotelic attachments may be corrected even prior to anaphase (Salmon et al., 2005; Cimini et al., 2006). On the other hand, there is no indication of activation of checkpoints in cases of anaphase bridging or formation of multipolar spindles. Hence these defects are potentially more dangerous as will be discussed further.

1.3 ANAPHASE BRIDGES

Anaphase bridging is a commonly observed phenomenon in cancer cells, which can be described as a chromatin fiber connecting the two chromosome masses together (Gisselsson et al., 2000) (Figure 3B). This abnormality was first described by Barbara McClintock in 1941 (McClintock 1942) and since then, it has been shown that bridges lead to structural and numerical chromosome changes which are strongly linked to tumorogenesis (Artandi et al., 2000; Stewenius et al., 2005). Furthermore, anaphase bridges have major contributions to CIN and have been reported in both cell culture (Gisselsson et al., 2000) and tissues (Montgomery et al., 2003).

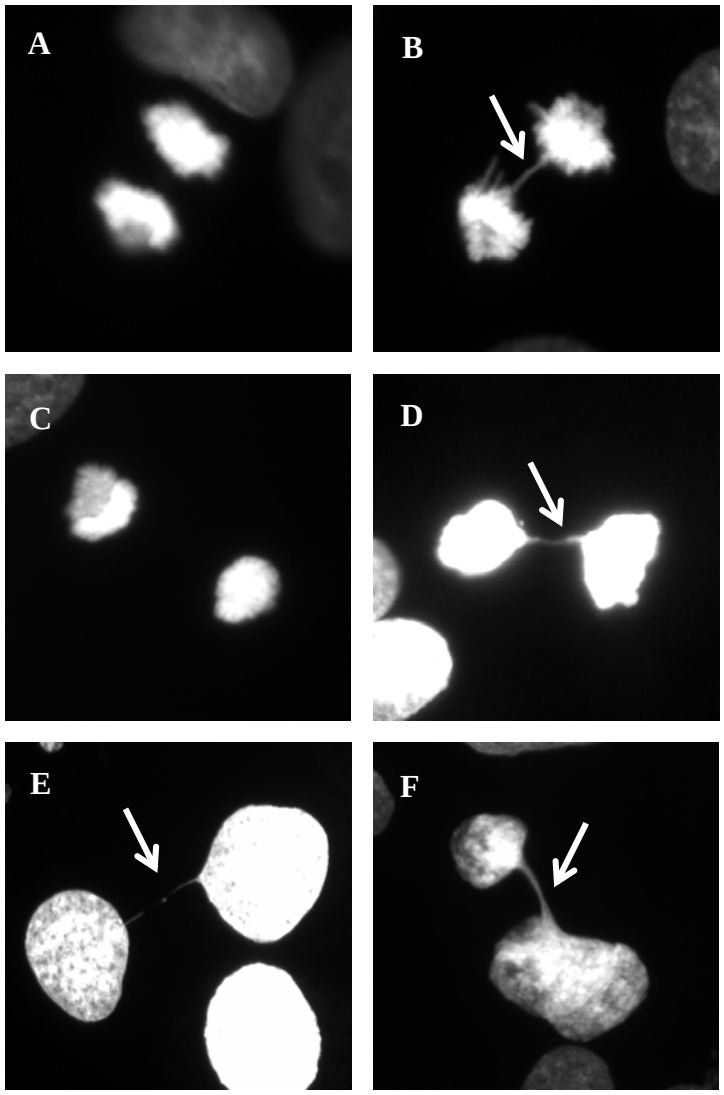


Figure 3 Examples of cells with bridges in UPCI:SCC103 cell line: normal anaphase (A), anaphase with a bridge (B). Bridges are usually resolved by telophase (C), however can sometimes persist to telophase (D) or interphase (E) and (F). Arrows indicate bridges. DAPI is used for DNA staining and images are taken with an Olympus camera with 100X magnification lens.

A bridge is thought to form from the fusion of two broken DNA ends, or as a result of shortened or absent telomere sequences (Fouladi et al., 2000; Gisselsson et al., 2001; Zhu et al., 2002). Telomeres are composed of tandem (TTAGGG) repeats at the end of the chromosomes, which span a region of 5-15 kb (Moyzis et al., 1988; de Lange et al., 1990). They form a specialized structure called a t-loop, and are stabilized by many telomeric proteins (Callen and Surralles 2004), which may either directly bind to telomeres or have a regulatory role in telomere protection. Some of these proteins have a dual function in both telomere maintenance and DNA damage repair, indicating a cross talk between telomeres and the DNA damage pathways (Nugent et al., 1998; Surralles et al., 2004). An average of 65 bp of telomeric sequence tends to be lost with each division in cells lacking telomerase or alternative ways to stabilize telomeres (Counter et al., 1992). Once the shortening process starts, the t-loop can no longer form and the protective proteins detach from the telomeres. In this case, the chromosome ends can act as free DNA breaks and may be fused to other ends with short telomeres. Supporting this, anaphase bridges have been shown to arise from telomere shortening, loss of telomeres or defects in telomerase (Artandi et al., 2000; Fouladi et al., 2000; Gisselsson et al., 2001; Rudolph et al., 2001; Lo et al., 2002). Moreover, there is a perfect correlation between telomere length in cancer cells and the frequency of endogenous bridges (Stewenius et al., 2005).

Telomeres might alternatively be lost with the occurrence of a DNA double stranded break (DSB) in the chromosome. This can also lead to a bridge, yet not necessarily through telomere-telomere fusions. How bridges form through the repair of double stranded breaks is the

main focus of this report. This gains particular importance, since many cancer cells exhibit stabilized telomeres.

1.3.1 Double Stranded DNA Repair Mechanisms

Cells are subjected to a number of DNA damaging events, threatening the genomic integrity. This damage can be induced by various agents, including endogenous factors such as free radicals formed as byproducts of metabolic functions, or stalled replication forks or exogenous factors such as ionizing radiation or mutagenic chemicals. Among the most severe lesions caused by these factors, DNA double stranded breaks (DSBs) are probably the most difficult to repair. Upon formation of a DSB, generally, the original ends are fused back together, restoring the chromosome's original structure (Rief and Lobrich 2002). However, misligation of two non-matching ends may also occur, resulting in an anaphase bridge and chromosomal instability (Mills et al., 2003; Pfeiffer et al., 2004).

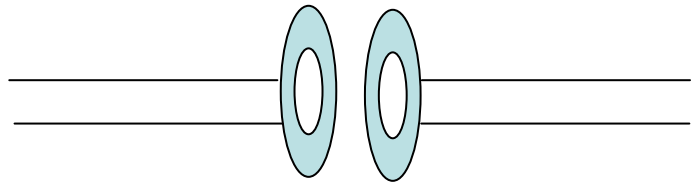
In order to deal with double stranded DNA breaks, mammalian cells have two major repair pathways: Non-homologous end joining (NHEJ) and homologous recombination (HR). While NHEJ requires little or no homology to fuse the broken ends, HR-dependent repair utilizes the homologous strand as a template. These mechanisms will be discussed further below:

1.3.1.1 Non Homologous End Joining (NHEJ)

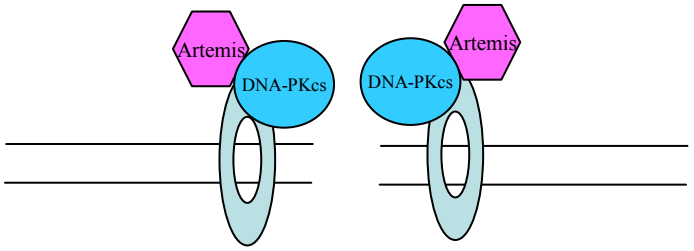
NHEJ is initiated by the binding of Ku proteins to the broken ends of the DNA (Smith and Jackson 1999) (Figure 4B). The Ku heterodimer, Ku80/70, which is composed of two subunits of molecular weight 86 and 73kDa, forms a hollow ring like structure large enough to

A) Double strand break

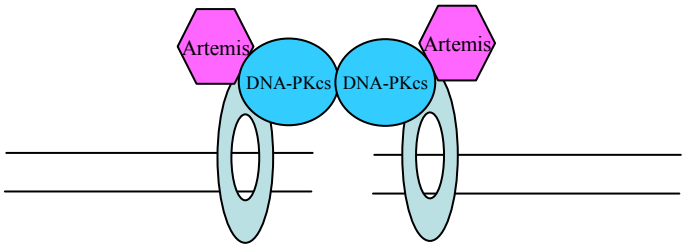
B) End Binding by Ku70/80



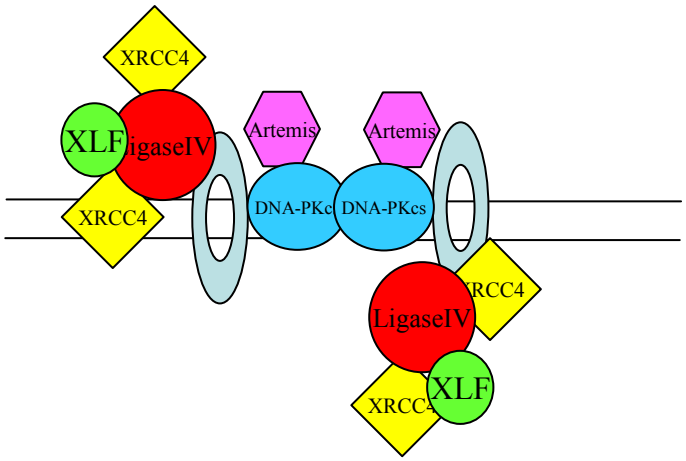
C) Recruitment of DNA-PKcs and Artemis



D) End-Bridging by DNA-PK and Phosphorylation of Artemis



E) Recruitment of Ligase IV/XRCC4/XLF



F) Ligation

Figure 4 NHEJ in mammalian systems. Upon formation of a DSB (A), Ku70/80 proteins are recruited to the damage site (B). The Ku heterodimer recruits DNA-PKcs, which is thought to mediate end-bridging. DNA-PKcs both phosphorylates and forms a complex with Artemis and this complex is involved in processing the 5' and 3' DNA overhangs (C, D). Finally, DNA Ligase IV/XRCC4/XLF complex is recruited to the lesion site (E) and the ends are ligated together (F). Figure adapted from (Hefferin and Tomkinson 2005).

accommodate the duplex DNA and protects DNA ends from being degraded (Walker et al., 2001). Upon binding, the complex translocates along the DNA possibly to allow further end processing (Ochem et al., 1997; Yavuzer et al., 1998). While the role of Ku in protecting DNA ends from degradation is well described, there is some ambiguity for its function as an alignment factor (Bliss and Lane 1997; Cary et al., 1997; Ramsden and Gellert 1998). Alignment and end bridging seem hard to reconcile, since Ku proteins are known to recruit another protein, DNA Dependent Protein Kinase catalytic subunit (DNA-PKcs) (Dvir et al., 1992; Gottlieb and Jackson 1993), which might also be involved in the alignment process (Figure 4C, D). It appears that the association of Ku proteins and DNA-PKcs is dependent on DNA. While DNA-PKcs can bind to DNA without the Ku proteins, the binding is enhanced by 100 fold in the presence of Ku (Dvir et al., 1992; Gottlieb and Jackson 1993; Suwa et al., 1994; Yaneva et al., 1997). The Ku heterodimer, together with DNA-PKcs, forms the active kinase unit, which is called the DNA-PK complex.

Deficiencies in NHEJ proteins, such as mutations in DNA-PKcs, result in a syndrome with severe combined immunodeficiency (SCID), since the NHEJ pathway is also required for RAG1/RAG2 endonuclease initiated V(D)J recombination (Jhappan et al., 1997; Taccioli et al., 1998). Recently a novel gene, which also causes the SCID phenotype, has been identified (Moshous et al., 2000; Moshous et al., 2001). The Artemis nuclease is found to associate with DNA-PKcs and is capable of hairpin opening, which is an intermediate structure formed during V(D)J recombination. The Artemis/DNA-PKcs complex exhibits nuclease activity, where 5' ends are blunted, and 3' overhangs are trimmed (Ma et al., 2002) (Figure 4D). Moreover,

phosphorylation of Artemis by DNA-PK appears to be crucial for this nuclease activity (Ma et al., 2002).

DNA-PK also appears to stimulate the last players of NHEJ: the XRCC4/DNA Ligase IV complex, which initiates the final ligation step between the two juxtaposed DNA ends (Chen et al., 2000) (Figure 4E, F). The activity of Ligase IV is strongly stimulated *in vitro* by its cofactor XRCC4, which is also shown to increase its stability (Critchlow et al., 1997; Grawunder et al., 1997; Bryans et al., 1999). XRCC4 is known to be phosphorylated by DNA-PKcs, however mutations in these sites do not interfere with its complementation of radiation sensitivity (Critchlow et al., 1997; Yu et al., 2003; Lee et al., 2004). Recently, a protein which has strong predicted structural similarity to XRCC4 has been identified. The XRCC4 like factor, XLF (or Cernunnos), can bind to XRCC4-Ligase IV complex and is possibly involved in this last step of break repair (Ahnesorg et al., 2006; Buck et al., 2006; Callebaut et al., 2006).

1.3.1.2 Phenotypes Associated with deficiencies in NHEJ components

Deficiency in Ku80 results in defects in V(D)J recombination, and increased sensitivity to irradiation (Nussenzweig et al., 1996; Zhu et al., 1996; Nussenzweig et al., 1997; Kabotyanski et al., 1998). Mice deficient in Ku80 exhibit growth retardation and shorter life span (Vogel et al., 1999). Moreover, spontaneous chromosome breaks appears to arise in fibroblasts derived from these animals, indicating insufficient response to DNA damage (Karanjawala et al., 1999). While heterozygous inactivation of Ku80 results in chromosomal instabilities such as translocations and fusions, homozygous mutation leads to cell death after a certain number of divisions (Li et al., 2002; Myung et al., 2004). Ku70 deficient mice manifest similar defects (Ouyang et al., 1997; Li et al., 1998).

Deficiencies in DNA-PKcs or Artemis show milder phenotypes without any growth retardation and similar incidences of T-cell lymphomas as wild type mice. However, as mentioned previously, both defects result in SCID phenotype (Jhappan et al., 1997; Taccioli et al., 1998; Moshous et al., 2000; Moshous et al., 2001) and lead to some sensitivity to DNA damaging agents (Jeggo 1998; Convery et al., 2005; Musio et al., 2005).

Knockout mice for Ligase IV and XRCC4 exhibit the most severe defects. Deletion of both genes results in embryonic lethality, possibly due to severe neurodegeneration (Barnes et al., 1998; Frank et al., 1998; Gao et al., 1998). Defects in neurogenesis are also seen in Ku80/70 deficient mice, but not in DNA-PKcs null animals (Gu et al., 2000). Lethality can be rescued by additional p53 deficiency, and these mice still suffer from high incidences of lymphomas indicating the importance of these proteins in protection from cancer (Frank et al., 2000; Gao et al., 2000).

1.3.1.3 Homologous Recombination (HR)

One of the earliest events in HR-dependent repair is converting the DSB ends to a recombination competent structure. This step involves degradation of single strands by the eukaryotic Mre11/RAD50/NBS1 (MRN) complex leaving a 3' protruding end, which is hundreds of bases long (Figure 5B) (D'Amours and Jackson 2002). This 3' single-stranded DNA (ssDNA), is pre-coated with Replication Protein A (RPA) (Figure 5C), which both resolves secondary structures and facilitates RAD51 loading on the DNA with the assistance of mediator protein RAD52 (Figure 5D) (Sung 1997; Shinohara and Ogawa 1998). Subsequently, this nucleoprotein filament invades the intact DNA duplex, and forms a structure called the

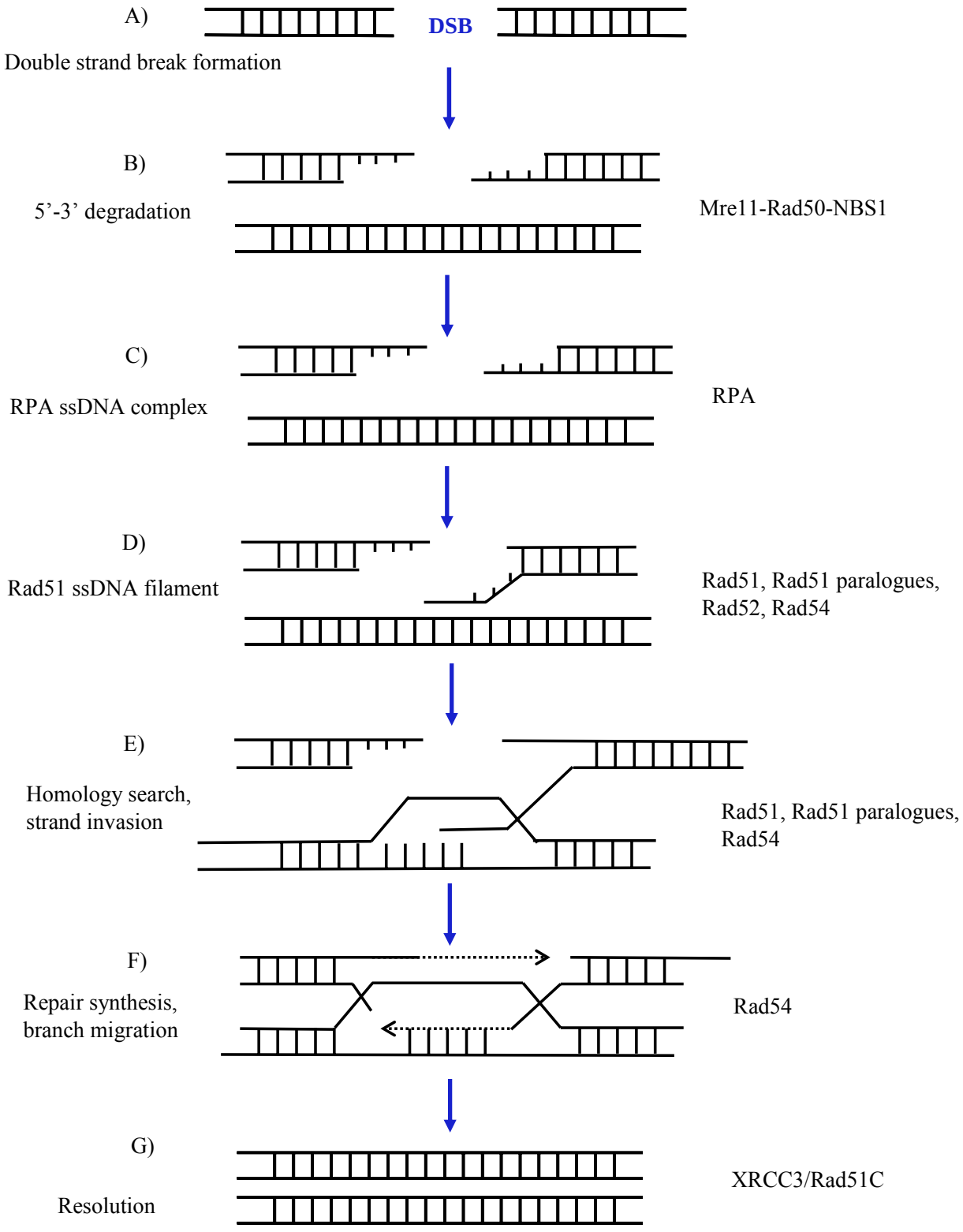

A)
DSB
Double strand break formation
B)
5'-3' degradation
Mre11-Rad50-NBS1
C)
RPA ssDNA complex
RPA
D)
Rad51 ssDNA filament
Rad51, Rad51 paralogues,
Rad52, Rad54
E)
Homology search,
strand invasion
Rad51, Rad51 paralogues,
Rad54
F)
Repair synthesis,
branch migration
Rad54
G)
Resolution
XRCC3/Rad51C

Figure 5 HR in mammalian systems. The DSB (A) ends are processed by the MRN complex (B). The 3' protruding end, which is precoated with RPA (C), is later loaded with RAD51 (D), which is facilitated and stabilized by RAD51 paralogues, RAD54 and the mediator RAD52 protein. Following strand invasion and branch migration (E-F), the broken strand is repaired by copying the intact template and finally the complex is resolved (G).

"D-loop". Strand displacement and branch migration are complicated tasks and involve many energy-dependent processes such as breaking internal hydrogen bonds, unwinding the DNA, and competing with other DNA binding proteins (Figure 5E). Hence, many proteins play roles during these processes including RAD51, and all five RAD51-like proteins, namely XRCC2, XRCC3, RAD51B (or RAD51L1), RAD51C (RAD51L2) and RAD51D (RAD51L3) (Albala et al., 1997; Rice et al., 1997; Cartwright et al., 1998; Dosanjh et al., 1998; Liu et al., 1998; Pittman et al., 1998; French et al., 2002). These RAD51 paralogues form four different complexes, XRCC2-RAD51D, RAD51B-RAD51C, XRCC3-RAD51C and RAD51B-RAD51C-RAD51D-XRCC2, which act to stimulate the activity of RAD51 in various aspects (Braybrooke et al., 2000; Kurumizaka et al., 2001; Sigurdsson et al., 2001; Henry-Mowatt et al., 2003; Yokoyama et al., 2003).

Other relevant proteins include RAD54, RAD54 paralogues, and the breast cancer associated proteins BRCA1/2 (Jasin 2002). RAD54 likely has a dual function in both enabling strand exchange and facilitating branch migration. It can stabilize RAD51 nucleofilament and enhance D-loop formation by introducing negative supercoils (Figure 5D-F) (Sigurdsson et al., 2002; Mazin et al., 2003). RAD54B, a RAD54 paralogue, also associates with RAD51 and colocalizes with RAD51 foci (DSB sites where repair proteins accumulate) (Tanaka et al., 2000). RAD54B has additive phenotypes with RAD54, indicating they have non-overlapping functions, however the exact role is yet to be discovered (Wesoly et al., 2006). Cells deficient for either BRCA1 or BRCA2 have impaired HR-dependent repair, and these proteins mediate their function probably in conjunction with RAD51 (Moynahan et al., 1999; Moynahan et al., 2001).

Once new DNA is synthesized by copying the homologous template, the intertwined molecules are resolved, possibly by the action of XRCC3/RAD51C complex (Figure 5G) (Brenneman et al., 2002; French et al., 2002; Liu et al., 2004).

1.3.1.4 Phenotypes Associated with deficiencies in HR components

Defects in HR proteins have a range of phenotypes. RAD51 appears as a key protein, since RAD51 disruption causes early embryonic lethality in mice, and mutant cell cultures do not proliferate even in a $p53^{-/-}$ background (Lim and Hasty 1996; Tsuzuki et al., 1996). On the other hand, knockout mice for RAD51 like proteins (XRCC2, RAD51B, and RAD51D) can progress until later stages in development (Shu et al., 1999; Deans et al., 2000; Pittman and Schimenti 2000). Both RAD54- and RAD54B-deficient mice are alive, but defective cells exhibit mild sensitivity to DNA damaging agents (Bezzubova et al., 1997; Essers et al., 1997; Wesoly et al., 2006). While RAD52 defects reduce HR, no effect on mutagen sensitivity is observed (Rijkers et al., 1998). BRCA1 or BRCA2 homozygous deletion results in embryonic death, like the loss of RAD51, and deficient cell lines are sensitized to DNA damage (Gowen et al., 1996; Connor et al., 1997; Ludwig et al., 1997; Shen et al., 1998; Scully et al., 1999).

1.3.1.5 The choice between NHEJ and HR

Both NHEJ and HR are important mechanisms in the repair of DSBs in all eukaryotes. However there seem to be differences in the choice of pathway between different organisms. While NHEJ is the prominent pathway in vertebrates, HR appears as the major mechanism in yeast (Critchlow and Jackson 1998; Dudasova et al., 2004). One reason for this choice could be due to the highly repetitive structure of the mammalian genome, which might make it harder to

find the correct template for HR to repair the damage. On the other hand, the yeast genome is smaller, nearly all genes are intronless and usually consist of a single copy.

NHEJ has been long considered as the error-prone or even "illegitimate" repair pathway, since the deletions can be harmful, if they occur in an essential gene or its regulatory sequences. However it should be noted that HR also runs the risk of creating rearrangements by recombining with related sequences in non-homologous chromosomes (albeit with reduced frequencies) (Richardson et al., 1998), or might lead to loss of heterozygosity by copying information from the homologous chromosome (Stark and Jasin 2003).

While both pathways seem to collaborate to maintain genomic stability, it also appears that there is a competition as to which repair pathway will be used to repair the damage. It has been shown that both NHEJ and HR can have access to the same DSB (Richardson and Jasin 2000). The HR proteins RAD51 and RAD52 can bind to DSB ends, possibly competing with the Ku complex of NHEJ (Baumann and West 1998; Haber 2000). In support of this model, reduction in NHEJ protein levels results in stimulation of HR-mediated repair (Fukushima et al., 2001; Pierce et al., 2001; Allen et al., 2002; Delacote et al., 2002). This stimulation only occurs if early NHEJ proteins, namely DNA-PKcs and Ku, are inhibited and not when late proteins are blocked such as XRCC4 inhibition (Pierce et al., 2001). Furthermore, consistently inactivated DNA-PKcs, which is a component of NHEJ, leads to decreased HR rates (presumably since it is still present at the break site, yet non-functional), while its absence leads to an increase in HR rates, indicating an "interactive competition" between the pathways (Allen et al., 2003). This selection between pathways is currently an area of intense investigation. It is at least partly

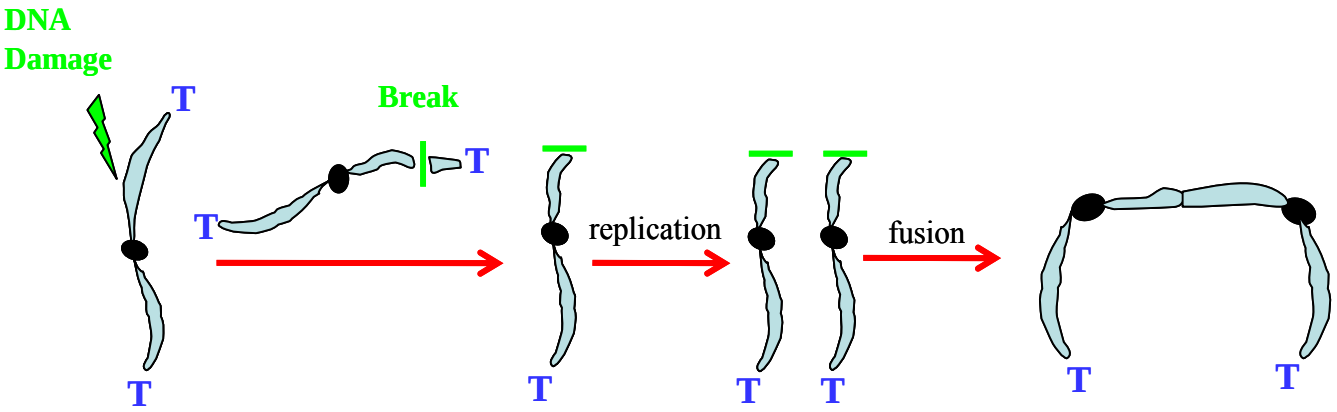
influenced by cell-cycle. NHEJ predominates throughout G1 to early S, and HR becomes more active during late S and mitosis, when the sister chromatids are available as template (Takata et al., 1998; Saintigny et al., 2001; Rothkamm et al., 2003).

1.3.2 Breakage-Fusion-Bridge (BFB) Cycles

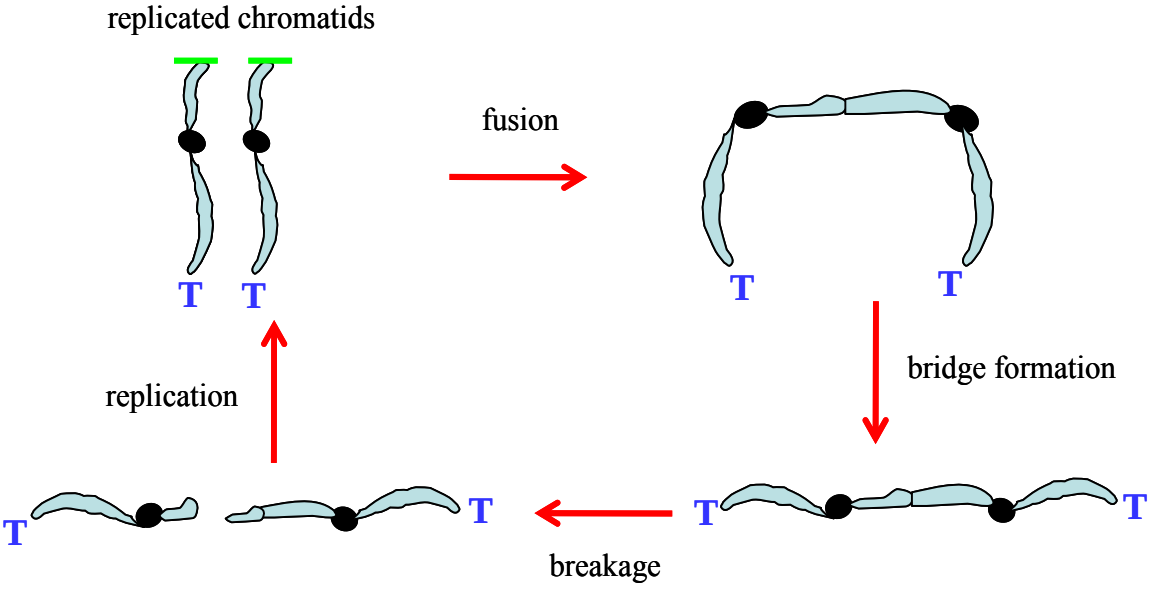
After the formation of a DSB, the ends are repaired by either of the pathways mentioned above. However, if the initial fusion occurs between either the sister chromatids (after replication) or with another chromosome, a dicentric chromosome will form with two centromeres (Figure 6A, B). Dicentrics have the potential to attach to both spindle poles resulting in a tug-of-war between the two spindle halves at anaphase (Gisselsson 2005). The bridge will eventually resolve by breaking, most likely not at the site it fused (Gisselsson et al., 2001; Hoffelder et al., 2004). In the next cell cycle, the telomere-less DNA ends are again primed to fuse after replication, which is known as the Breakage-Fusion-Bridge (BFB) cycle, and can result in loss or amplification of genetic information or translocation of chromosome arms. Fusion events following DNA replication, as described, is known as “chromatid-type” BFB cycles (Figure 6B).

In cases where fusion events precede DNA replication, the chromosome will replicate producing two dicentrics. These dicentrics may segregate either in a parallel fashion or by producing two anaphase bridges (Figure 6C). If they segregate in parallel, each daughter cell will inherit a dicentric chromosome, which will face the same scenario in the next cell cycle. In cases of bi-directional segregation, the bridges will form, and eventually break (Hoffelder et al., 2004).

A


DNA
Damage
Break
T
T
T
T
T
T
T
T
T
T
replication
fusion

B


replicated chromatids
T
T
fusion
T
T
bridge formation
T
T
breakage
T
T
replication

C

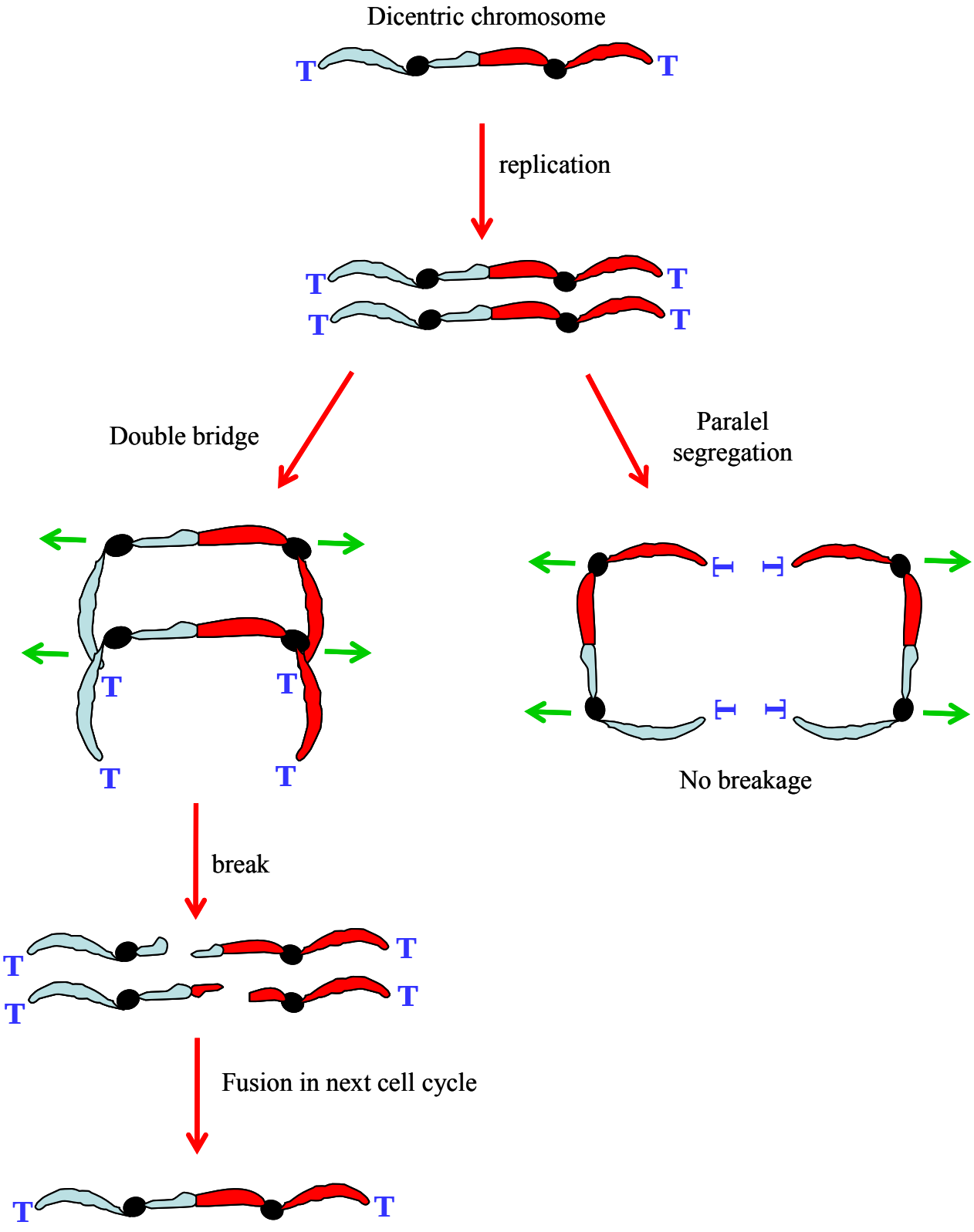

Dicentric chromosome
T
T
replication
T
T
T
T
Double bridge
Paralel segregation
T
T
T
T
T
T
T
T
No breakage
break
T
T
T
T
Fusion in next cell cycle
T
T

Figure 6 Breakage-Fusion-Bridge cycles. Double stranded break formation followed by replication may result in bridging between sister chromatids (A). Chromatid type BFB cycles (B). Chromosome type BFB cycles and possible outcomes of chromosome segregation. See text for details (C). Green lines indicate the sites of break, "T" stands for telomeres. Centromeres are represented by black circles, and different chromosomes are color coded by red and blue.

The cells will have sticky telomere-free DNA ends, which may fuse back and enter a "chromosome type" BFB cycle.

1.3.3 Fate of Anaphase Bridges

It is now known that the breakage along the anaphase bridge typically occurs at multiple sites, leading to formation of micronuclei and extensive loss of DNA material (Gisselsson et al., 2001; Hoffelder et al., 2004). In an extreme case, the entire bridge may be fragmented, resulting in loss of whole chromosome arms, which may explain the high frequencies of isochromosomes or whole-arm translocations in cancer cells (Gisselsson et al., 2005; Stewenius et al., 2005). Furthermore, these rearrangements also result in amplifications of low copy genes. For example, there is evidence indicating that both cyclinD1 and NuMA genes have been amplified via breakage-fusion-bridge cycles (Jiang et al., 1992; Huang et al., 2002). Alternatively, a bridge might result in detachment of sister chromatids from the spindle, which would give rise to loss of the whole chromosome (Gisselsson et al., 2005; Stewenius et al., 2005). Lastly, anaphase bridging might interfere with cytokinesis, resulting in a binucleate cell (Stewenius et al., 2005). After another round of DNA replication and centrosome duplication, a binucleate has the potential to divide in a multipolar fashion, which will eventually cause further chromosomal instability (Luo L and Saunders WS, unpublished data).

Although bridges usually break in anaphase from the tension of the spindle pulling forces or during cytokinesis, in rare cases they will persist until telophase or even after cytokinesis resulting in formation of an interphase bridge seen as persistent chromatin strands connecting

daughter cells (Figure 3C-F). It is not yet known whether these cells continue BFB cycles, function as a binucleate cell or cease proliferation.

1.4 MULTIPOLAR SPINDLES (MPS)

During mitosis, each daughter cell is ensured to obtain the correct amount of DNA through carefully controlled attachments of the chromosomes to the highly organized spindle fibers and symmetric separation in a bipolar fashion. However, divisions with more than two poles are also observed which leaves the cells with abnormal numbers of chromosomes (Figure 7) (Wunderlich 2002). Multipolar spindle (MPS) formation is a defect observed in many cancer cell lines, and is associated with an increase in centrosome number (Saunders 2005; Stewenius et al., 2005).

1.4.1 Centrosome Structure and Duplication

Centrosomes are the microtubule organizing centers in mammalian cells. The major structural elements of centrosomes are a pair of barrel shaped centrioles surrounded by an amorphous framework of proteins called the pericentriolar material (PCM). Centrioles are small cylindrical organelles (~200-400nm) composed of 9 triplet longitudinal fibrils and radial spokes connecting fibrils to the center of the centriole (Figure 8) (Dutcher 2001; Dutcher 2001). Among the major components of the PCM are the γ-tubulin ring complexes, the site of microtubule nucleation (Zheng et al., 1995), and the Sfi1p and centrin fibers, which make connections between different components of the centrosome, mediating overall structure and centriole

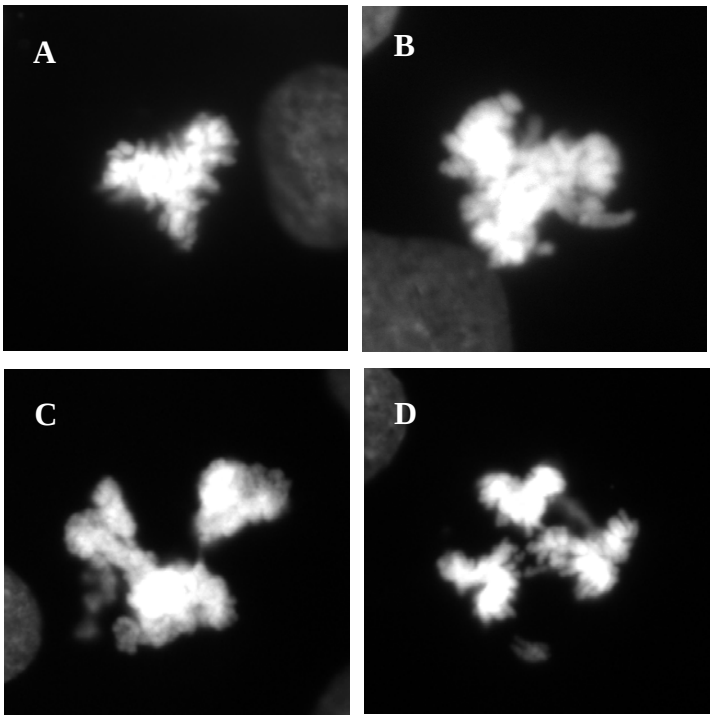


Figure 7 Multipolar mitoses in metaphase (A, B) and anaphase (C, D) observed in UPCI:SCC103 cell line. Notice that bridging usually accompanies multipolarity. DAPI is used for DNA staining and images are taken with an Olympus camera with 100X magnification lens.

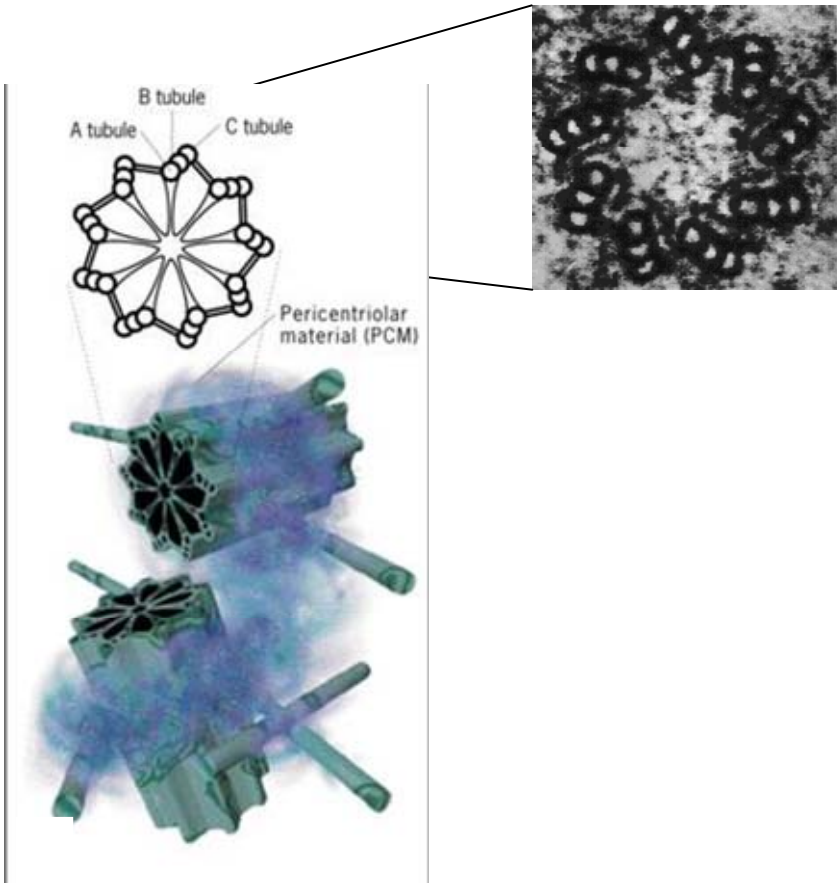


Figure 8 Centrosome structure. Centriole configuration with an array of nine microtubule triplets is shown in cartoon and in enlarged view by electron microscopy. Magnification ~ 305,000X. Figure from: http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/Cytoskeleton.html and http://io.uwinnipeg.ca/~jfranck/Bio3221_Pwrpt_lectures/Nov10_lecture_files/slide0016_image016.jpg

duplication (Salisbury 2004). Other structural components include several coiled coil proteins (Salisbury 2003), such as pericentrin (Doxsey et al., 1994; Dictenberg et al., 1998), Cep135 (Ohta et al., 2002), AKAP-450 (Keryer et al., 1993; Witczak et al., 1999), and ninein (Bouckson-Castaing et al., 1996), which regulate centrosomal activity.

Since centrosomes play a critical role in equal segregation of the chromosomes, they have to be duplicated once and only once in the cell cycle to ensure bipolarity. In G1, cells typically have two centrioles, which are orthogonally oriented relative to each other. Once cells pass the G1 checkpoint, the centrioles separate slightly, and nascent procentrioles start emerging perpendicular to the mother centrioles at the proximal end (Adams and Kilmartin 2000; Khodjakov et al., 2002). Elongation of procentrioles continues throughout G2 and is completed by the end of mitosis. Centrosomal maturation continues by recruitment of the PCM proteins during the G2/M phase and completion takes ~1.5 cell cycles (Dictenberg et al., 1998). A number of phosphorylation and ubiquitin dependent degradation events also take place to regulate the centrosome duplication cycle (Freed et al., 1999; Fry et al., 2000; Wojcik et al., 2000; Hinchcliffe and Sluder 2001; Meraldi and Nigg 2002).

The centriole pairs and the associated PCM, which forms the new centrosomes, migrate away from each other shortly before mitosis, ensuring bipolar spindle formation.

1.4.2 Centrosome amplification and MPS

Extra numbers of centrosomes have been reported in many different tumor types including breast, lung, brain, gall bladder, bone, pancreas, colorectal, head and neck cancers

(Lingle et al., 1998; Pihan et al., 1998; Weber et al., 1998; Carroll et al., 1999; Lingle and Salisbury 1999; Sato et al., 1999; Gustafson et al., 2000; Kuo et al., 2000; Pihan et al., 2001; Sato et al., 2001). Moreover, changes in centrosome number is strongly correlated with aneuploidy and CIN in many cases (Lingle et al., 1998; Sato et al., 1999; Ghadimi et al., 2000; Pihan et al., 2003).

Centrosomes can potentially be amplified by three different mechanisms (Nigg 2002). The first model involves several rounds of duplication within a single cell cycle. Since centrosome duplication requires a long time, there has to be a delay in cell cycle progression for this model to be correct. Indeed, when cells are treated with drugs, such as hydroxyurea or aphidicolin to stall DNA replication (Balczon et al., 1995; Meraldi et al., 1999), or when RAD51 deficient cells are arrested in G2 phase after irradiation (Dodson et al., 2004), centrosomes continue to duplicate in the absence of DNA replication and completion of the cell cycle. In the second model, over-replication occurs due to a failure of cytokinesis. Abortion of cell division arises from a variety of reasons some of which are: persistence of DNA damage, an inactive spindle-assembly checkpoint or abnormalities in mitotic progression (Meraldi et al., 2002; Millband et al., 2002). For example, it has been shown that a reduction in the activity of the myosin light chain kinase, which functions in contractile ring formation and completion of cytokinesis, is a major source of cytokinesis failure in a variety of cancer cell lines (Matsumura 2005) (Wu Q. and Saunders WS., unpublished results). Thirdly, cell fusion might also result in supernumerary centrosomes. This phenomenon has been observed following treatment of cells with X-Ray or UV or overexpression of Rad6 in epithelial cells (Kura et al., 1978; Brathen et al., 2000; Shekhar et al., 2002). Regardless of which initial event leads to an amplification in

centrosome number, the main outcome is the increased likelihood of formation of multipolar spindles (Lingle et al., 1998; D'Assoro et al., 2002; Lingle et al., 2002).

It has been observed that both MPS and centrosome amplification appear together in cancer cells, suggestive of a link between these abnormalities (Gisselsson et al., 2002). Despite the intimate correlation, cancer cells do not always undergo multipolar mitosis when they acquire extra centrosomes. A typical example is the N1E-115 cell line, where supernumerary centrosomes coalesce to form a bipolar metaphase plate (Ring et al., 1982). Similar results are also observed in UPCI:SCC114 cells indicating that there are mechanisms clustering the centrosomes allowing bipolar division (Quintyne et al., 2005). There is evidence suggesting that coalescence is achieved through a dynein-dependent mechanism, since this microtubule binding motor is reduced in the spindle of most cancer cells. Restoring dynein to the spindle results in centrosomal clustering and bipolar division (Quintyne et al., 2005).

1.4.3 Other mechanisms leading to MPS

Although centrosomal amplification is a major mechanism in MPS formation, multipolarity can be induced in the absence of changes in centrosome number. Overexpression of Nek2 kinase, which plays a role in centriole separation through phosphorylation of linker proteins between the centrioles, has been shown to provoke centrosome splitting and multipolarity (Fry et al., 1998; Fry et al., 1998). Centrosomal splitting can also occur via treatment of microtubule destabilizing drugs such as nocodazole, colcemid, or disorazole (Jean et al., 1999; Meraldi and Nigg 2001) (Acilan C, Saunders WS, unpublished observations). Moreover, the splitting effect can be amplified with the combined action of nocodazole and

overexpression of Nek2, indicating that the effect can be additive (Meraldi and Nigg 2001). Furthermore, treatment of cells with reagents other than microtubule destabilizers or cold-shocking the cells have also been reported to induce splitting of centrosomes and MPS (Schliwa et al., 1983; Callaini and Marchini 1989; Kojima and Czihak 1990).

In addition, there is evidence indicating that incomplete replication or DNA damage can result in centrosomal splitting in both Drosophila and mammalian cells (Hut et al., 2003). Treatment of cells with γ-irradiation has also shown to induce multipolarity and once again centrosome overduplication has been proposed as the mechanism. In chapter 5, we shall provide evidence suggesting other mechanisms as to how γ-irradiation might lead to MPS.

1.4.4 MPS and tumorogenesis

During a multipolar division, a balanced distribution of chromosomes to the daughter cells is very unlikely, even if the sister chromatids segregate accurately. In point of fact, it has been shown that the division of sister chromatids after a tripolar mitosis is almost random (Stewenius et al., 2005). Under this assumption, some of the progeny cells will inherit no copies of certain chromosomes (nullisomy) and it is likely that clonal expansion will not be favored in cells containing nullisomies. So why do cancer cells exhibit multipolar mitoses but are not eliminated by selection? It can be speculated that the majority of the daughter cells will not undergo further cell divisions, which will be an evolutionary dead end, but a rare event can grant a selective advantage. This would hold true especially after a multipolar division in binucleate cells, which might provide extra number of chromosomes. Moreover, a genetic variation leading to *in vivo* resistance to chemotherapeutic drugs or an ability to stimulate angiogenesis in a tumor

could also favor multipolar divisions. Alternatively, formation of MPS can be a secondary event without any particular advantage. Cancer cells frequently lack cell cycle checkpoints and abnormal mitotic figures could be expected to occur in these backgrounds. Supernumerary centrosomes are only associated with a minority of cells contributing ~1–15% of the population. It is possible that those that divide in a multipolar fashion are eliminated from the culture and a steady frequency of supernumerary centrosomes is achieved by *de novo* amplification of centrosomes.

1.5 CORRELATION BETWEEN MULTIPOLAR SPINDLES AND ANAPHASE BRIDGES

Both anaphase bridges and multipolar spindle formation can lead to chromosomal instability and massive changes in chromosomal structure and number. They are usually found concomitantly driving the cancer cell to aneuploidy (Gisselsson et al., 2004). Moreover, there is a strong positive correlation between the frequency of MPS and the level of bridging in many different tumors tested (r=0.96) (Gisselsson et al., 2002). The basis for such a correlation could be a single event, environmental or genetic, causing both of these defects. For instance, treatments such as ionizing radiation and X-ray exposure have been shown to induce both (Scott and Zampetti-Bosseler 1980; Gisselsson et al., 2001; Sato et al., 2001). Furthermore, loss of p53 results in an increase in centrosome number probably through deficiencies in the regulation of the centrosome duplication cycle and failure of cytokinesis. p53 might also control centrosome cycle through transactivation independent ways such as physical binding to the centrosomes (Carroll et al., 1999; Tarapore and Fukasawa 2002). Moreover, p53 loss also affects the

frequency of anaphase bridges in backgrounds with deficient DNA repair pathways or telomerase enzyme (Zhu et al., 2002). Consistently, expression of human papillomavirus proteins E6 or E7, which bind to and inactivate p53 and pRb tumor suppressor proteins, can both trigger formation of anaphase bridges and lead to supernumerary centrosomes and multipolar mitoses (Duensing and Munger 2002; Schaeffer et al., 2004). Alternatively, a correlation might exist due to a dependence of anaphase bridges or multipolar spindles on one another. We have observed that when cells undergo multipolar anaphase, they usually contain bridges connecting some or all poles to each other (Figure 7C, D). Thus, within a mixed population of normal and aberrant division, these events tend to occur together. It is known that anaphase bridges do not necessarily resolve and persistence through interphase is observed (Figure 3D-F). In such instances, the bridge might interfere with cytokinesis, which would lead to an increase in centrosome number, hence multipolar spindles. While any of the above, alone, could explain the observed correlation, they might also co-exist strengthening the link between these defects.

2.0 CHAPTER II: ROLE OF DNA REPAIR PATHWAYS IN THE FORMATION OF ANAPHASE BRIDGES

2.1 INTRODUCTION

2.1.1 Oxidative stress and DNA damage

There are a number of DNA damaging events and oxidative damage constitutes a significant source of DNA damage in all aerobic organisms. Oxidative damage can occur through exogenous sources such as ionizing radiation (IR) (Skov 1984; Teoule 1987; Nikjoo et al., 1994) or from endogenous sources such as production of H_2O_2 as a result of metabolic processes associated with redox reduction reactions in the mitochondria (Chance et al., 1979).

While H_2O_2 does not react with DNA directly, it results in production of $^{\bullet}OH$ radicals, which are highly reactive with DNA, through the Fenton reaction (Imlay et al., 1988).

$$Fe^{2+}+H_2O_2\rightarrow {}^{\bullet}OH+Fe^{3+}.$$

Metals, such as Cu^{+} and Fe^{2+}, catalyze this transition, and both of these metals are abundant in cellular fluids, and are also associated with DNA in trace amounts. Interestingly,

DNA damage through H_2O_2 requires the presence of these metals, consistent with Fenton reaction as the primary source of reactive oxygen species (Blakely et al., 1990). $^{\bullet}OH$ can result in nearly 100 different types of DNA damage including formation of single stranded DNA breaks (SSB) (Michalik et al., 1995). It can also indirectly lead to double stranded breaks (DSB) possibly due to close proximity of SSBs, when H_2O_2 is present at higher concentrations (Dahm-Daphi et al., 2000; Jackson and Loeb 2001).

IR can also result in different DNA lesions, including DSBs (Hutchinson 1985). On the other hand, unlike H_2O_2, ionizing radiation can exert its effects directly on DNA, by radiation energy (Teoule 1987; Ward 1988). Ionization can release 6-7 times more energy than needed to break a covalent C-C bond (Hall 1994), and DSBs can be induced by IR, even at low doses (Dahm-Daphi et al., 2000). The effects of IR may also be indirect through production of reactive oxygen species by ionizing water molecules (Skov 1984; Teoule 1987; Ward 1988; Nikjoo et al., 1994).

2.1.2 Secondary effects induced by H_2O_2

While H_2O_2 is a useful experimental reagent to induce DNA damage, this treatment has other side effects, such as damaging cells through oxidation of membrane lipids or proteins (Howe et al., 2004; Huang et al., 2004). Moreover, it is known to stimulate apoptosis by altering Ca^{2+} homeostasis (Lin et al., 2004; Shin et al., 2004; van Rossum et al., 2004). In contrast, direct H_2O_2 exposure inhibits CaM-kinase activity, hence might have an inhibitory effect on apoptosis as well (Franklin et al., 2006). Furthermore, certain oncogenes, such as K-*ras*, are shown to be activated by oxidative stress, which suggests a tumor promoting role for H_2O_2 (Jackson 1994).

H_2O_2 treatment is able to influence cell volume as well by changing membrane potential (Bychkov et al., 1999) or by activation of membrane serine-threonine phosphatases (Bize and Dunham 1995; Bize et al., 1998). In addition, the cytoskeletal network is affected by hydrogen peroxide, which can either selectively alter the structure of the cytoskeletal proteins (Aksenov et al., 2001) or rearrange cellular actin networks (Huot et al., 1998). H_2O_2 also induces formation of focal adhesion complexes possibly through activation of the SAPK2/p38 pathway (Dalle-Donne et al., 2001).

2.1.3 Anaphase Bridges

Anaphase bridging, a commonly observed segregation defect in cancer cells, is one of the major sources of genomic instability (Gisselsson et al., 2000) (Montgomery et al., 2003). It has been proposed that bridging may have hazardous consequences like gene deletions, amplifications or translocations, and is known to be strongly linked to carcinogenesis (Artandi et al., 2000; Stewenius et al., 2005). Anaphase bridges can theoretically be produced by several mechanisms such as telomeric fusions, or repair of DNA double-stranded breaks (Bryant 1984; Artandi et al., 2000; Fouladi et al., 2000; Gisselsson et al., 2001; Rudolph et al., 2001; Lo et al., 2002; O'Hagan et al., 2002). While a significant amount of evidence has been obtained on how bridges form as a result of telomeric fusion, relatively little is known about bridge induction after DSB formation.

In an attempt to determine how and why cells form bridges following DSB induction, we first set out to determine what DNA repair pathways the cells use to heal the DNA damage that cause bridges. Preliminary experiments were performed using hydrogen peroxide to induce

DSBs. To exclude the possible side effects of H_2O_2 mentioned above, the results were confirmed and expanded using IR.

Our studies have indicated that neither of the two major DNA repair pathways in mammalian cells, HR or NHEJ, alone, is required for bridge formation. In fact, the NHEJ pathway seems to play a role in the prevention of bridges. Moreover, it appears that cancer cells that have intrinsically high NHEJ activity are less likely to form bridges.

2.2 RESULTS

2.2.1 Screening genetic mutants of DNA repair proteins using H_2O_2 to induce DSBs for deficiencies in formation of anaphase bridges

Whilst cells usually fuse the correct broken ends efficiently, the presence of dicentric chromosomes and anaphase bridges indicate that incorrect fusions also occur. In order to explore the mechanisms behind anaphase bridging in response to DSBs, we hypothesized that formation of bridges is an enzymatic process, which involves the fusion of broken DNA ends via repair proteins. Initially, we tested this hypothesis by using genetic mutants for numerous proteins involved in either NHEJ or HR, the major DSB repair pathways in vertebrates. The DSBs were induced by H_2O_2 treatment in unsynchronized cultures. Cells were fixed 24 hours following treatment, which is approximately one cell-cycle duration after the breaks were introduced. Anaphase bridges were scored based on DAPI staining and exclusively DNA links that are entirely continuous between the condensed chromosome masses were counted as a bridge. Consistent with previous reports, in all cell lines tested, treatment of H_2O_2 resulted in increase bridges, albeit with varying frequencies (Figures 9-11) (Thomas et al., 2003).

2.2.1.1 Mutant cell lines exhibit similar levels of bridging in HR-deficient backgrounds in response to H_2O_2 treatment

We began by examining whether HR plays a role in bridging. Figure 9 illustrates the induction of anaphase bridges in HCT116 cells and the RAD54B mutant derived from this cell

line by targeting all three copies of the gene (HCT116 cells harbor three alleles of RAD54B). The loss of RAD54B expression was verified by immunoblotting (Figure 13). RAD54B deletion results in decreased HR rates and varying levels of sensitivity in response to different DNA damaging reagents (Miyagawa et al., 2002; Wesoly et al., 2006). In this HR mutant, we observed that anaphase bridges were induced to similar extents compared to the parental line, indicating that HR pathway was not required for bridge formation.

2.2.1.2 Mutant cell lines exhibit enhanced levels of bridging in NHEJ-deficient backgrounds in response to H_2O_2 treatment

In order to test whether NHEJ plays a role in bridging, we used Ku80 deficient Chinese Hamster Ovary (CHO) and the DNA-PKcs deficient human glioblastoma (MO59) cell lines. For both cell lines, the parental wild type cells showed an induction of bridges upon H_2O_2 treatment. Strikingly however, the level of anaphase bridge formation in either NHEJ deficient cell line was higher than seen in wild type cells (Figure 10). This result suggested that NHEJ was not required in the formation of bridges, but rather it played an efficient role in their prevention.

2.2.1.3 There is no additive effect for induction of anaphase bridges with deficiencies in different proteins of NHEJ pathway

DNA-PKcs is a member of phosphotidylinositol-3 (PI-3) kinase family (although there is no evidence for its function as a lipid kinase like PI-3 kinase) (Smith et al., 1999). PI-3 kinases, including DNA-PKcs, are known to be inhibited by wortmannin (Hosoi et al., 1998). Given this tool, we wanted to test whether inhibition of DNA-PKcs by wortmannin will have similar effects on bridging in other cell lines as well as in cells that are already defective for NHEJ. As predicted, anaphase bridges were enhanced upon induction of DSBs in wild type cells after drug

treatment. Nevertheless, inhibition of DNA-PKcs in cells with an existing mutation in a NHEJ protein, Ku80, did not further augment bridge frequency, indicating that there is no additive effect between the NHEJ proteins for the induction of anaphase bridging (Figure 11). However, it should be noted that wortmannin is not specific for DNA-PKcs and among the other PI-3 kinases, activity of DNA repair proteins, such as ATM or ATR, is also expected to be reduced.

2.2.1.4 p53 is not required for bridge formation

The tumor suppressor protein, p53, has a critical role in many signal transduction pathways including DNA repair, distinguishing between growth arrest or apoptosis (Niida and Nakanishi 2006; Sun 2006) p53 has been shown to inhibit deletion of bases during repair related joining events by NHEJ and has been proposed to influence NHEJ during site-directed, but not IR induced DSB repair (Tachibana 2004; Dahm-Daphi et al., 2005).

To test whether p53 is required for bridge formation, we assayed for bridging in p53 knockout mouse epithelial cells (Figure 12). Upon H_2O_2 treatment, anaphase bridges were greatly induced from ~5% to an average of 80%. Likewise, another segregation defect, formation of micronuclei that occurs as a consequence of bridging, was increased in accordance. Consistent with this data, it has been reported by our lab that treatment of cells with cigarette smoke condensate also induces anaphase bridge formation at a higher frequency in p53 deleted backgrounds (Luo et al., 2004).

The induction of bridges in p53 deleted cells suggests that this protein is probably not required for bridge formation. However, this experiment lacks control cells with wild type p53. Hence these results are inconclusive for a definitive role for p53 in bridge formation.

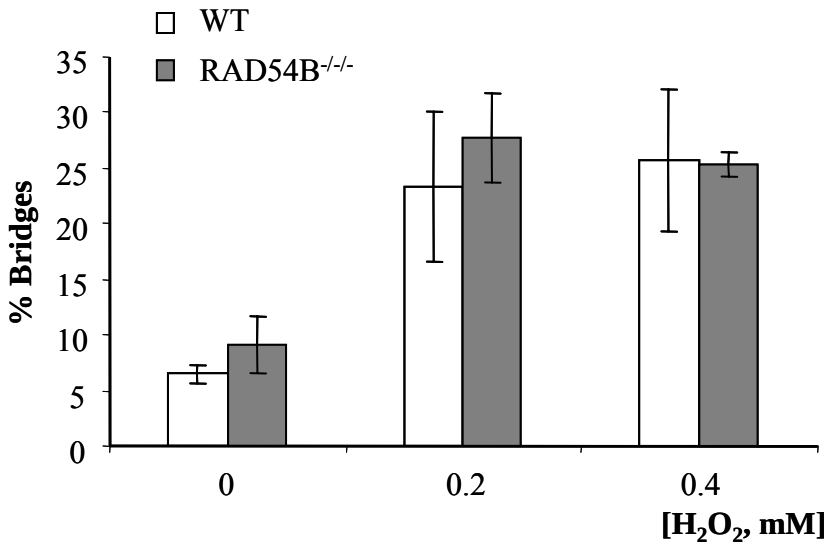
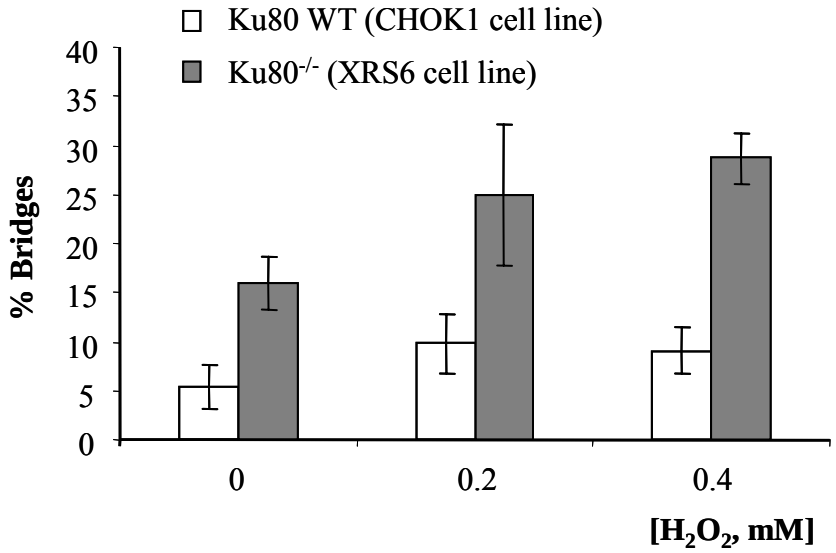


Figure 9 Induction of anaphase bridges in human colon cancer cell line HCT116 following H_2O_2 treatment. A RAD54B-deficient cell line was created by sequential gene targeting of all three copies of the RAD54B gene in HCT116 (wild type, WT) cells. Cells were treated in culture medium containing H_2O_2 at the indicated concentrations, switched to regular medium following 10 minutes of incubation and fixed in cold methanol 24 h after H_2O_2 treatment. Cells were scored for the percentage of bridges in the total anaphase population.

A)


Ku80 WT (CHOK1 cell line)
Ku80-/- (XRS6 cell line)
% Bridges
40
35
30
25
20
15
10
5
0
0
0.2
0.4
[H2O2, mM]

B)

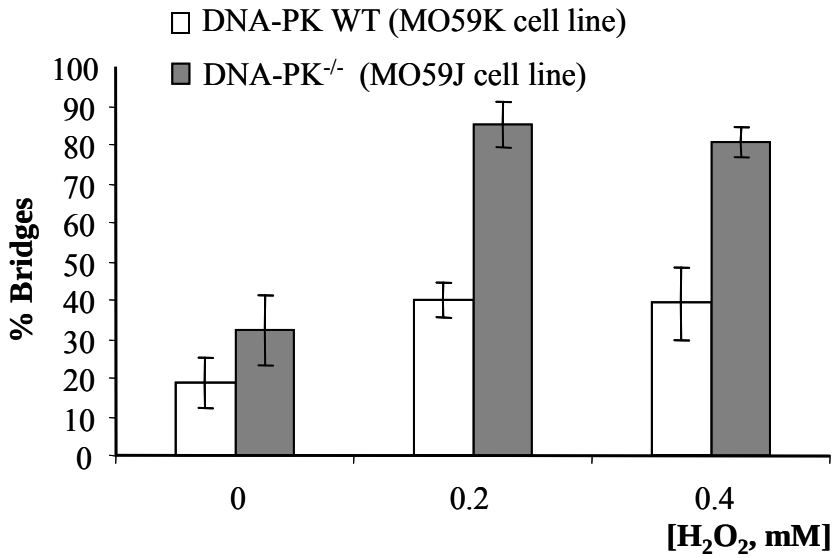
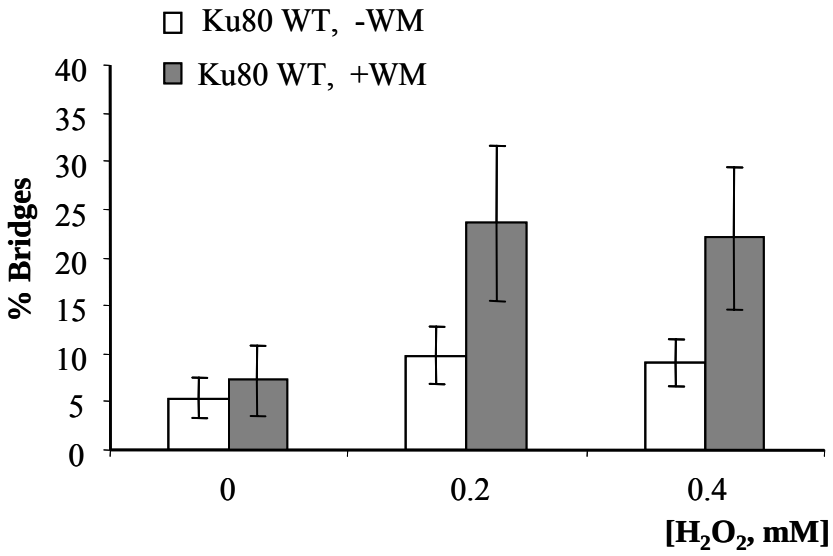

DNA-PK WT (MO59K cell line)
DNA-PK-/- (MO59J cell line)
% Bridges
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
[H2O2, mM]

Figure 10 Induction of anaphase bridges in cells deficient for NHEJ proteins after H_2O_2 treatment. Ku80 (A) or DNA-PKcs deficient (B) cells are treated with H_2O_2 as described in Figure 9, and scored for anaphase bridging. The XRS-6 cell line is derived from the parent Chinese Hamster Ovary (CHOK1) cells, and is deficient for Ku80 expression (A). MO59K is the sister cell line for MO59J, which fails to express the catalytic subunit of DNA-PK. The two cell lines were established from the same tumor, and otherwise exhibit similar genomic instability (Allalunis-Turner et al., 1993) (B).

A)


Ku80 WT, -WM
Ku80 WT, +WM
% Bridges
40
35
30
25
20
15
10
5
0
0
0.2
0.4
[H2O2, mM]

B)

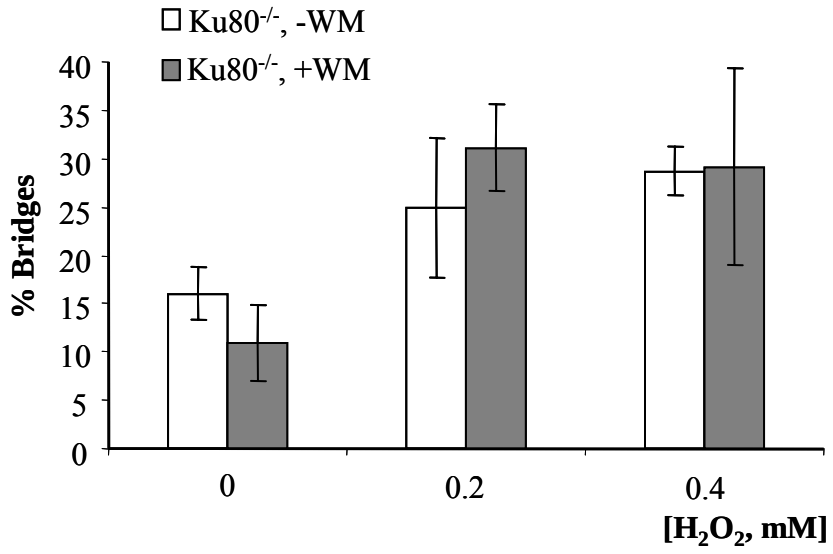

Ku80-/-, -WM
Ku80-/-, +WM
% Bridges
40
35
30
25
20
15
10
5
0
0
0.2
0.4
[H2O2, mM]

Figure 11 Inhibition of DNA-PK by wortmannin (WM) enhances bridge induction. Wild type (WT) CHOK1 (A), or Ku80 deficient ($Ku80^{-/-}$) XRS-6 (B) cells are treated with H_2O_2 as described previously, but grown in wortmannin (WM) containing medium, where indicated. While inhibition of DNA-PKcs by wortmannin induces bridge formation in a background wild type for NHEJ (A), an additive induction is not observed in Ku80 deficient backgrounds (B).

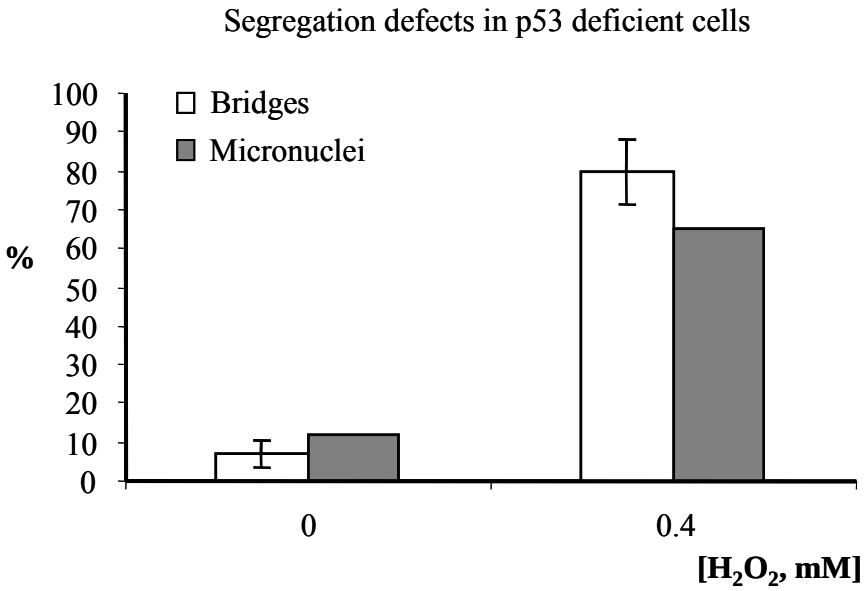


Figure 12 KE epithelial cells that are derived from p53 knockout mouse were treated with H_2O_2 as described previously. Cells are scored for bridge formation and presence of micronuclei. Both of these defects were induced following DSB induction.

2.2.2 Role of DNA repair pathways involved in anaphase bridging

Although formation of anaphase bridges is most likely a result of the DNA damaging effects of H_2O_2, in order to rule out other secondary causes, IR was also used as an additional source of DNA damaging agent.

Since cell lines used in these initial experiments were coming from different genetic backgrounds and species, cross comparison of the data was hard to judge. Hence we used deletion mutants of HR pathway or siRNA to reduce the levels of NHEJ proteins in the same HCT116 background in the following sets of experiments.

2.2.2.1 HR is not required for bridge formation

A reduction in homologous recombination is generally tested by two different assays: targeted integration frequency and sister chromatid exchange (SCE) rate. The two mutants used in our assays were once again the RAD54B mutant, which is known to display reduced targeted integration frequencies (>10 fold) with no change in sister chromatid exchange rates (Miyagawa et al., 2002), and the XRCC3 mutant, which exhibits reduced SCE rates without altered targeted integration frequencies (Yoshihara et al., 2004). Neither the XRCC3 nor the RAD54B mutant cell lines express the corresponding proteins as confirmed by immunoblotting (Figure 13).

Consistent with our results using H_2O_2, ionizing radiation treatment led to similar levels of anaphase bridging in either mutant background relative to the parental HCT116 cell line ($p<0.0001$, both mutants) (Figure 14A). To rule out a possible influence of known deficiency of

MLH1 (Aaltonen et al., 1993; Branch et al., 1995), a DNA mismatch repair protein, or unknown mutations in the HCT116 cell line, we also examined parental and HR-defective RAD51C mutant Chinese Hamster Ovary cells and again found no difference in bridge induction with mutation of HR ($p<0.08$) (Figure 14B).

2.2.2.2 NHEJ is essential for prevention of bridges induced by IR

To explore the role of NHEJ in anaphase bridging, we targeted both Ku80 and XRCC4 proteins using siRNA. These targets represent early and late acting proteins in the pathway (Figure 4). siRNA transfection of HCT116 cells efficiently reduced the Ku80 and XRCC4 expression as judged by immunoblotting (Figure 15). The knockdown was also confirmed by immunostaining of the cells for these proteins (Figure 16). These cells clearly showed decreased staining upon siRNA treatment and only cells with no visible staining were scored. The average transfection efficiency was between 90-95%, hence most of the population was included.

In both of the targeted knockdowns, we observed a significant increase in anaphase bridge formation ($p<0.0001$, both knockdowns) (Figure 17A). To exclude the possibility that these findings were specific for HCT116, we established similar conclusions using Ku80 and XRCC4-deficient CHO cells, where enhanced levels of bridging are seen ($p<0.0001$, both mutants) (Figure 17B, C). These observations, in combination with our findings using H_2O_2 treatment, reveal that NHEJ activity is critical to prevent bridge formation in response to DNA damage.

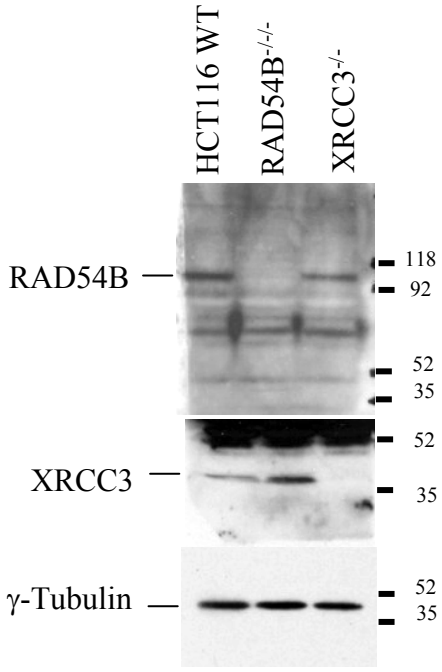
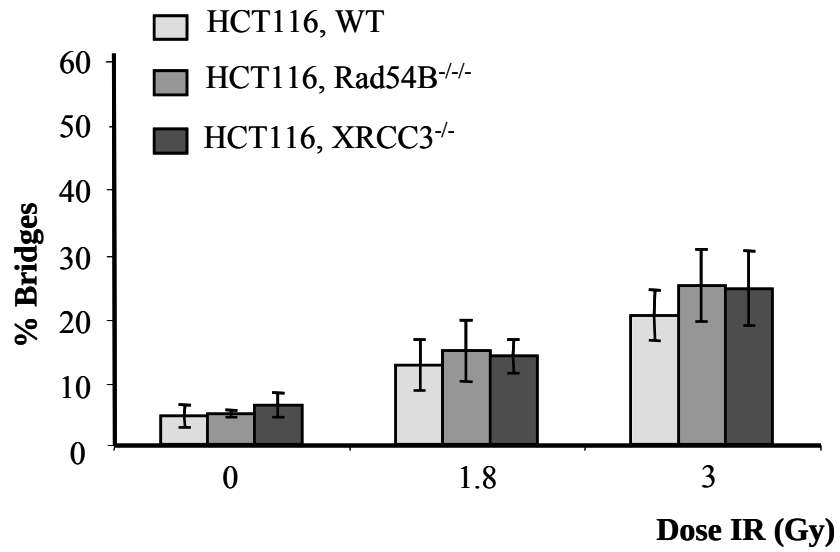


Figure 13 HR-deleted cell lines RAD54B$^{-/-/-}$ (HCT116 cells that are disrupted in all three copies of the RAD54B gene) and XRCC3$^{-/-}$ (HCT116 cells that are disrupted in both copies of the XRCC3 gene) do not express RAD54B, or XRCC3 proteins, respectively. Whole cell extracts were immunoblotted and both non-specific bands and γ-tubulin were used as loading controls.

A)


HCT116, WT
HCT116, Rad54B-/-/-
HCT116, XRCC3-/-
% Bridges
60
50
40
30
20
10
0
0
1.8
3
Dose IR (Gy)

B)

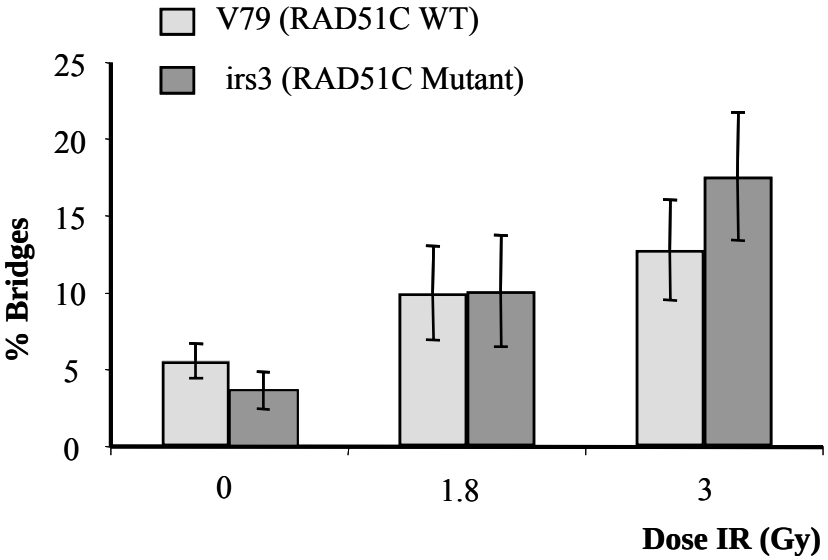

V79 (RAD51C WT)
irs3 (RAD51C Mutant)
% Bridges
25
20
15
10
5
0
0
1.8
3
Dose IR (Gy)

Figure 14 Anaphase bridges were induced to similar levels in HR mutants by IR. (A) HCT116 cells, or RAD54B or XRCC3 mutants in that background, were treated with IR, fixed after one day, and DAPI stained. Percentage of bridges was calculated from the total anaphases. (B) Mutation of the HR component RAD51C did not change bridge-induction in CHO cells. Wild type and the RAD51C mutant cells were IR-treated and scored for anaphase bridges.

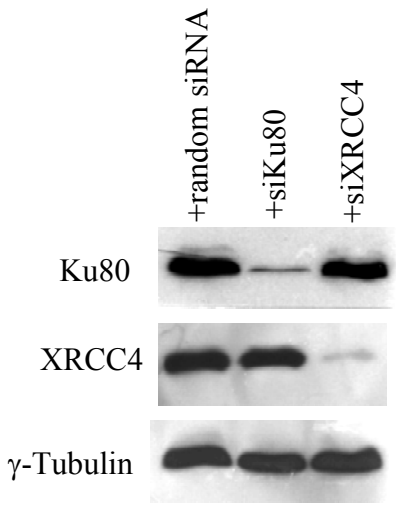
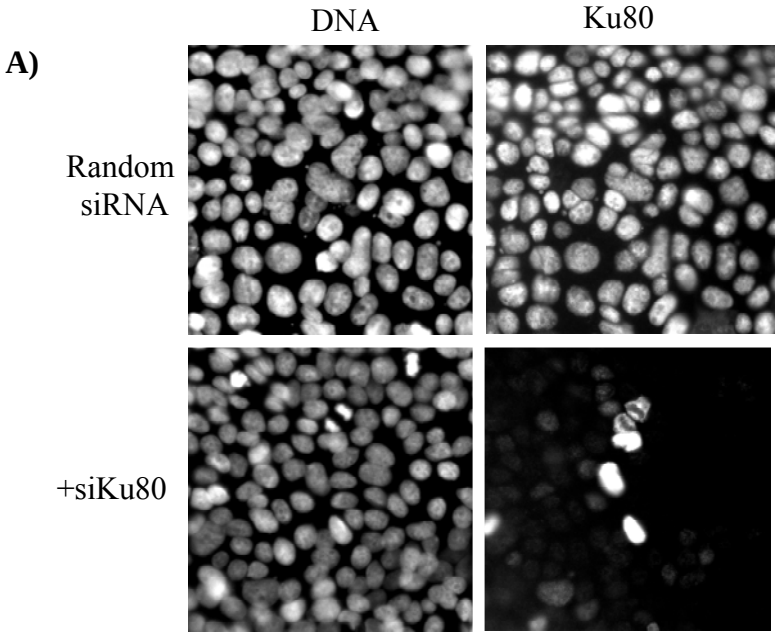


Figure 15 Both Ku80 and XRCC4 levels are reduced after siRNA treatment. Immunoblots of HCT116 cells were transiently transfected with siRNA for Ku80 (siKu80) or XRCC4 (siXRCC4) and harvested three days after transfection using RIPA buffer.


A)
DNA
Ku80
Random siRNA
+siKu80

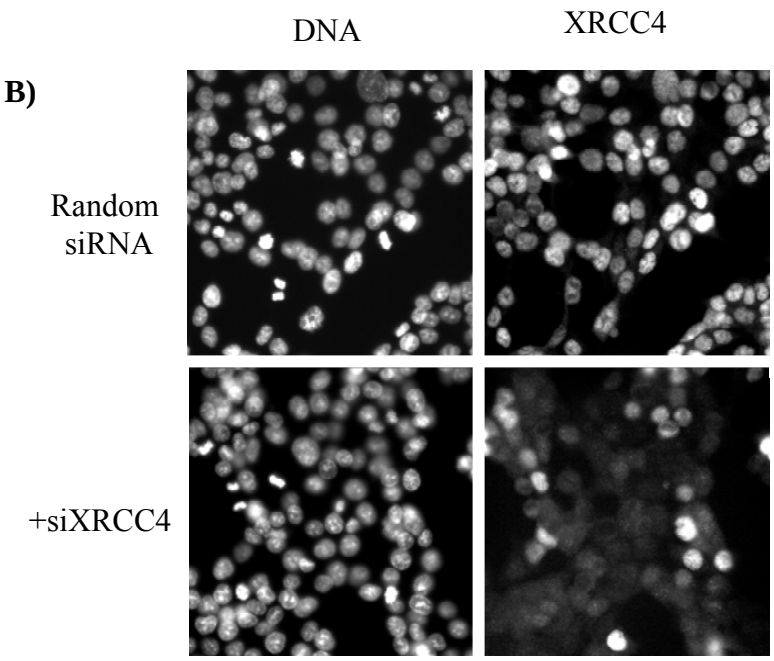
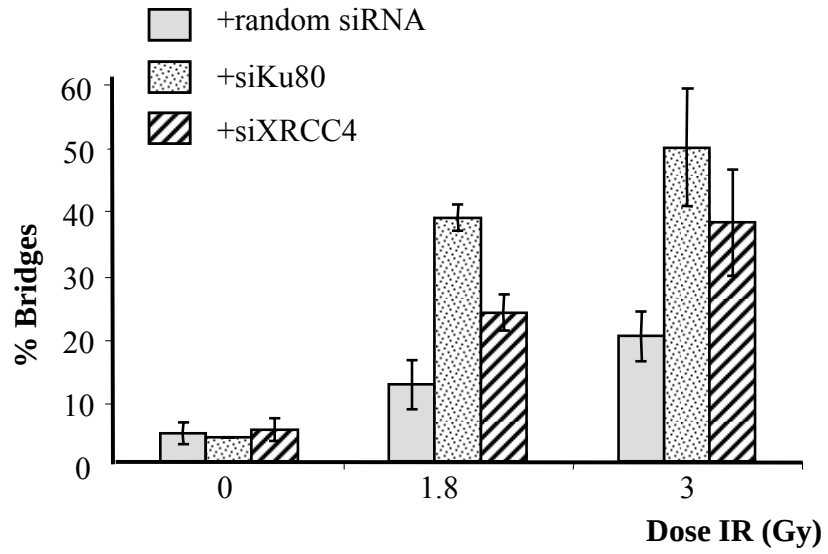
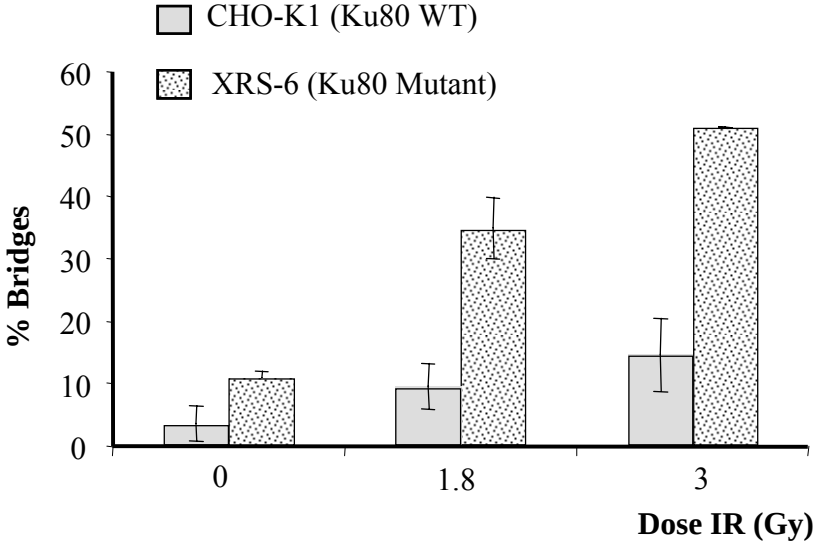

B)
DNA
XRCC4
Random siRNA
+siXRCC4

Figure 16 HCT116 cells were fixed in methanol and stained for Ku80 (A) or XRCC4 (B) three days after transfection with either siKu80 or siXRCC4, respectively. siRNA treatment results in reduction in the expression of both of these proteins as determined by fluorescence intensity. Only anaphases with reduced intensity were scored for bridging.

A)


+random siRNA
+siKu80
+siXRCC4
% Bridges
60
50
40
30
20
10
0
0
1.8
3
Dose IR (Gy)

B)


CHO-K1 (Ku80 WT)
XRS-6 (Ku80 Mutant)
% Bridges
60
50
40
30
20
10
0
0
1.8
3
Dose IR (Gy)

C)

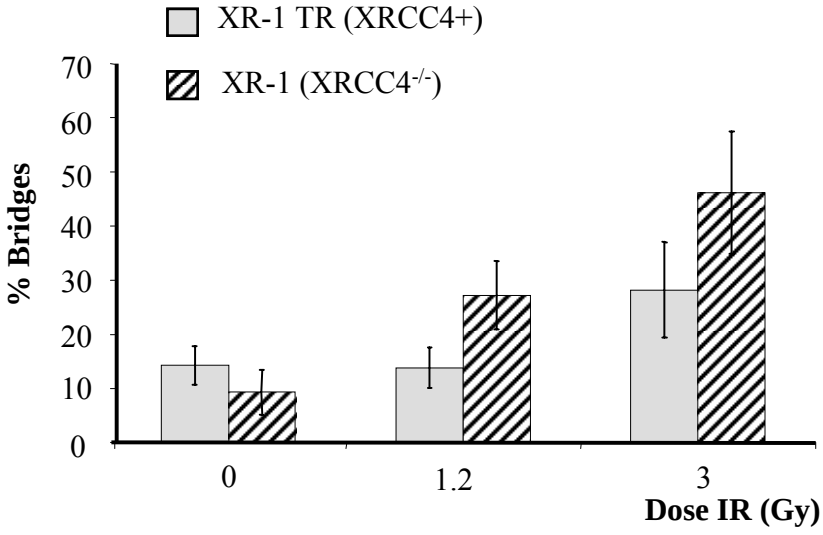

XR-1 TR (XRCC4+)
XR-1 (XRCC4-/-)
% Bridges
70
60
50
40
30
20
10
0
0
1.2
3
Dose IR (Gy)

Figure 17 Enhanced induction of anaphase bridges in siRNA-treated cells or genetic mutants upon IR treatment. (A) HCT116 cells were transiently-transfected with either siKu80 or siXRCC4. Cells were treated with indicated doses of IR three days after transfection. Cells were fixed one day after IR treatment and stained for DNA to count bridges and antibodies to XRCC4 or Ku80 to measure knockdown. Only anaphases with no visible staining were scored for bridges. The transfection efficiency was ~ 90%. (B) Bridge induction in the CHO-K1 parental line or the same background with a Ku80 mutation (XRS-6). (C) Bridge induction in CHO cell lines that were either $XRCC4^{-/-}$ or $XRCC4^{-/-}$ stably-transfected with an XRCC4 expression plasmid (XRCC4+).

2.2.2.3 HR appears to form bridges, when NHEJ is compromised

It has been previously reported that HR levels are increased in NHEJ deficient mutants (Fukushima et al., 2001; Pierce et al., 2001; Allen et al., 2002; Delacote et al., 2002). In order to explore whether HR is the source of bridging in the absence of NHEJ, we used siRNA knockdown of NHEJ proteins (Ku80 and XRCC4) in HR deleted backgrounds (XRCC3 and RAD54B) in all pair wise combinations and examined their capacity to form bridges. Consistently, the increased bridge phenotype by NHEJ induction was reduced back to wild type levels ($p<0.001$, all combinations) (Figure 18). We conclude that the increased bridge induction from loss of NHEJ requires HR.

To confirm these observations, we performed similar experiments in chicken DT40 cells. Again, Ku70 deficiency resulted in elevated levels of bridging ($p<0.02$), while RAD54 mutation alone showed no further increase than wild type. In both cell lines, the HR/NHEJ double deficiency led to a decrease in the high anaphase bridge phenotype seen in NHEJ mutants alone ($p<0.01$) (Figure 19).

2.2.2.4 NHEJ/HR double deficient cells can still repair DSBs

To distinguish whether the reduction of bridging in these double mutants is due to persistent breaks or by an unknown repair mechanism which does not lead to bridges we tested the presence of DNA breaks by phosphorylated H2AX (γ–H2AX) staining, an established marker for DSBs (Rogakou et al., 1998; Sedelnikova et al., 2002). While almost all cells were

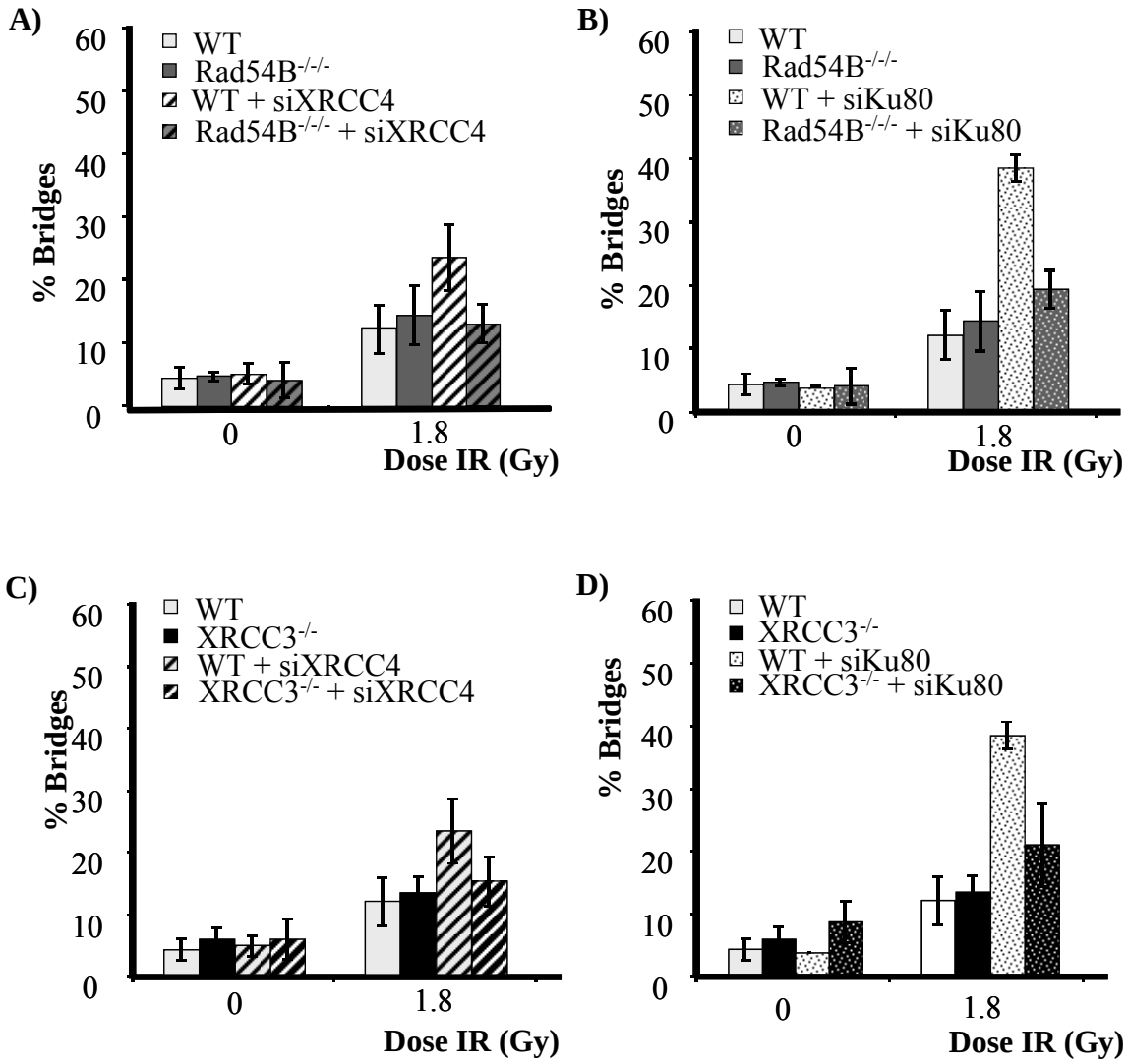


Figure 18 (A-D) Reducing NHEJ activity in HR-deleted backgrounds mostly eliminates the increased bridge frequency from loss of NHEJ in all pair-wise combinations tested ($p<0.001$, all combinations). Cells were treated with IR three days after transfection, fixed one day later and stained with DAPI and antibodies to XRCC4 or Ku80. Only anaphases with no visible staining (~ 90% of total cells) were scored for bridges.

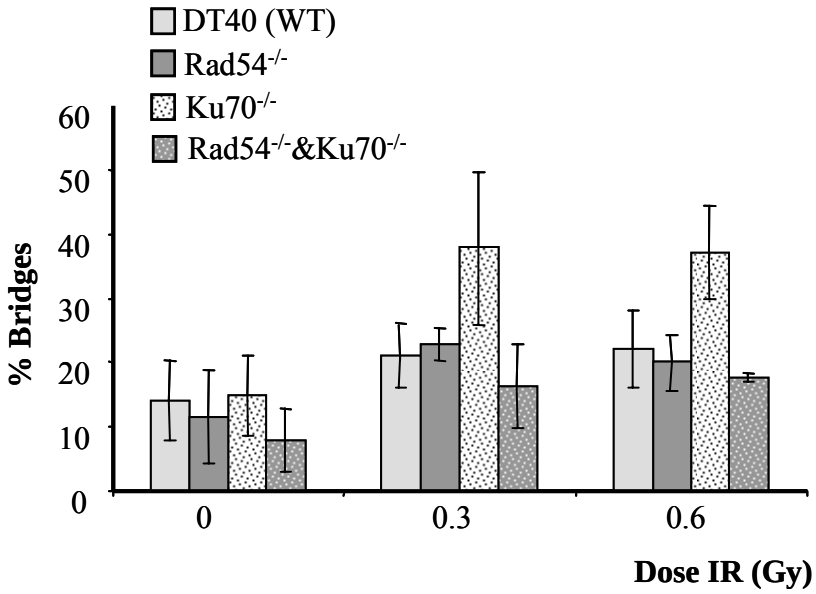


Figure 19 Chicken DT40 cells also show HR-dependent induction of bridges in NHEJ mutants. Wild type, RAD54-deficient, Ku70-deficient, or RAD54/Ku70 double-deficient DT40 cells were treated with the indicated doses of IR and scored for anaphase bridge formation. As observed with human cells, HR-deficiency did not change bridge induction, and mutational loss of NHEJ enhanced the bridge levels, while the HR/NHEJ double mutants showed levels of induction similar to the parental line. Both DIC optics and DAPI staining were needed to distinguish anaphase bridges in these cells.

positive for foci formation 20 minutes after IR, the staining disappeared by 24 hours, at the time anaphase bridging is scored (Figure 20). Even though the differences between these samples were dramatic, quantification for the presence of γ–H2AX foci was not very obvious due to the differences in the size of the foci, which were scattered on many focal planes. Yet, approximate scores for cells containing foci are shown in Figure 21.

Although these results suggest that DSB repair still occurred in the HR/NHEJ defective background, breaks might simply lose γ-H2AX phosphorylation without repair. In order to exclude this possibility, we tested other DNA double strand break markers, 53BP1 and phosphorylated-ATM (P-ATM), which also form foci at the site of DSBs (Figures 22, 23) (Anderson et al., 2001; Bakkenist and Kastan 2003). Once again, the majority of the foci disappeared by 24h in every combination tested (siRNA knockdown of XRCC4 and Ku80 in RAD54B and XRCC3 deficient backgrounds).

2.2.3 Decrease in anaphase bridges does not appear to be due to a decrease in mitotic index

It has been previously shown that cells that are defective for Ku80, XRCC4 or XRCC3 are sensitive to ionizing radiation (Giaccia et al., 1990; Nussenzweig et al., 1997; Brenneman et al., 2000). Moreover, cells deficient for both repair pathways exhibit reduced proliferation (Gorski et al., 2003; Couedel et al., 2004; Mills et al., 2004). Correspondingly in our assays, if cells with damage do not undergo mitosis, they would not form a bridge in anaphase, and this could potentially account for the decrease in anaphase bridging in cells compromised for both pathways.

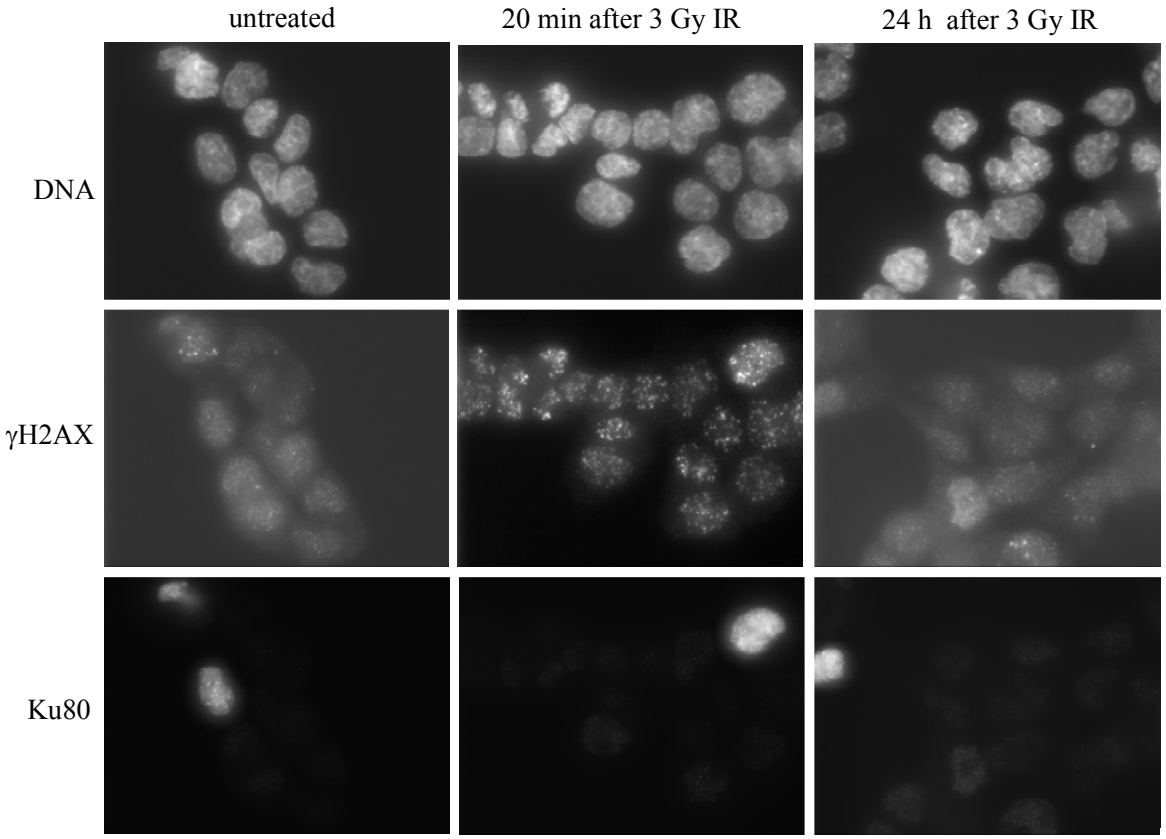


Figure 20 γ-H2AX staining was transient in HR/NHEJ defective cells. RAD54B$^{-/-/-}$ cells were transfected with siKu80 and monitored for the presence of γ-H2AX foci 20min or 24h after IR treatment. Similar results are obtained for all pair-wise HR/NHEJ double deficiencies (data not shown). The formation of foci in repair defective backgrounds was similar to the wild type HCT116 cells. See Figure 21 for quantification.

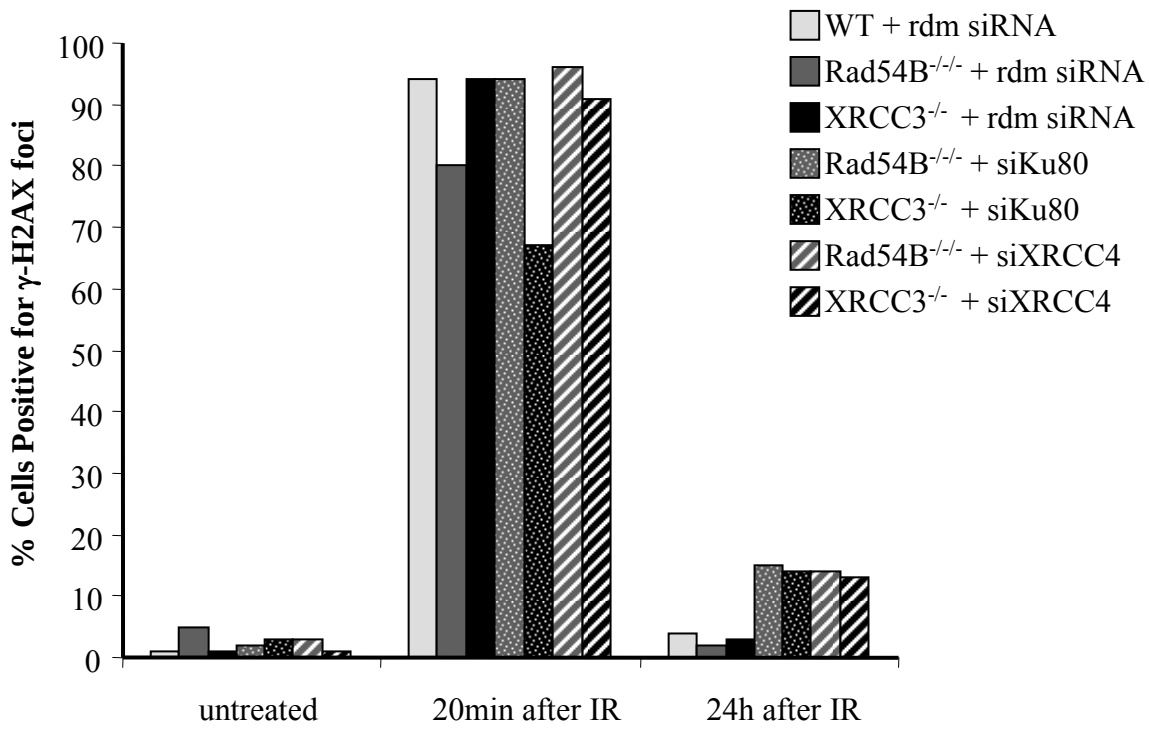


Figure 21 Cells were scored for formation of γ-H2AX foci following IR treatment. Majority of the breaks disappear by 24h in HR/NHEJ defective cells. Similar levels of foci appearance were observed in repair defective backgrounds compared to the wild type HCT116 cells 20min after IR. Although both the number of foci/cell and the number of cells containing foci decreased after 24h, the percentage of cells with γ-H2AX foci was slightly more in NHEJ/HR deficient cells. The percentage of cells that contain foci is shown in the graph. Approximately 500 cells were scored for each experiment.

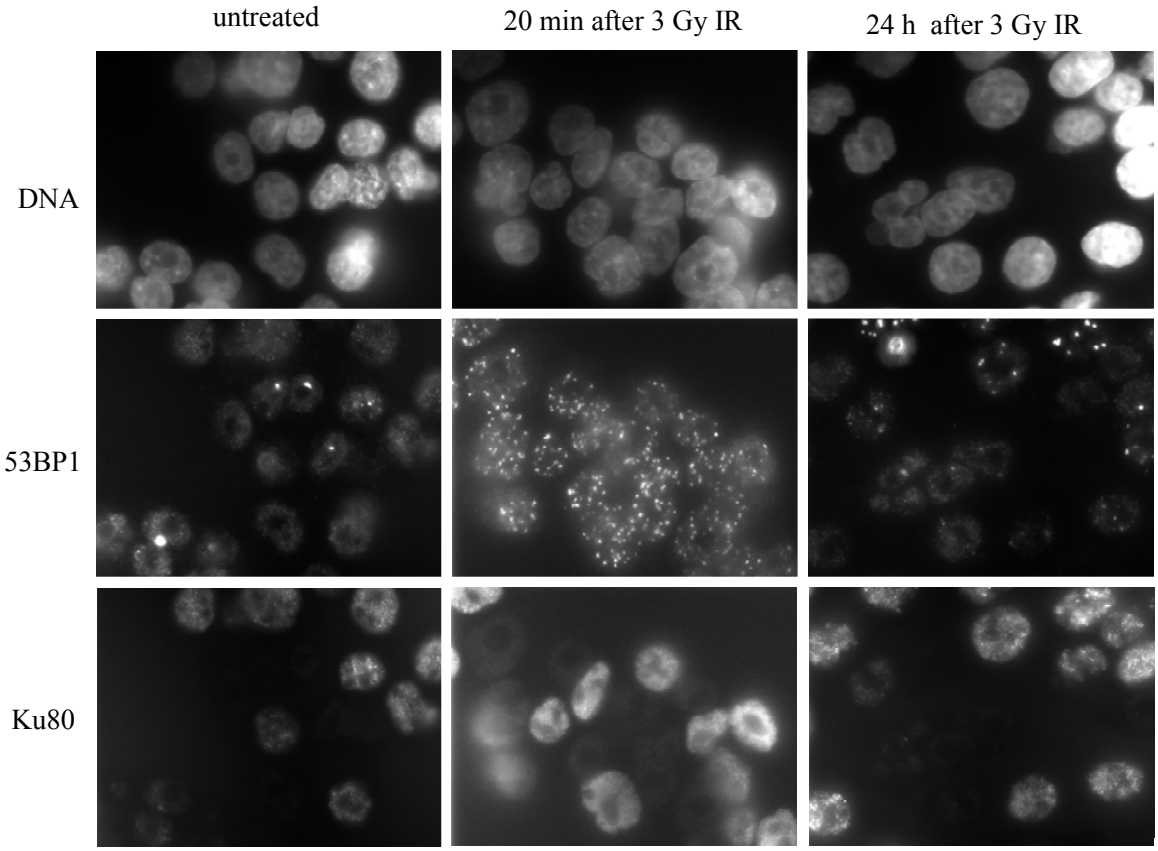


Figure 22 53BP1 staining was transient in HR/NHEJ defective cells. RAD54B$^{-/-/-}$ cells were transfected with siKu80 and monitored for the presence of 53BP1 foci 20min or 24h after IR treatment. Similar results are obtained for all pair-wise HR/NHEJ double deficiencies (data not shown).

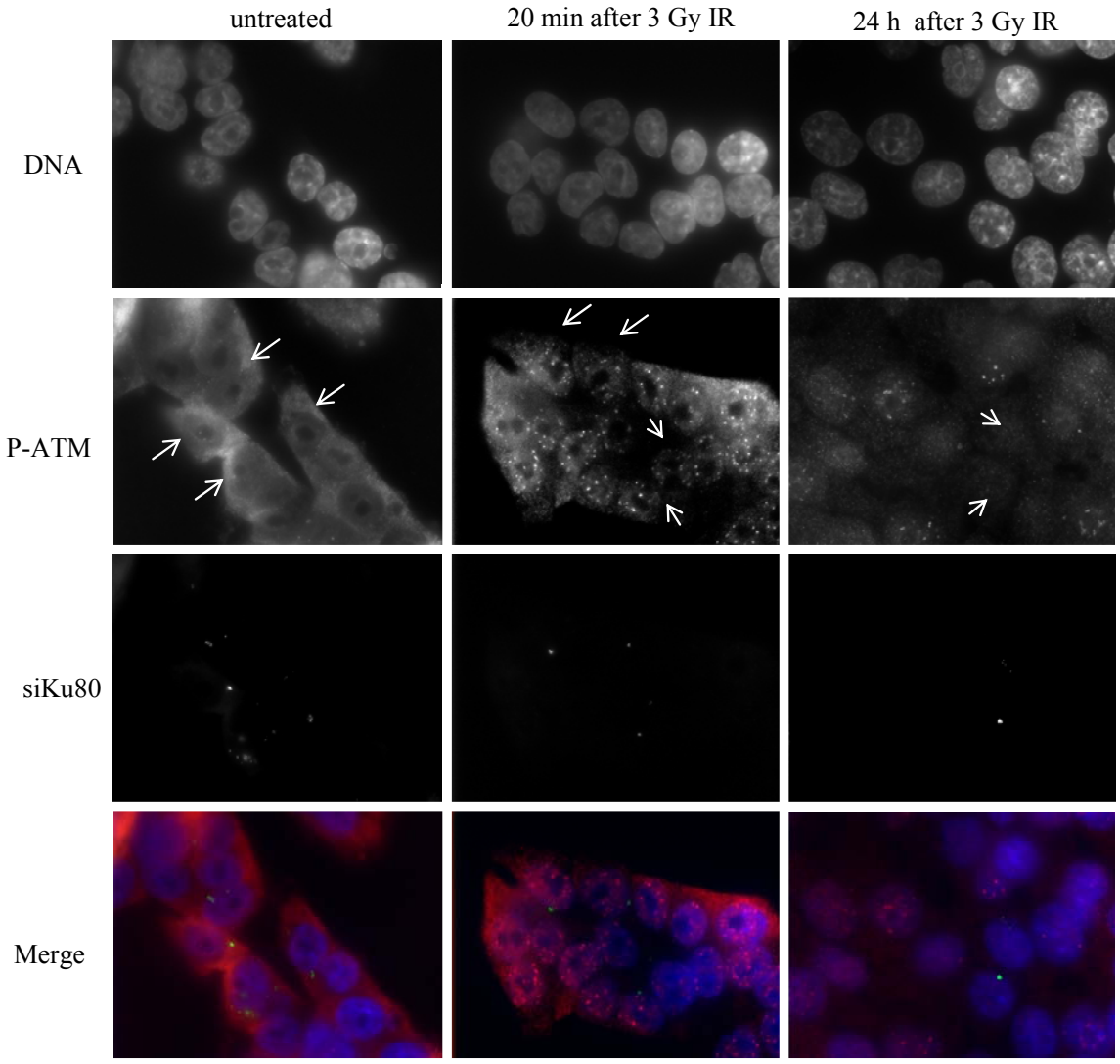

untreated
20 min after 3 Gy IR
24 h after 3 Gy IR
DNA
P-ATM
siKu80
Merge

Figure 23 P-ATM staining was transient in HR/NHEJ defective cells. RAD54B$^{-/-/-}$ cells were transfected with siKu80 and monitored for the presence of P-ATM foci 20min or 24h after IR treatment. Both P-ATM and Ku80 antibodies were from the same species, hence a fluorophore-labeled random siRNA was used as a cotransfection marker (third panel labeled siKu80). Labeled siRNA is 100% associated with knockdown of Ku80 as determined on a separate slide (data not shown), but probably underestimates the number of knocked down cells since only accumulation of siRNA label can be detected by immunofluorescence. Arrows indicate transfected cells. Similar results are obtained for all pair-wise HR/NHEJ double deficiencies (data not shown).

In order to explore whether there is a reduction in proliferating cells, we scored mitotic indices before and after ionizing radiation treatment. While there was a mild decrease in XRCC4 deficient backgrounds, cell division was mostly not affected despite the repair deficiencies (Figure 24). Moreover we did not detect a reduction in cell proliferation in any background during the course of the experiment (data not shown). These results suggest that the decrease in anaphase bridge frequency in repair deficient cells for both pathways cannot simply be explained by a decrease in mitotic population. Consistent with our results, XRCC3 deletion in the same HCT116 cell line has been previously published not to cause sensitivity to ionizing radiation (Yoshihara et al., 2004). Hence it appears that HCT116 cells lack the checkpoints required to arrest cell division.

Since the anaphase bridges are expressed as a percentage in the total anaphase population (number of cells scored in each category is displayed in Table 1), and the fact that mitotic index is not affected in double deficient cells, the decrease in anaphase bridging in these cells does not appear to be due to a decrease in the anaphase population.

2.2.4 In vitro NHEJ ligation assay

Since NHEJ clearly plays a role in the prevention of bridges, can we explain the anaphase bridge variation in cancer cell lines by differences in NHEJ activity? We used an *in vitro* ligation reaction to quantify NHEJ activity in different tumor cell lines (Diggle et al., 2003). Linearized plasmid DNA was incubated with whole cell extracts (WCE) and religation measured by the appearance of dimers and trimers. (The presence of WCE blocked monomeric ligation by an unknown mechanism, as shown by comparison of T4 DNA ligase +/- WCE, (Figure 25D)).

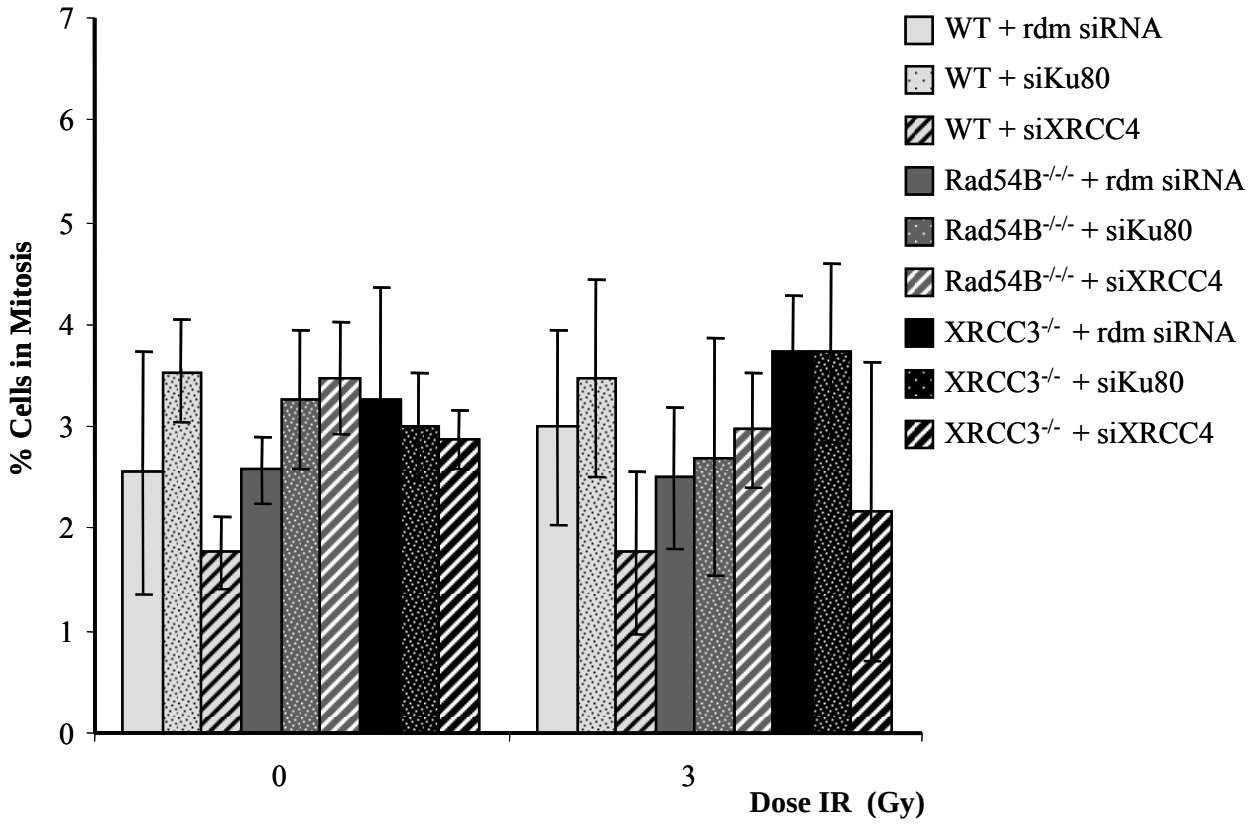


Figure 24 Mitotic indices for HCT116 parental cells and for repair deficient backgrounds. HCT116 cells do not show growth abnormalities in repair defective backgrounds upon ionizing radiation treatment. Each experiment was repeated three times and 500-600 cells were scored per slide from five different positions on the coverslip. (rdm siRNA: random siRNA, siKu80: siRNA to Ku80, siXRCC4: siRNA to XRCC4)

		# of coverslips			Total # of anaphases		
HCT116 genotype	**siRNA**	**0 Gy**	**1.8 Gy**	**3 Gy**	**0 Gy**	**1.8 Gy**	**3 Gy**
WT	*random*	6	5	6	650	660	664
WT	*Ku80*	3	5	5	234	490	467
WT	*XRCC4*	4	6	6	313	525	494
RAD54B-/-/-	*random*	4	7	6	503	1038	624
RAD54B-/-/-	*Ku80*	4	5	5	330	313	298
RAD54B-/-/-	*XRCC4*	4	6	6	336	451	331
XRCC3-/-	*random*	7	4	6	750	446	949
XRCC3-/-	*Ku80*	4	6	6	304	563	523
XRCC3-/-	*XRCC4*	6	9	9	513	860	674
DT40 Genotype							
WT		9	11	11	954	706	810
RAD54		4	6	6	269	336	302
Ku70		9	11	6	518	468	194
RAD54/Ku70		10	10	3	396	296	97
Name cell line	*Genotype*						
V79	WT	5	7	5	902	1079	847
irs3	RAD51C def	6	7	5	1194	985	941
CHO-K1	WT	6	5	6	801	835	565
XRS6	Ku80 def	5	3	2	639	208	97
XR-1 TR	WT	10	6	4	710	316	227
XR-1	XRCC4 def	11	7	4	649	307	271

Table 1 Total number of coverslips and cells scored for each experiment. (WT: wild type, def: deficiency)

Although this assay has been previously shown to measure NHEJ activity (Baumann and West 1998; Diggle et al., 2003), to confirm the specificity of the assay we tested various NHEJ inhibitors.

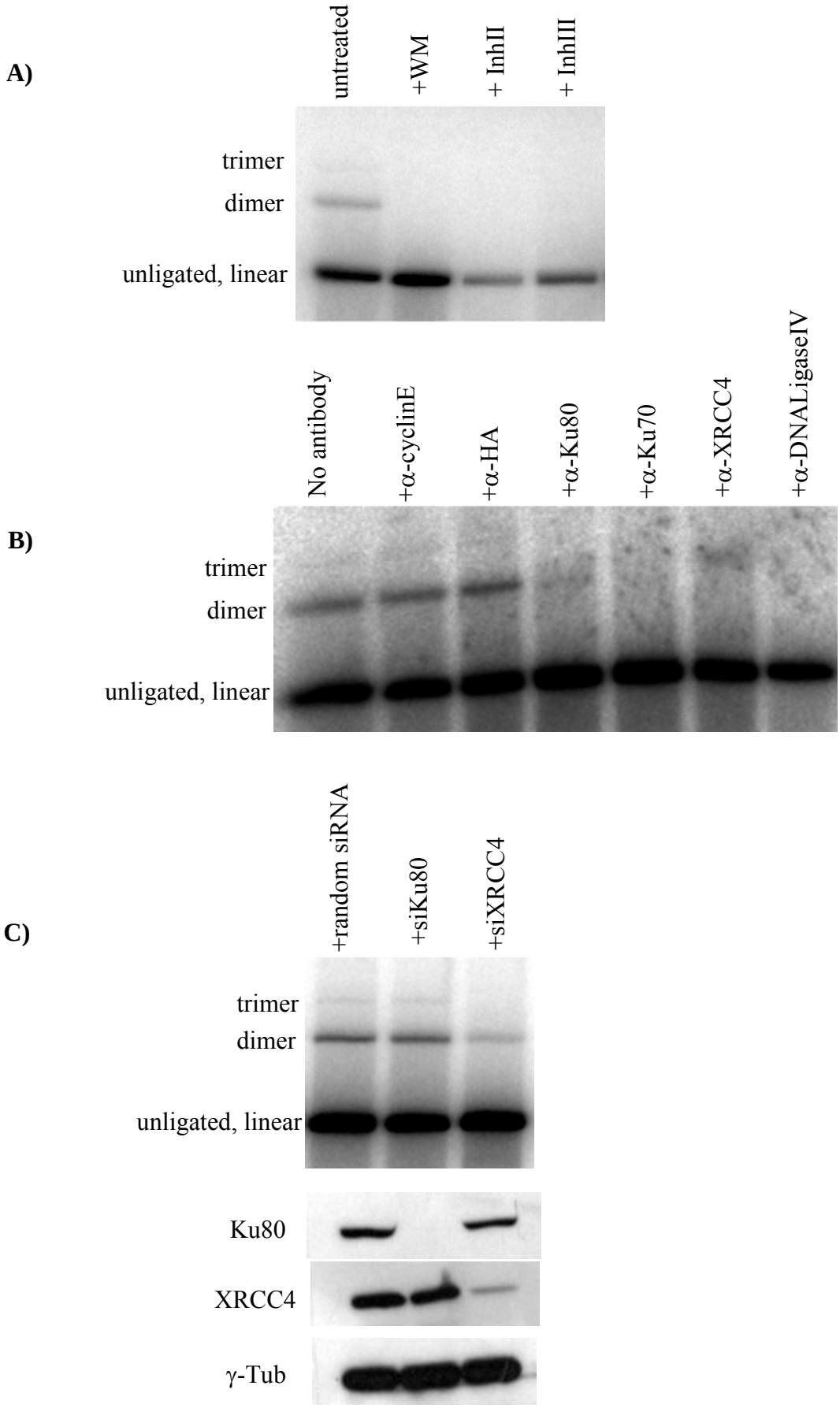
Addition of PI3-kinase inhibitors (to block DNA-PKcs activity), or antibodies specific to NHEJ proteins reduced ligation (Figure 25A, B). Interestingly, while siRNA to XRCC4 diminished ligation, siRNA to Ku80 did not reproducibly decrease ligation, which could be due to residual Ku80 after knockdown (Figure 25C). Consistent with other reports, addition of Ku80 antibodies effectively reduced NHEJ activity with very little ligation (Figure 25B, lane 4) (Baumann and West 1998). The difference could be due to residual Ku80 from the untransfected population or incomplete siRNA knockdown. Alternatively, addition of antibodies might interfere with the activity of other proteins that are in complex with Ku80 and result in a reduction in the NHEJ activity. While this assay could be less sensitive to Ku80 levels, because it was dependent on NHEJ components and we believe reflects cellular NHEJ activity. As expected, the NHEJ activity of HR mutants was undiminished (Figure 25E).

2.2.4.1 NHEJ activity is correlated with the induction of bridges in cancer cells

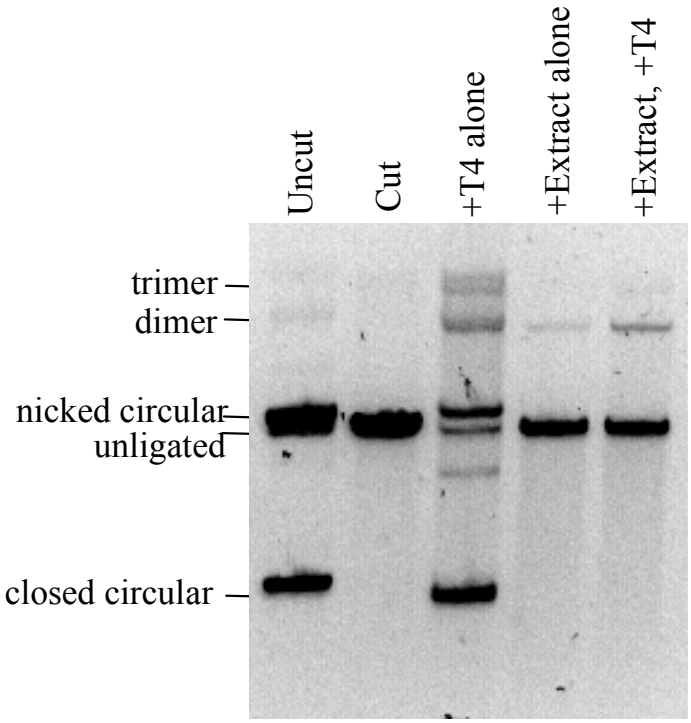
We next tested for a correlation between NHEJ activity and bridge induction for eight tumor-derived and two control cell lines (Figure 26). The RPE-hTERT normal control cell line had very few anaphase bridges prior to treatment and arrested in interphase following IR treatment (Figure 26A and data not shown. Total cell counts are shown in Table 2.). We therefore utilized HEK-293, an immortalized nonmalignant cell line, for a reference. Anaphase bridges in this line increased from ~20% to ~40% after IR exposure. Among the tumor-derived cell lines, the frequency of both pre-existing and induced bridges varied dramatically (Figure

26A, 0Gy), suggesting that the pathway leading to bridge formation has varied levels of activity in different cancer cells. The induced bridge levels are calculated as the difference of anaphase bridge frequency between 1.8Gys and untreated levels, since some cell lines do not increase bridge frequency at doses higher than 1.8Gys (data not shown). To compare levels, we defined NHEJ activity as the ratio of multimers to all plasmid bands.

It appears that the NHEJ activity is generally inversely proportional to the induction of bridges (Figure 27, $p < 0.07$). The relationship is not strictly linear; perhaps reflecting the genetic variance of these independent cell lines. We find no correlation between pre-existing bridges and NHEJ activity (data not shown). These results suggest that the level of induced bridges depends on the NHEJ activity of the cancer cells, although the correlation is rather weak.


A)
untreated
+WM
+ InhII
+ InhIII
trimer
dimer
unligated, linear
B)
No antibody
+α-cyclinE
+α-HA
+α-Ku80
+α-Ku70
+α-XRCC4
+α-DNALigaseIV
trimer
dimer
unligated, linear
C)
+random siRNA
+siKu80
+siXRCC4
trimer
dimer
unligated, linear
Ku80
XRCC4
γ-Tub

D)


Uncut
Cut
+T4 alone
+Extract alone
+Extract, +T4
trimer
dimer
nicked circular
unligated
closed circular

E)

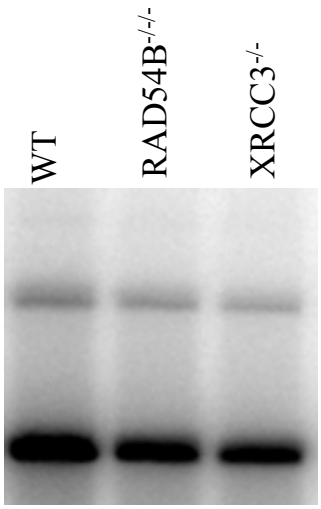
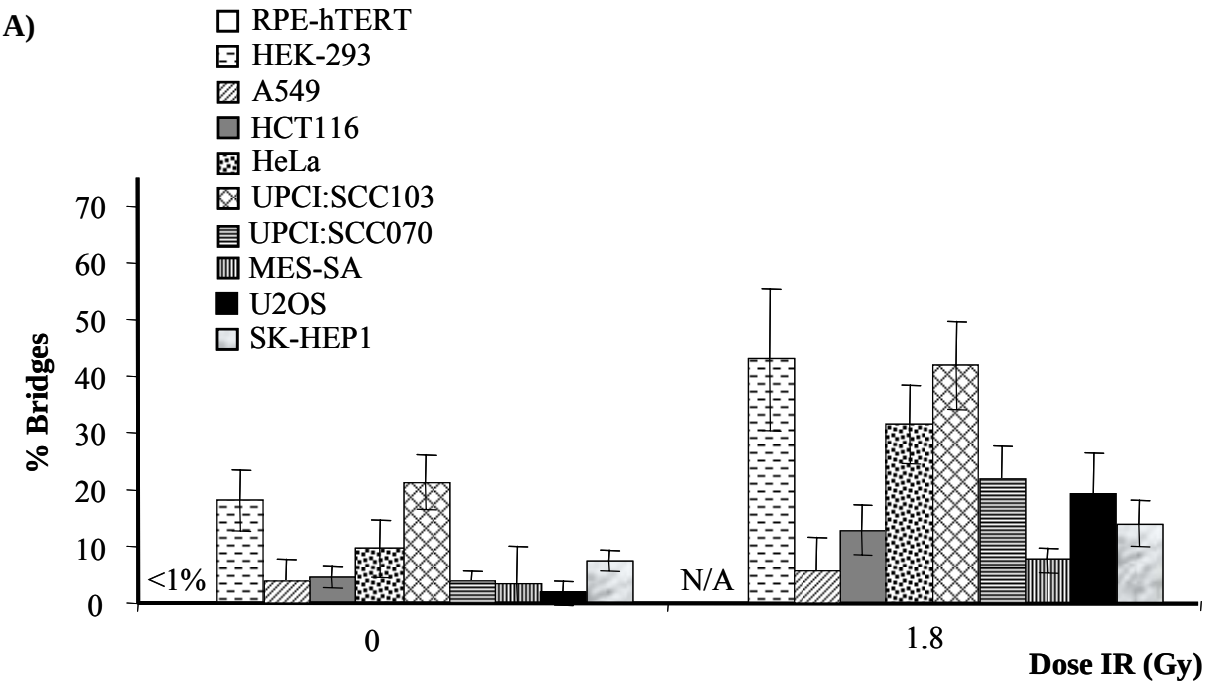

WT
RAD54B-/-/-
XRCC3-/-

Figure 25 (A) Addition of DNA-PK inhibitors abolished ligation capability of WCEs. WM: Wortmannin, InhII/ InhIII: DNA-PK Inhibitor II or III. Forty μg of total protein each reaction. (B) Addition of antibodies specific for NHEJ proteins reduced NHEJ activity. (C) WCEs prepared from cells that were transfected with siXRCC4, but not siKu80, show reduced ligation activity. DNA gels of NHEJ ligation on top, immunoblots of WCEs below. (D) Intramolecular ligation is inhibited by WCE. Equal amounts of WCE and T4 ligase were used in all indicated lanes. Monomeric circles, like the supercoiled band near the bottom of the gel, can reform with the addition of T4 ligase, but not when WCE is present. WCE also seems to suppress the total ligation activity of T4 ligase. DNA was visualized with SYBR-Green (Molecular Probes). (E) HR mutants have no change in NHEJ ligation efficiency.

A)


RPE-hTERT
HEK-293
A549
HCT116
HeLa
UPCI:SCC103
UPCI:SCC070
MES-SA
U2OS
SK-HEP1
% Bridges
70
60
50
40
30
20
10
0
<1%
N/A
0
1.8
Dose IR (Gy)

B)

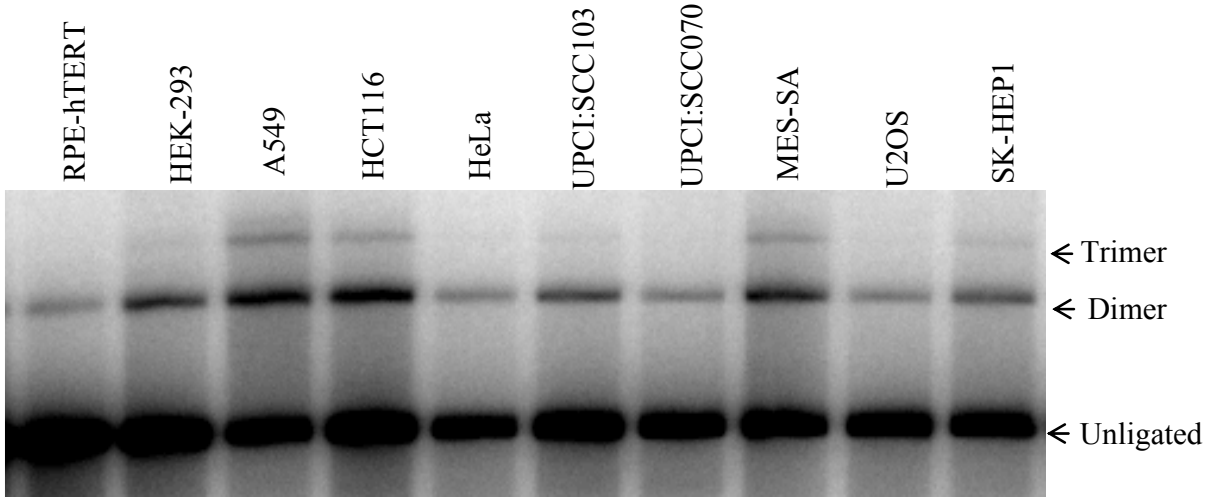

RPE-hTERT
HEK-293
A549
HCT116
HeLa
UPCI:SCC103
UPCI:SCC070
MES-SA
U2OS
SK-HEP1
Trimer
Dimer
Unligated

Figure 26 (A) Bridge induction varies between different cell lines. The indicated cell lines were treated with IR, fixed 1 day after treatment, and stained with DAPI to visualize bridges. The RPE-hTERT are primary retinal pigmented epithelial cells expressing human telomerase. (B) NHEJ activity varies between different cell lines. WCEs from the indicated cell lines were used for end joining reactions, as described in Materials and Methods.

Name cell line	# of coverslips		Total # of anaphases	
	0 Gy	1.8 Gy	0 Gy	1.8 Gy
293	4	5	422	652
A549	6	4	280	137
HCT116	6	5	650	660
HeLa	4	4	208	197
UPCI:SCC 103	6	5	393	232
UPCI:SCC 70	6	7	492	482
MES-SA	4	6	282	251
U2OS	4	4	139	143
SKHEP	6	8	554	570

Table 2 Total number of coverslips and cells analyzed for each experiment.

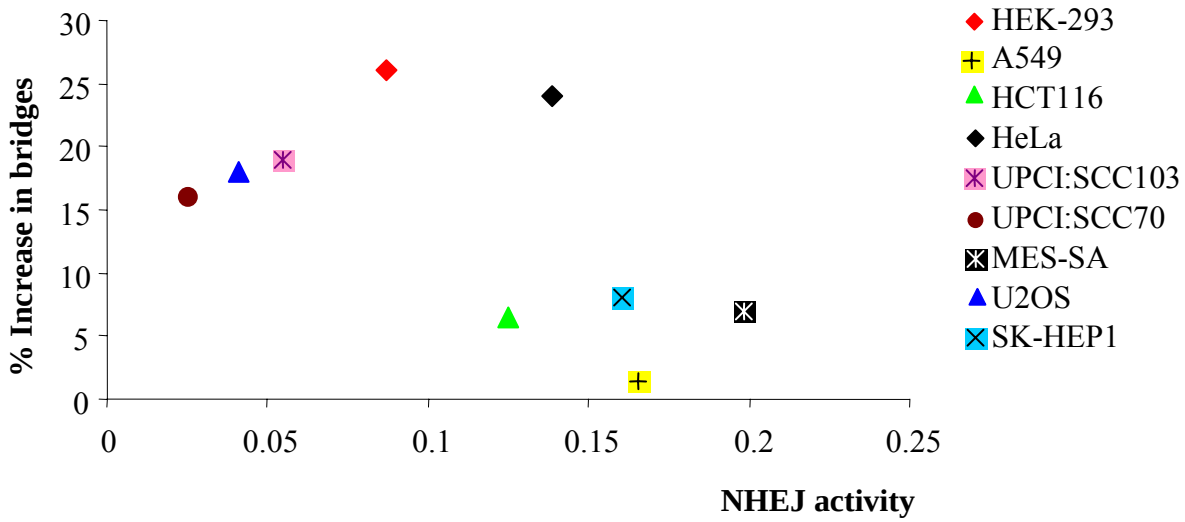


Figure 27 Correlation between NHEJ activity *in vitro* and induction of bridges by IR. The ligation activity of WCEs from the cell lines in Figure 26 (except RPE-hTERT cells) was quantified as described in the text. The median value of NHEJ for each cell line was plotted versus the median percent increase in bridges at 1.8 Gy of IR. The values in this graph were the median for 4-7 experiments for NHEJ activity and 4-12 experiments for bridge frequency. Each experiment gave similar results, but the absolute values of the different data points varied substantially from day-to-day ($p<0.07$).

2.3 DISCUSSION

In this study, we investigated which DNA repair pathway contributes to formation of anaphase bridges using cells compromised in NHEJ, HR or both pathways. Our data support a role for NHEJ as an essential pathway in preventing the formation of chromatin bridges, which lead to gross chromosomal changes. This is consistent with previous observations of chromosomal instability in NHEJ mutants, such as non-reciprocal translocations, or deletions (Difilippantonio et al., 2000; Gao et al., 2000; Bassing et al., 2002; Rooney et al., 2003), some of which could be a consequence of anaphase bridges. Based on our data, HR is not required for the formation of bridges. However surprisingly, in the absence of NHEJ, the HR pathway apparently repairs DSBs in such a way that a chromatin bridge forms in anaphase (Figure 28).

It has been previously demonstrated that DSBs may be repaired by an NHEJ-independent pathway resulting in formation of dicentric chromosomes and subsequent translocations and gene amplifications in cells deficient for XRCC4 or Ligase IV (Zhu et al., 2002) or Ku80/70 (Difilippantonio et al., 2002). The authors have proposed mechanisms which involve incursion of the DSB end into an intact template followed by break induced replication. Replication of the broken chromatid leads to fusion of the sister chromatid ends, formation of dicentrics and cycles of breakage-fusion-bridge. Furthermore, the authors also reported that these chromosomal changes can occur in the absence of both Ku80 (NHEJ) and RAD54 (HR), which implies that intact homologous recombination is not required for bridge formation in the absence of NHEJ. However, these studies did not examine the change in the frequency of dicentrics (or

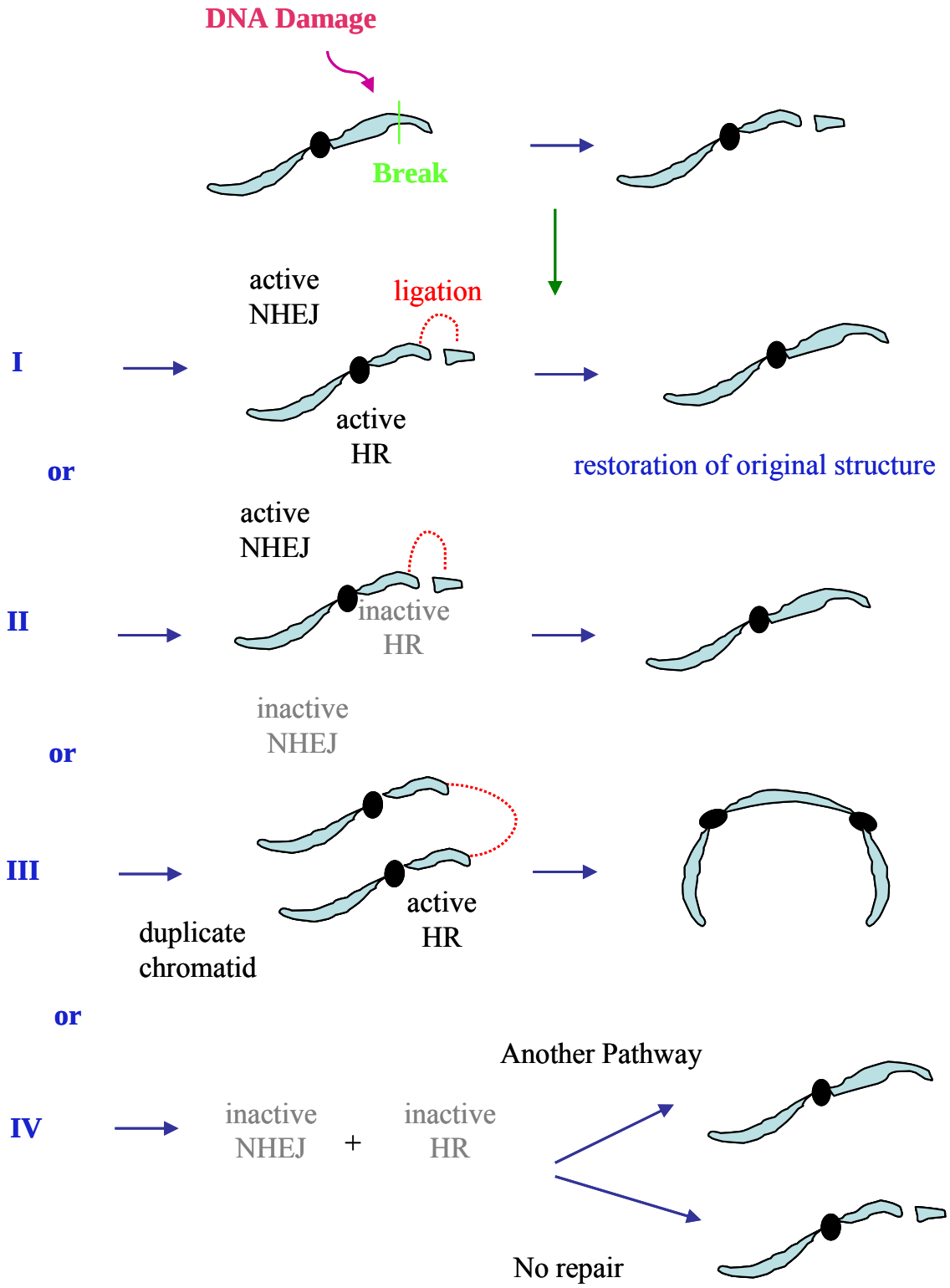

DNA Damage
Break
active
NHEJ
ligation
I
active
HR
restoration of original structure
or
active
NHEJ
II
inactive
HR
inactive
NHEJ
or
III
active
HR
duplicate
chromatid
or
Another Pathway
IV
inactive
NHEJ
+
inactive
HR
No repair

Figure 28 Cells with active NHEJ and HR remove DSBs without forming anaphase bridges (I). NHEJ can still repair the breaks efficiently without bridging in the absence of HR (II). However when NHEJ is compromised, HR repairs the damage, but forms anaphase bridges during this repair (III). In the absence of both pathways, wild type levels of anaphase bridges are observed, which could indicate either the presence of a third repair pathway or persistence of breaks (IV). Our data suggest that majority of the DSBs are repaired, hence a slow acting third pathway could act in the absence of both pathways.

translocations) following DNA damage. It should be noted that despite the reduction in bridging in NHEJ/HR compromised cells, there is still a basal level of induction in bridging which could account for the genomic aberrations observed in these studies.

The mechanism for HR-driven bridge formation in the NHEJ mutants is currently unknown. One possibility is suggested by the observations that a reduction in NHEJ protein levels results in a stimulation of HR-mediated repair (Fukushima et al., 2001; Pierce et al., 2001; Allen et al., 2002; Delacote et al., 2002). The stimulation is greatest if early NHEJ proteins, namely DNA-PKcs and Ku70/80, are diminished, and less if the late-acting XRCC4 is inhibited (Pierce et al., 2001). Not surprisingly then, we found Ku80-deficiency leads to a higher level of bridging compared to lack of the late-acting XRCC4 (Figure 17A-C). Moreover, studies on the motility of free chromosome ends have indicated that immobilization of the ends requires Ku80 (personal communication with Dr. T. Misteli). This result is consistent with the increase in bridging in the absence of Ku80, since formation of an anaphase bridge would require loss of positional constraints for the fusion of incorrect ends. Perhaps enhanced levels of HR crossover intermediates form in the NHEJ-deficient cells which cannot be completely resolved, thus leading to bridges in anaphase. Further analysis will be required to test this model.

The tendency of HR to form anaphase bridges in NHEJ-deficient backgrounds may explain why patients with increased frequencies of HR also carry elevated cancer risk and chromosomal instability (Bishop and Schiestl 2000; Bishop and Schiestl 2001; Thompson and Schild 2002; Bishop and Schiestl 2003; Griffin and Thacker 2004). Loss of heterozygosity, amplifications, or translocations are genetic events common during carcinogenesis, and are

associated with HR. For example, in acute myleloid leukemia, duplication within the *ALL-1* gene involves recombination between *Alu* sequences (Schichman et al., 1994). In chronic myeloid leukemia, the nuclear protein Bcr is translocationally juxtaposed to the Abl kinase, and has been shown to have shared homology at the break site, possibly indicating HR as the mechanism (Jeffs et al., 1998). A future line of investigation would be to test whether diminished NHEJ activity is necessary for these HR-driven illegitimate recombinational changes.

Recently, attention has been focused on mice that are deficient for both HR and NHEJ repair pathways. Derived cell lines have higher frequencies of DSBs as shown by elevated γ-H2AX and chromosomal instability (Gorski et al., 2003; Couedel et al., 2004; Mills et al., 2004). The reduction in bridge formation in our cell lines does not appear to be a consequence of persistent breaks since γ-H2AX-foci disappear after IR. Moreover, other proteins involved in DSB repair, 53BP1 and phospho-ATM, are also no longer present at the break sites. However, we cannot discount the possibility that breaks may lose DSB repair markers without completing repair.

While the disappearance of repair foci and the existence of bridges in the double mutants suggest the possibility of a third pathway to repair the damage, it is also possible both NHEJ and HR may function despite absence of these repair proteins albeit maybe with reduced accuracy or efficiency. A third NHEJ-independent repair pathway has previously been proposed (DiBiase et al., 2000; Wang et al., 2001; Wang et al., 2001). Whilst there is evidence that DNA ligase III and PARP-1 might be involved in this alternative pathway (Wang et al., 2005; Wang et al., 2006), it is not yet well characterized. The back up NHEJ path efficiently is shown remove the majority

of the DSBs in the absence of canonical NHEJ factors and is shown to operate with 20-30 fold slower kinetics (Wang et al., 2001; Wang et al., 2003). This path could potentially account for the repair we see in HR/NHEJ deficient backgrounds, since bridges are scored after 1 cell cycle (~24h). More detailed kinetic studies of anaphase bridge induction in NHEJ/HR deficient backgrounds, where foci disappearance rate is observed between 20min and 24h are required to initially explore this possibility.

In our studies, we also found that intrinsic differences in NHEJ activity might explain why cells form varying levels of bridges in response to IR. However, this correlation is not very strong ($p<0.07$), possibly due to genetic differences in these cancer cells, such as aneuploidy. Since the number of chromosomes in each cell may influence the number of bridges observed, we obtained the karyotypes of these commonly used cell lines (from HyperCLDB and ATCC Cell line databases and personal communication with Dr Susanne Gollin). These results indicate that HCT116 and MES-SA cells display near diploidy, cell lines such as HEK-293, A549, HeLa, UPCI:SCC070, U2OS and SK-HEP1 are close to triploid, and UPCI:SCC103 cells are near pentaploid. Among these, while both HEK-293s and SK-HEP1 cells are hypotriploid, HEK-293 cells show the highest induction of bridges after IR (~26%), and SK-HEP1 cells exhibit only a mild increase (~8%). On the other hand, the pentaploid cell line, UPCI:SCC103, shows an intermittent value with a ~19% raise. Hence total chromosome number is not likely to be the source for the differences in anaphase bridging, however the correlation between induction of bridges and NHEJ activity might be weakened by this added variable.

3.0 CHAPTER III: ANALYSIS OF NHEJ IN INDUCTION OF ANAPHASE BRIDGES AND END JOINING ACTIVITY IN CANCER CELLS

3.1 INTRODUCTION

As discussed in Chapter II, cancer cells which lack proper checkpoint signaling continue cell cycle progression despite defects such as anaphase bridges. Briefly, we hypothesized that anaphase bridging is a consequence of incorrect repair of double stranded breaks (DSBs), defined as fusion of two non-contiguous DNA ends, and we wanted to explore which repair pathway is involved in misfusion. Between the two major repair mechanisms in mammalian cells, non homologous end joining (NHEJ) and homologous recombination (HR), our findings indicated that NHEJ plays an important role in the prevention of bridges and that the intrinsic NHEJ activity in cancer cells (as determined by an *in vitro* ligation assay) negatively correlates with the induction of anaphase bridges.

In this chapter, I shall discuss our studies in determining whether overall NHEJ activity or the expression of any one of the NHEJ proteins has a correlation with the bridging potential. To answer this question, we examined the levels of various NHEJ proteins and compared their

expression to bridge induction as well as NHEJ activity. This analysis does not take into account other changes in activity that are not caused by elevated expression.

During NHEJ mediated repair, the ligation is initiated with the recruitment of Ku80 and Ku70 proteins to the damage site, which protect the DNA ends from degradation and allow further processing (Smith et al., 1999). Subsequently, Ku80/70 recruits DNA Protein Kinase catalytic subunit (DNA-PKcs), and together they form the DNA-PK complex (Dvir et al., 1992; Gottlieb and Jackson 1993). DNA-PKcs both phosphorylates and associates with Artemis and this complex is involved in processing the 5' and 3' DNA overhangs (Ma et al., 2002). Other targets of DNA-PKcs include XRCC4 and Ligase IV, which function in the final ligation step of NHEJ. XRCC4/Ligase IV complex seals the break along with the associated XLF protein (See Figure 4 for illustration of the pathway) (Critchlow et al., 1997; Chen et al., 2000; Ahnesorg et al., 2006; Buck et al., 2006).

XRCC4 is a small (38kDa), nuclear protein, which tightly binds to Ligase IV and increases its stability (Critchlow et al., 1997; Bryans et al., 1999). This binding also enhances the activity of Ligase IV *in vitro*, possibly at the adenylation step (Grawunder et al., 1997). The deficit of XRCC4 causes sensitivity to ionizing radiation (IR), defects in proliferation, and increased genomic instability including chromosomal translocations (Gao et al., 1998; Yan et al., 2006). Moreover, cells which lack XRCC4 show enhanced levels of anaphase bridges after ionizing radiation (Chapter II).

We report here our analysis of the NHEJ components involved in bridge formation. Among these proteins, endogenous XRCC4 expression was correlated with resistance to anaphase bridging. Furthermore, the levels of XRCC4 demonstrated a correlation with the NHEJ activity of tumor cells, suggesting a limiting role of XRCC4 for NHEJ. However, elevation of XRCC4 by itself was insufficient to prevent bridges in cancer cell lines that exhibited lower XRCC4 levels. These results suggest that although XRCC4 appears to have an important role for bridge formation, it is not the only factor that determines induction of bridging. Moreover, coexpression of XRCC4 with its interacting partner, DNA Ligase IV, did not affect the increased bridging phenotype, suggesting that factors other than XRCC4/Ligase IV are limiting in these cells.

3.2 RESULTS

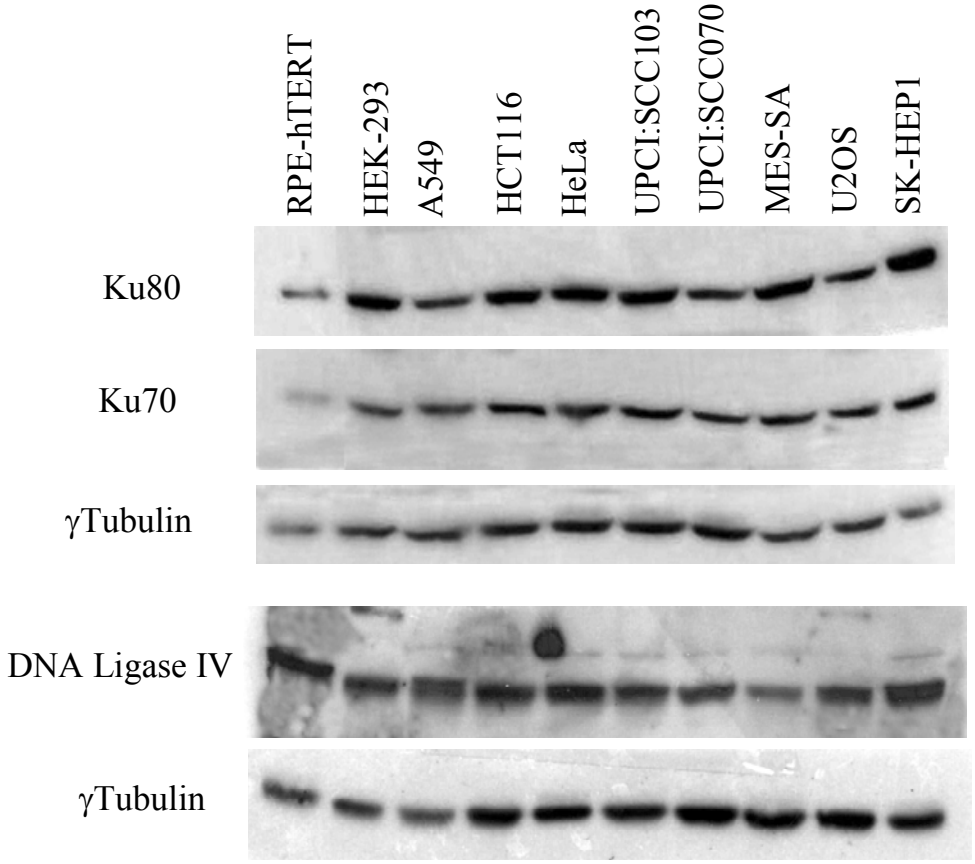
3.2.1 XRCC4 expression is correlated with both the induction of bridges and NHEJ activity

To determine which NHEJ component, if any, correlates with induction of bridges, expression of various NHEJ proteins was assessed in commonly used cancer cell lines. Immunoblotting for DNA-PKcs did not give consistent results, possibly due to transfer inefficiencies of this high molecular weight protein (~420 kDa) (data not shown). DNA Ligase IV, Ku80 and Ku70 expression varied little between cell lines (Figure 29A), as reported formerly (Kasten et al., 1999; Carlomagno et al., 2000; Merel et al., 2002). The levels of XRCC4 expression on the other hand, varied substantially (Figure 29B). Moreover, this variation was inversely correlated with the frequency of induced bridges after IR treatment ($p<0.04$, Figure 30A). Next we determined the NHEJ activity of these cell lines as described previously (Chapter II) and compared with XRCC4 expression. These results indicated that the intrinsic changes observed in XRCC4 expression was correlated with NHEJ activity *in vitro* ($p<0.05$; Figure 30B).

3.2.2 Restoring XRCC4 in cells with low endogenous levels does not rescue the increased bridging phenotype

To test if XRCC4 levels were limiting for the induction of bridges, we exogenously-expressed XRCC4 in two low-expressing cell lines (HeLa and UPCI:SCC103). Overexpression

A)


RPE-hTERT
HEK-293
A549
HCT116
HeLa
UPCI:SCC103
UPCI:SCC070
MES-SA
U2OS
SK-HEP1
Ku80
Ku70
γTubulin
DNA Ligase IV
γTubulin

B)

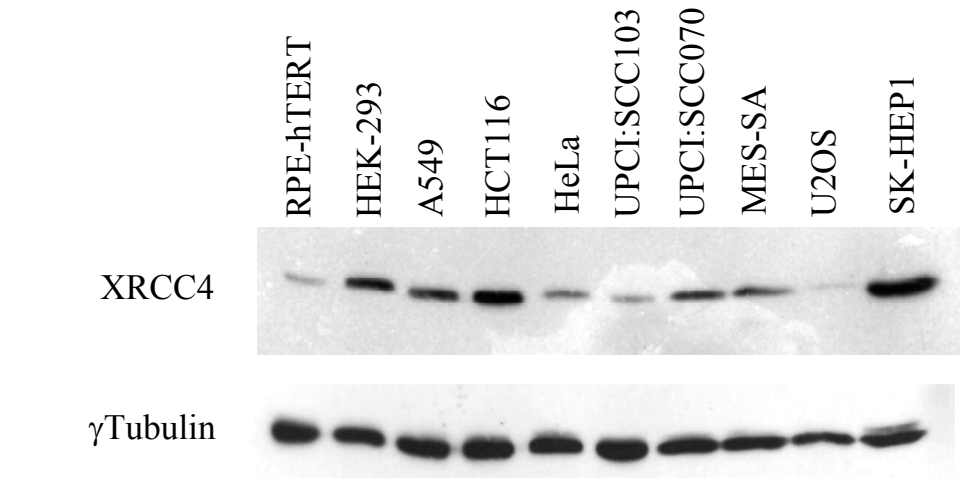
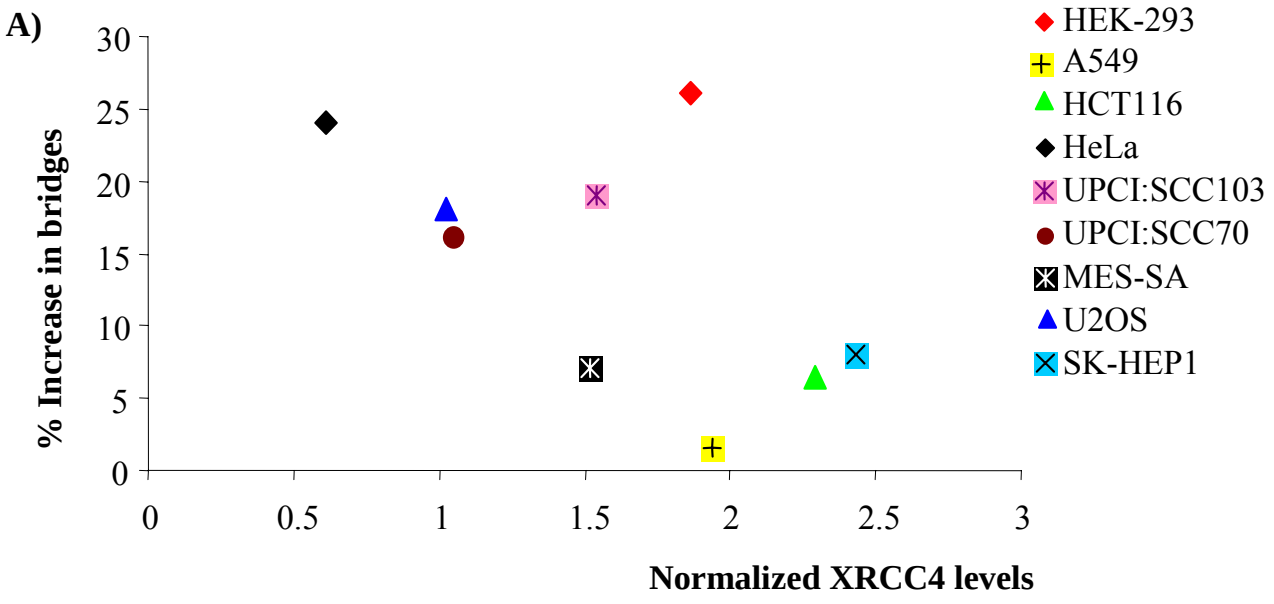

RPE-hTERT
HEK-293
A549
HCT116
HeLa
UPCI:SCC103
UPCI:SCC070
MES-SA
U2OS
SK-HEP1
XRCC4
γTubulin

Figure 29 (A) Ku80, Ku70, and DNA Ligase IV-expression levels were relatively constant, (B) while XRCC4 levels show variation among different cancer cell lines. Twenty μg of total whole cell extracts (WCEs) (also used in NHEJ activity assays in Chapter II) were immunoblotted and γ-tubulin was used as a loading control.


A)
% Increase in bridges
30
25
20
15
10
5
0
0
0.5
1
1.5
2
2.5
3
Normalized XRCC4 levels
HEK-293
A549
HCT116
HeLa
UPCI:SCC103
UPCI:SCC70
MES-SA
U2OS
SK-HEP1

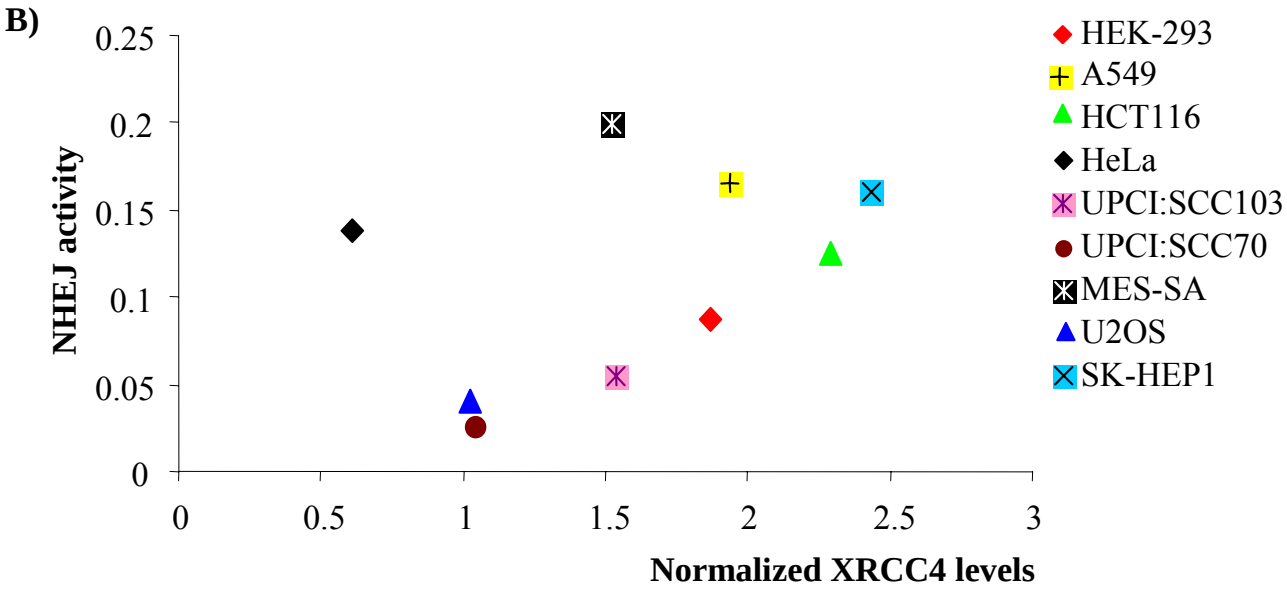

B)
NHEJ activity
0.25
0.2
0.15
0.1
0.05
0
0
0.5
1
1.5
2
2.5
3
Normalized XRCC4 levels
HEK-293
A549
HCT116
HeLa
UPCI:SCC103
UPCI:SCC70
MES-SA
U2OS
SK-HEP1

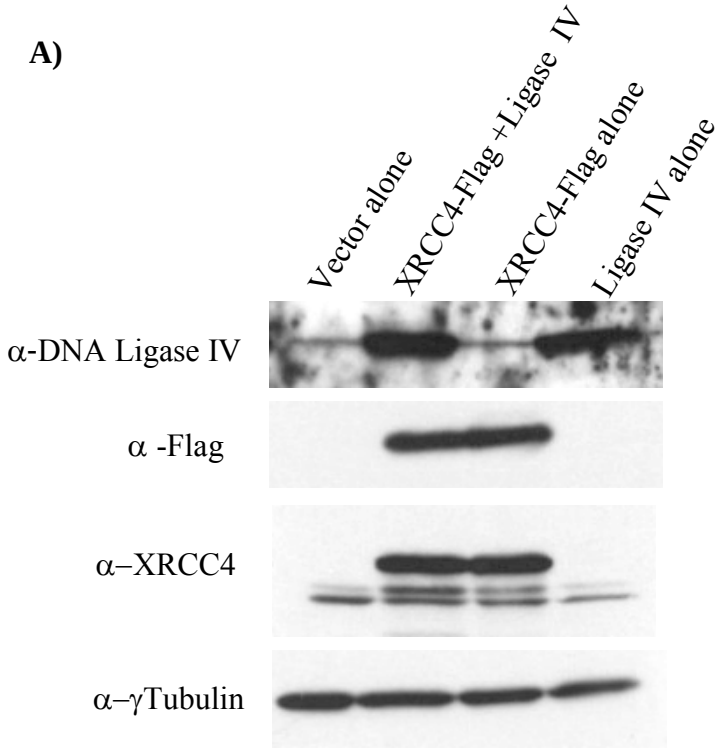
Figure 30 (A) Inverse correlation between XRCC4 levels and bridge induction. XRCC4-expression levels were quantified using Image Guage software, normalized for γ-tubulin levels, and the median plotted against the increase in bridges after ionizing radiation. (B) NHEJ-dependent ligation was positively correlated with XRCC4 levels. The values in these graphs were the median for 4-7 experiments for NHEJ activity and 4-12 experiments for bridge frequency and 3-7 experiments for XRCC4 expression. While individual experiments gave similar trends, there was a variation in the absolute values of experiments performed on different days.

was confirmed by both immunoblotting and immunofluorescence (Figure 31A, B and data not shown). Based on counting cells for the presence of Flag-tag staining, elevated expression of XRCC4 did not reduce bridge formation in response to IR in either tested cell line (Figure 32). Exogenously-expressed XRCC4 was able to suppress the elevated bridge phenotype of an $XRCC4^{-/-}$ CHO mutant (Figure 33). These observations suggest that the exogenously-expressed XRCC4 was active, but could not complement the bridging defect of the low-expressing cell lines.

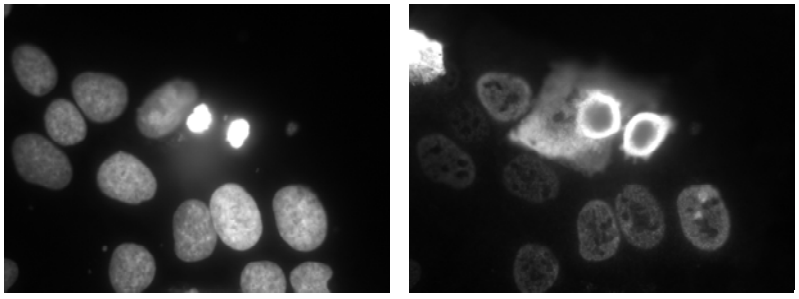
This result indicates the presence of other variables playing a role in determining bridge frequency. To test whether this variable is DNA Ligase IV, the binding partner of XRCC4, we coexpressed XRCC4 and Ligase IV (Figure 31A and data not shown). Transfection was controlled by the presence of GFP cotransfection marker for Ligase IV overexpression. However, we did not control for the activity of Ligase IV expressed from our construct. Simultaneous expression of these proteins did not reduce anaphase bridging (Figure 32) indicating that Ligase IV levels are not limiting in these cell lines.

While the levels of XRCC4 were correlated with bridge induction, other unknown variables apparently influence bridge frequency, since restoring XRCC4 levels cannot rescue increased anaphase bridging. Thus, we believe XRCC4 is a potentially useful marker for anaphase bridges from DNA damage, but is not limiting bridge formation in the tested cancer cell lines.

A)



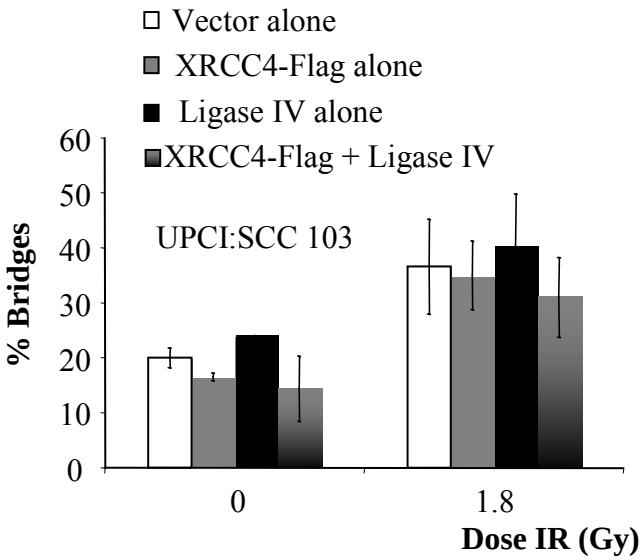
B)



DNA XRCC4-Flag

Figure 31 (A) Expression levels of DNA repair proteins were increased after transfection with p3XFlag-XRCC4 and/or pTSIGN-Ligase IV plasmids. Cells were lysed 1 day after transfection and immunoblotted for DNA Ligase IV, Flag-tag, XRCC4 or γ-tubulin. (B) UPCI:SCC103 cells were transfected with p3XFlag-XRCC4 and stained with anti-Flag antibodies. Cells with Flag-tag expression were used for quantification of bridges. Similar results were obtained for HeLa cells.

A)


Vector alone
XRCC4-Flag alone
Ligase IV alone
XRCC4-Flag + Ligase IV
UPCI:SCC 103
% Bridges
60
50
40
30
20
10
0
0
1.8
Dose IR (Gy)

B)

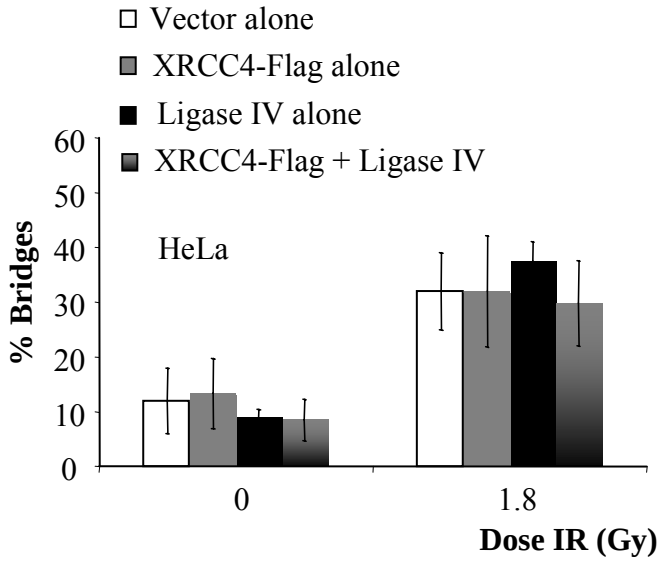

Vector alone
XRCC4-Flag alone
Ligase IV alone
XRCC4-Flag + Ligase IV
HeLa
% Bridges
60
50
40
30
20
10
0
0
1.8
Dose IR (Gy)

Figure 32 Overexpression of XRCC4 does not rescue the bridge induction phenotype. Both UPCI:SCC103 (A) and HeLa (B) cells were transfected with p3XFlag-XRCC4 alone, pTSIGN-Ligase IV (with H2B-GFP marker) or both plasmids. Cells were scored for their bridge forming potential based on either the presence of Flag-tag or the cotransfection marker.

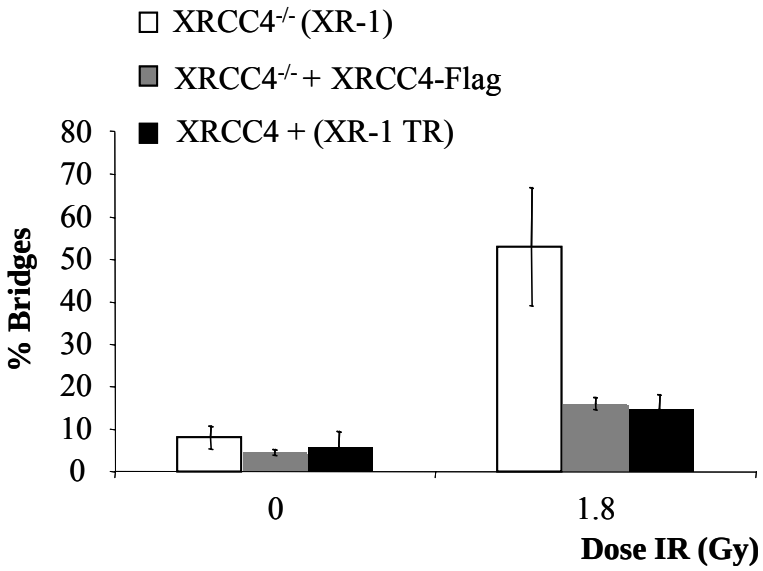


Figure 33 Overexpression of XRCC4 corrected the increased bridging phenotype in XRCC4$^{-/-}$ CHO cells. XR-1 (XRCC4$^{-/-}$ CHO cell line) cells were transfected with p3XFlag-XRCC4 and were scored for their bridge forming potential based on the presence of Flag-tag. XR-1 TR: XR-1 cells stably transfected with wild type XRCC4 gene.

3.3 DISCUSSION

In this study, we tested whether intrinsic genetic differences can explain why cells form various levels of bridges in response to ionizing radiation. Our first candidates were NHEJ proteins, since our previous studies suggested a preventative role for NHEJ in anaphase bridging (Chapter II). Because there is no direct quantitative analysis for activity for each of the components, we assayed for the expression of NHEJ proteins. Among the NHEJ players tested, Ku80, Ku70, and DNA Ligase IV demonstrated similar expression between different cell lines, while XRCC4 levels displayed variation. A variation in NHEJ proteins in cancer cells had previously been of interest to other laboratories for various reasons. In agreement with our findings, both Ku70 and Ku80 levels appeared to be similar, and differences in XRCC4/Ligase IV levels were detected, although different cell lines were used in these studies (Kasten et al., 1999; Carlomagno et al., 2000; Merel et al., 2002). However, these groups failed to find correlations between expression of proteins and the phenotypic differences such as radiosensitivity of the patients or *in vitro* ligation activity. Here we report that the reduced expression of XRCC4 is correlated with the induction of anaphase bridging. Moreover, the ligation capacity of the cancer cells also appears to positively correlate with XRCC4 levels.

If XRCC4 was the only factor playing a role in determining the tendency to form bridges, restoring XRCC4 levels in cell lines with low endogenous expression would be expected to rescue the increased frequencies of bridging. However, increasing XRCC4 expression alone does not significantly change the induction of anaphase bridges, indicating that XRCC4 probably acts

with other factors in this process. Coexpression of XRCC4 with a potential candidate factor, DNA-Ligase IV, did not change resistance to bridging suggesting DNA Ligase IV is not limiting for these cells. Interestingly, the interaction between XRCC4 and Ligase IV has been reported to affect the stability of Ligase IV and low XRCC4 expression corresponded to low Ligase IV levels (Bryans et al., 1999). The authors demonstrated that increasing XRCC4 levels in these cell lines stabilizes Ligase IV and enhances its expression (Bryans et al., 1999). In contrast to previous reports, the change in XRCC4 expression did not correlate with the levels of DNA Ligase IV, but different cell lines were used in these studies. These results suggest that cancer cells might have alternative mechanisms to stabilize Ligase IV.

Based on assays using nuclear extracts, it has been previously suggested that XRCC4/Ligase IV complex is rate limiting for NHEJ (Nick McElhinny et al., 2000; Huang and Dynan 2002). This study further suggests that a natural correlation exists for the intrinsic expression of XRCC4 and the intrinsic ligation capacity of the extracts supporting a limiting role for this protein. This is in contrast to Merel et al. (2002) who found that XRCC4 levels did not correlate with the *in vitro* NHEJ activity from different breast cancer lines. The differences between the studies may be due to different methodology or different source material for the malignant lines.

NHEJ factors are potential tools both as diagnostic markers and targets for chemotherapy and radiotherapy, since deficiencies in these proteins factors confer sensitivity to DNA damaging agents. Among the NHEJ proteins, XRCC4 has been previously demonstrated to acquire mutations in cancer cells and small nucleotide polymorphisms (SNPs) in the XRCC4 gene have

been associated with elevated cancer risk (Fu et al., 2003). Moreover, XRCC4 has recently been proposed as a diagnostic tool in non-BRCA1/2 heritable breast cancer cases (Allen-Brady et al., 2006). Based on our studies, among the tested NHEJ proteins, XRCC4 seems to be correlated with anaphase bridging, which is additional justification as a testing its importance for diagnostic tool.

4.0 CHAPTER IV: THE APOPTOTIC PATHWAYS AND THE ABILITY TO SENSE DNA DAMAGE ARE INTACT IN ORAL CANCER CELLS WITH HIGH ANAPHASE BRIDGES.

4.1 INTRODUCTION

Cancer development is a consequence of DNA damage. Normal cells with intact checkpoints and repair machinery either arrest until the damage is corrected or undergo programmed cell death if the damage exceeds the cells' repair capacity. On the other hand, cancer cells continue proliferation despite the existing defects and frequently are resistant to apoptotic stimuli.

Most of the current cancer therapies, including chemotherapy and radiation treatment, are based on triggering apoptotic pathways and selective death of cancer cells. Once the decision to die is made, several morphological and biochemical changes occur. Commonly, these can be characterized as fragmentation of DNA, a change in cell size, membrane blebbing, or chromosomal condensation (Hengartner 2000). Other intracellular changes include, but are not limited to, induction of tumor suppressor proteins such as p53, activation of caspases, inhibition

of both Bcl-2 family proteins and inhibitor of apoptosis proteins (IAPs) (Fulda and Debatin 2006).

While most therapies are based on killing cells by apoptosis, cancer cells may have disturbed apoptotic pathways, which contribute to failure of the treatment. These disturbances could be at various levels, including overexpression of anti-death proteins, inactivation of the proteins involved in apoptotic signaling pathways or inability to sense damage initially. In support of this, cancer cells which cannot undergo apoptosis have been reported in the literature (Zhao et al., 1997; Alam and Ratner 2001). However, apoptosis is not the only form of cell death and these cells can still be effectively eradicated through other means, involving necrosis, mitotic catastrophe, or autophagy (Abend 2003; Brown and Wilson 2003; Okada and Mak 2004).

A characteristic element of anti-tumor strategies that trigger apoptosis is combining different drugs and killing cancer cells selectively with minimal damage to rest of the organism. Therefore, various approaches have been taken in order to increase the cytotoxicity of the damaging reagents and tendency of cancer cells to respond with apoptosis. For example, mutual action of S-phase specific poisons and irradiation has been shown to sensitize cells to apoptosis (Gorczyca et al., 1993). Moreover, triggering cell differentiation following DNA damage increases programmed cell death and has been proposed as another anti-tumor strategy (Del Bino et al., 1994; Darzynkiewicz 1995). Anti-apoptotic proteins, such as Bcl family members, have been attractive targets for cancer therapy. Consequently, small, membrane permeable chemicals inhibiting the activity of anti-apoptotic proteins have been identified and combining this treatment with chemotherapy has been shown to increase cell death (Wang et al., 2000; Real et

al., 2004). All of these strategies have taken numerous facts about cancer cells into consideration. Cancer cells undergo several rounds of cell division and have inactive checkpoints, which make them more vulnerable to selective killing upon damage in S-phase, especially given that normal cells are arrested in G1. In many cases, tumor cells may be de-differentiated and make extensive amounts of anti-death proteins and become highly sensitive to apoptosis when the conditions are reversed.

As described in the previous chapters, anaphase bridging is a common phenomenon in cancer cells. We hypothesized that bridging occurs as a result of erroneous repair of the broken ends by fusion of two wrong ends. Consequently, cells escape from the inhibitory effects of DSBs since there are no further telomere-free DNA ends. Hence anaphase bridging can be considered a method of uninterrupted cell cycle progression without undergoing arrest or apoptosis. If this is true, a combined therapy of induction of DSBs by radiation treatment, and blocking repair proteins involved in bridging could serve as another potential strategy to increase apoptosis in tumors with high frequencies of anaphase bridges.

With this idea in mind, initial studies were performed in order to test whether the apoptotic pathways are intact in cancer cells which exhibit high levels of bridges following DSB induction. UPCI:SCC103 and UPCI:SCC040 cell lines were subjected to H_2O_2 treatment and assayed for their ability to sense DNA damage, induce proteins involved in cell death signaling and execute apoptosis.

4.2 RESULTS

4.2.1 Oral cancer cells can sense DNA damage

UPCI:SCC103 and UPCI:SCC040 cells were treated with H_2O_2 and the occurrence of DSBs was confirmed by the detection of TUNEL positive cells (data not shown). DSB marker γ-H2AX was used to observe cellular response to DNA damage via formation of foci. Cells were fixed 30min and 1h after H_2O_2 treatment (Figure 34). Both cell lines showed intense γ-H2AX foci after induction of DSBs at these time points, suggesting that pathways involved in sensing DNA damage are intact.

4.2.2 Cancer cells show induction of p53 and change in subcellular localization following DNA damage

p53 has been often considered as the "guardian of the genome" as it plays an important role in making the decision between cell cycle arrest and cell death in response to DNA damage and various cellular stresses. It acts as a signal transducer and results in changes in the expression level of hundreds of genes via its ability to function as a transcription factor. These genes include apoptotic genes such as BAX, Bcl-L, FAS1, and genes involved in cell cycle regulation such as p21 WAF1/CIP1, GADD45, CyclinD1 or MDM2, which negatively regulates p53 activity (reviewed in (Tokino and Nakamura 2000)).

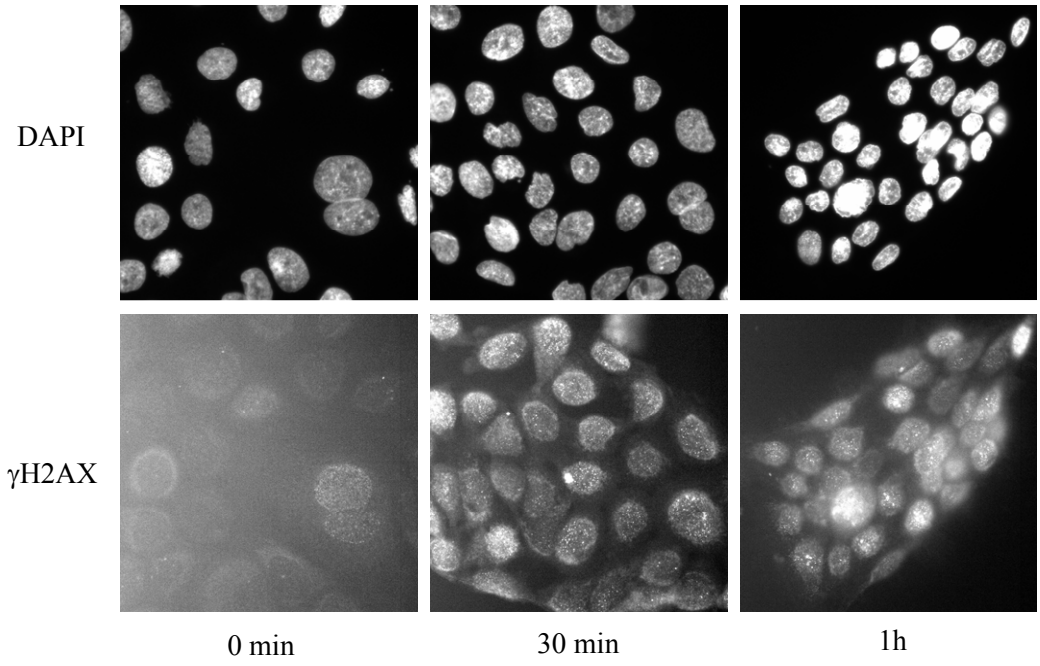


Figure 34 Cancer cells appear to be able to sense the DNA damage as judged by γ-H2AX foci formation. UPCI:SCC103 cells were treated with 4mM H_2O_2 for 10min, and fixed either 30min or 1h after treatment and stained for γ-H2AX and DNA. Foci formation was observed in almost all cells.

p53 activity can be modulated by different mechanisms: an increase in levels, either by enhanced half life or by new transcription, post-translational modifications such as phosphorylation, acetylation, ubiquitination, cis/trans isomerization, neddylation, sumolytion, methylation, ribosylation and O-glycosylation events or a change in subcellular localization from the cytoplasm to the nucleus (Liang and Clarke 2001; Lacroix et al., 2006).

During the cell cycle, in normal cells, p53 is located mostly in the cytoplasm through G1, translocates into the nucleus in G2/ early S, and shuttles back to the cytoplasm in late S (Shaulsky et al., 1990). MDM2 protein, which binds to and promotes p53 degradation by ubiquitination (Haupt et al., 1997), is involved in transportation of p53 from the nucleus to the cytoplasm (Roth et al., 1998; Tao and Levine 1999). Upon damage, MDM2 is sequestered in the nucleolus, which frees p53 to activate its target genes and p53 accumulates in the nucleus (Tao and Levine 1999).

Mutations affecting p53 function are very frequent in cancer cells. Indeed, loss of p53 is correlated with drug resistance and predisposes mice to tumor formation (Attardi and Jacks 1999; Wallace-Brodeur and Lowe 1999). Hence, we assayed for p53 activity and localization in UPCI:SCC103 cells. While p53 levels increased only slightly upon treatment with H_2O_2, there was significant augmentation of p21 levels, which is a target of p53 (Figure 35). Interestingly, p53 appeared to be sequestered in a subnuclear area in all untreated cells. After DNA damage, p53 was distributed diffusely in the nucleus and cytoplasm (Figure 36). While the intensity of immunofluorescence staining for p53 increased after treatment, the enhancement was mostly

detected in the cytoplasm. These results suggest that p53 may be active; however we cannot totally argue that it is fully functional, since the increase observed in its expression is not very compelling and p21 is only one of the many targets of p53. More experiments testing p53 regulations have to be performed to test its activity and the genomic DNA be sequenced.

4.2.3 Oral cancer cells can undergo apoptosis upon DNA damage

Next we tested the ability of these cells to mediate apoptosis. As mentioned previously, one characteristic feature of programmed cell death through apoptosis is DNA fragmentation, which can be detected as formation of DNA ladders following gel electrophoresis. Cells were treated with H_2O_2 and collected after 3, 6 and 9 days. It is important to note that the concentration of H_2O_2 used in this assay was 10 times higher than what was used to induce anaphase bridges.

We observed that H_2O_2 treatment under these conditions can lead to apoptosis starting from 3 days after exposure (Figure 37). Cell death mostly occurred in cells collected from the floating population and to lesser extent, at 9 days after treatment, in the attached cell population (Figure 37, lane 8). This result indicates that at least some of the oral cancer cell lines tested in this study can undergo apoptosis. However, there is also an apoptosis resistant population, which is indicated by no ladder formation in the attached cells. It will be interesting to see whether these cells can be sensitized further when repair proteins involved in bridge formation are blocked.

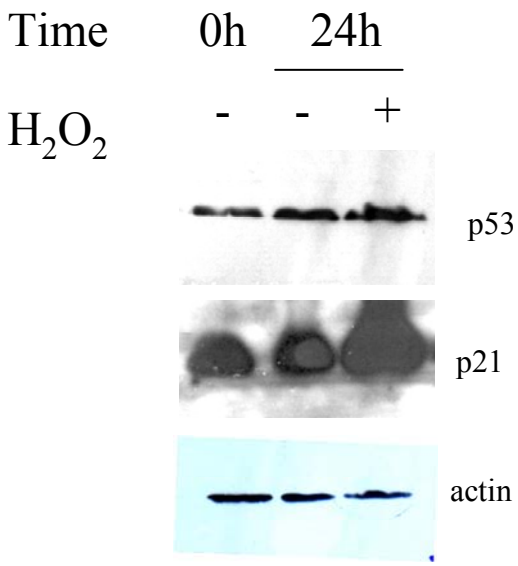


Figure 35 While p53 levels are only slightly increased upon H_2O_2 treatment, p21 induction is more pronounced. UPCI:SCC103 cells are subjected to 4mM H_2O_2 and lysed in RIPA buffer 24h after treatment. 100μg of total cell lysate is used for immunoblotting for p53 and p21. Actin is used for a loading control.

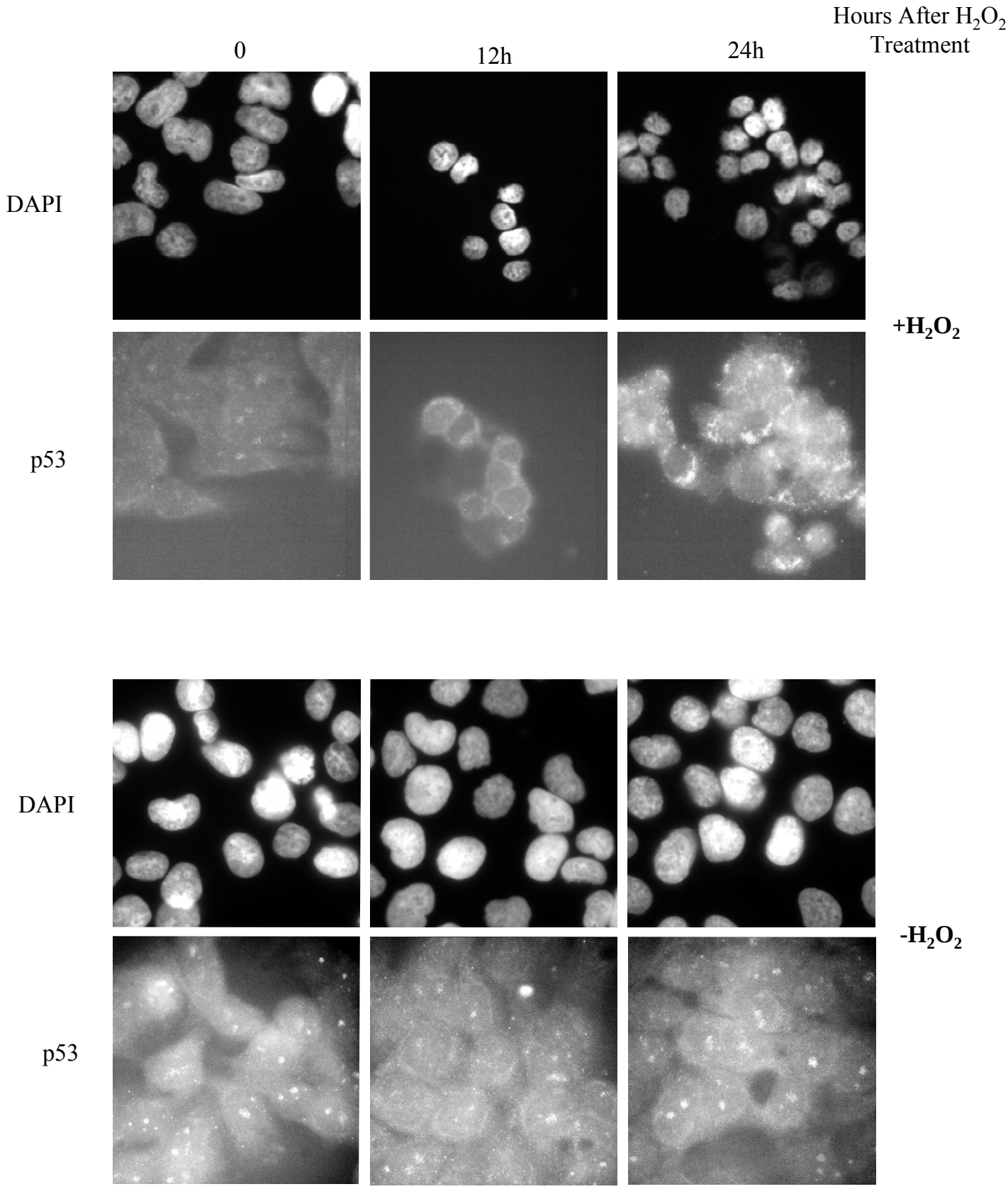
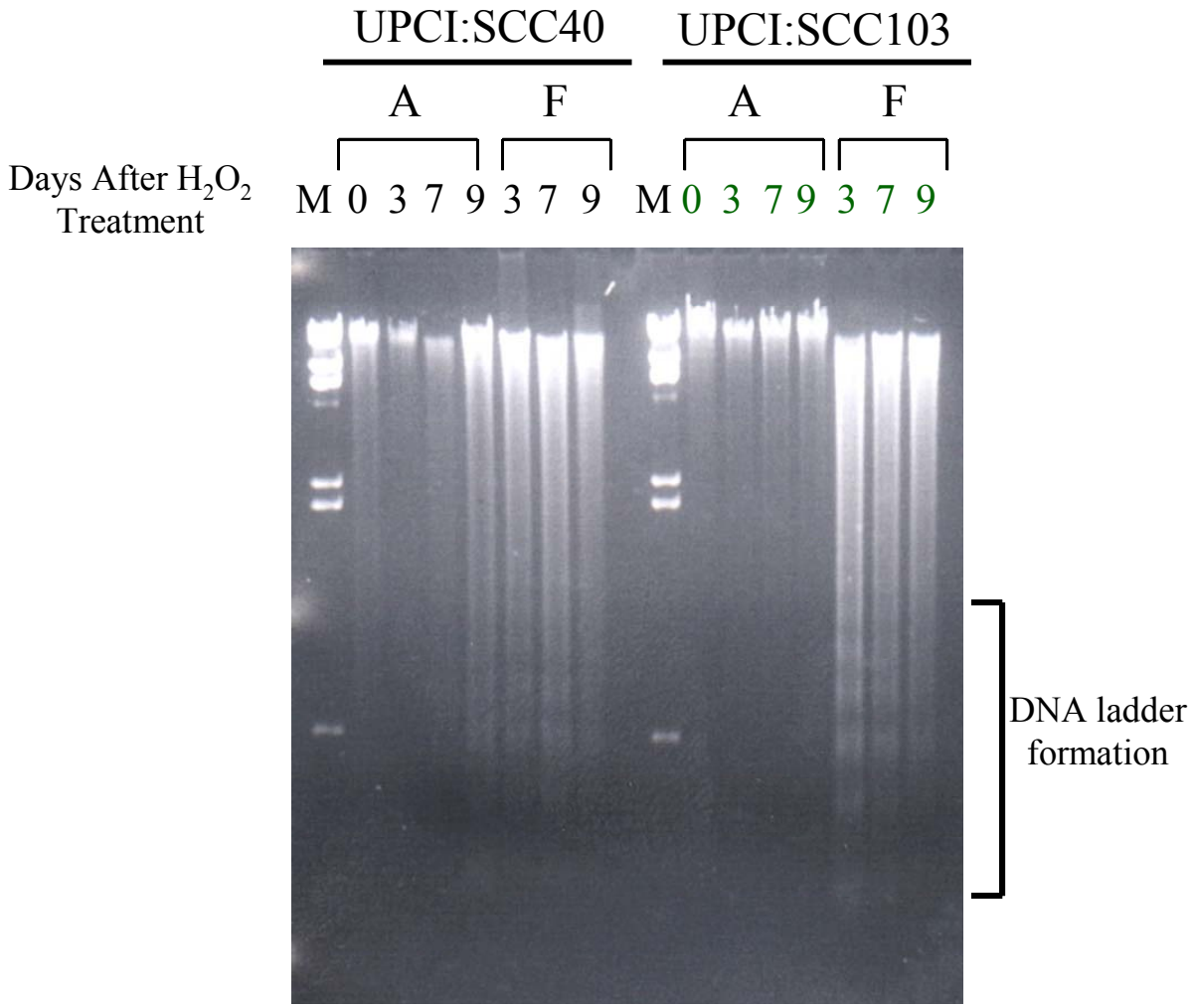

Hours After H_2O_2 Treatment
0
12h
24h
DAPI
p53
+H_2O_2
DAPI
p53
-H_2O_2

Figure 36 UPCI:SCC103 cells were treated with 4mM H_2O_2, fixed at indicated times and stained with anti-p53 antibodies. Upon H_2O_2 treatment, subcellular localization of p53 was predominantly nuclear.



A: Cells that are **A**ttached on the plate

F: Cells that **F**loat

Figure 37 UPCI:SCC103 and UPCI:SCC040 cells are treated with 4mM H_2O_2, lysed at indicated times after treatment. The genomic DNA is isolated and electrophoresed on agarose gels. Apoptotic DNA ladder formation is observed mostly in floating cells.

4.3 DISCUSSION

This chapter summarizes my preliminary studies on the apoptotic capacity of cancer cells with high bridge frequencies. We hypothesized that anaphase bridging may allow cells to bypass apoptotic pathways that are activated in response to DNA damage and continue cell cycle progression. Our initial data indicate that these cell lines can sense DNA damage and undergo apoptosis, yet further experiments will be needed to test this hypothesis.

Our results demonstrated that while the cell lines under analysis were capable of DNA ladder formation, a subpopulation of cells was still attached with very little cell death. This could either indicate that cells are still resistant to apoptosis or that the dose of H_2O_2 used was not sufficient to trigger apoptosis. Similar experiments using control cells with normal checkpoints and apoptotic pathways should be able to resolve this question. Moreover, as mentioned previously, H_2O_2 treatment both leads to and inhibits apoptosis through mechanisms other than damage to DNA (Lin et al., 2004; Shin et al., 2004; van Rossum et al., 2004; Franklin et al., 2006). Since our focus is apoptosis as a result of DNA damage, confirming these results by using other means, such as ionizing radiation would strengthen these conclusions. Lastly, whilst there is an increase in p53 and p21, there could still be mutations elsewhere in the apoptotic pathways. Assays for caspase activation or cytochrome c release from mitochondria would provide further evidence for the integrity of cell death signaling in these cancer cells.

Assuming intact apoptosis, which repair proteins should be blocked in order to test our hypothesis? While we collected significant information on how bridges form, unfortunately the induction of bridges was not completely eliminated in any of the DNA repair mutants. Even in the absence of both pathways, although the level of bridging was reduced compared to NHEJ mutants alone, the induction was still at wild type levels, which suggests the existence of a third unknown pathway contributing to bridge formation. Hence the aim of testing our hypothesis by inhibiting anaphase bridge formation is not as straightforward as we initially assumed. Based on what we know about bridges, we can speculate that the double deficient cells or wild type cells will undergo apoptosis more frequently than NHEJ deficient cells, since they form fewer bridges in response to damage. However, such an experiment relies on the assumption that repair deficiencies will only change anaphase bridge frequency and will not interfere with any of the apoptotic pathways. Yet, there appears to be a cross-talk between both repair mechanisms and apoptotic signaling. For example, physical interactions have been reported between p53 and the HR proteins, RAD51 and RAD54 (Linke et al., 2003). HR rates increase in the absence of p53 and this p53 mediated limitation of HR is thought to be through p53-RAD51 interactions (Linke et al., 2003). More recent studies demonstrated that the proapoptotic proteins, Bax and Bid, can inhibit HR (Dumay et al., 2006). Furthermore, overexpression of RAD51 provides increased resistance to ionizing radiation (Vispe et al., 1998) and elevated levels of RAD51 have been observed in cancer cells, which might contribute to their insensitivity to apoptotic stimuli (Raderschall et al., 2002). As for NHEJ, similar associations have been observed. Both Ku and DNA-PKcs have been demonstrated to regulate apoptosis, but not cell-cycle arrest (Wang et al., 2000; Sawada et al., 2003). Deficiencies in NHEJ or HR result in apoptosis, and defects in both pathways are associated with more cell death than either one alone (Barnes et al., 1998; Gao et

al., 1998; Raderschall et al., 2002; Couedel et al., 2004). These observations are consistent with our hypothesis suggesting a role for anaphase bridging in prevention of apoptosis. The increased apoptosis phenotype observed in double deficient cells could partially be due to the decrease in the frequency of bridges. Yet, these experiments should be performed where the expected increase in apoptosis and decrease in bridges are confirmed in the same set of experiments.

Lastly, a comparison of cell death rates between cell lines with low and high levels of bridge induction could be informative; however other differences in genetic backgrounds would have to be considered during evaluations.

5.0 CHAPTER V: EVIDENCE FOR CENTROSOMAL SPLITTING AND MULTIPOLAR SPINDLE FORMATION AS A RESULT OF IONIZING RADIATION

5.1 INTRODUCTION

It has been commonly accepted that genomic instability and cancer are closely related phenomena. Almost every type of cancer has previously existing and ongoing rearrangements in the genome. These chromosomal alterations can arise from numerous events including errors during cell division and cell cycle progression (Charames and Bapat 2003). Of these errors, segregation defects such as multipolar spindles (MPS) play a major role in chromosomal instability, leading to tumor development.

MPS can be described as abnormal mitotic figures observed when cell division takes place with more than two poles (Figure 7). Multipolarity is a commonly observed defect in cancer cells and is mainly associated with supernumerary centrosomes (Saunders 2005; Stewenius et al., 2005). Increase in centrosome number is reported in many carcinomas including breast, lung, brain, gall bladder, bone, pancreas, colorectal, head and neck cancers (Lingle et al., 1998; Pihan et al., 1998; Weber et al., 1998; Carroll et al., 1999; Lingle and Salisbury 1999; Sato et al., 1999; Gustafson et al., 2000; Kuo et al., 2000; Pihan et al., 2001; Sato et al., 2001). Hence centrosomal amplification and formation of aberrant mitotic spindles have been proposed to be

involved in tumorogenesis, as these defects can lead to missegregation of chromosomes and genomic instability (Doxsey 1998).

Centrosomes are the microtubule organizing center (MTOC) of the cells, which play an important role in cell-cycle progression. Centrioles, which are small barrel shaped structures and the pericentriolar material (PCM), amorphous protein mass surrounding the centrioles, constitute the major elements of centrosomes. Each centriole is composed of nine longitudal microtubule triplets and proteins such as centrin, cenexin and tektin (Rieder et al., 2001). On the other hand, the PCM contains proteins involved in microtubule nucleation and anchoring such as γ-tubulin, pericentrin and ninein (Rieder et al., 2001).

Normally, centrosome duplication starts after the G1 checkpoint and maturation of the centrosomes continues until the next cell cycle (Dictenberg et al., 1998; Adams and Kilmartin 2000; Khodjakov et al., 2002). During prophase, centrosomes begin migrating to opposite ends of the cells and mitotic spindles form. After cytokinesis, each cell receives only one centrosome. This is a carefully controlled process, since precise duplication ensures bipolar spindle formation and proper segregation of chromosomes.

An increase in centrosome number can be a consequence of over-replication of centrosomes, failure of cell division or cell-cell fusion (Nigg 2002). While it is not currently known which mechanism contributes most in tumorogenesis, the final outcome of all these events is the tendency to form multipolar spindles (Lingle et al., 1998; D'Assoro et al., 2002; Lingle et al., 2002).

In Chapter I, the correlation between multipolar spindles and anaphase bridges was discussed. In an attempt to find whether there is a direct causal relationship between bridging and multipolarity, we performed experiments where either defect was induced and asked whether the frequency of the other segregation defect changed in parallel. While we failed to find a direct cause and effect relationship between these abnormalities, our studies indicated that ionizing radiation (IR), but not H_2O_2, leads to formation of MPS in human embryonic kidney cells. These preliminary studies demonstrated that IR results in an increase in multipolarity as fast as 30 minutes after treatment. Moreover, this effect appears to be through centrosomal splitting/fragmentation as judged by centrosomal markers such as γ-tubulin and centrin-2.

5.2 RESULTS

5.2.1 DNA damage by ionizing radiation, but not H_2O_2 increases MPS formation

Anaphase bridges can be induced via either H_2O_2 or ionizing radiation, possibly due to the double strand breaks induced by these agents. To test whether another segregation defect, multipolar mitosis, which has a strong positive correlation with anaphase bridges in cancer cells (Gisselsson et al., 2002 117) can be induced by DNA damage as well, we treated human embryonic kidney (HEK-293) cells with both of these agents.

Upon H_2O_2 treatment, while anaphase bridges were induced as expected (data not shown), we failed to see an elevation in the level of MPS (Figure 38). On the other hand, IR treatment did show a slight, but notable and reproducible induction in multipolarity (Figure 39). Consistent with our results, previous reports have suggested a role for gamma-irradiation on centrosome overduplication (Scott and Zampetti-Bosseler 1980; Sato et al., 2000; Sato et al., 2000). These studies have demonstrated that occurrence of supernumerary centrosomes depends on p21Waf1/Cip1 and p27 (Sato et al., 2000; Sugihara et al., 2006) and that in some cell lines, centrosome amplification appears to increase only in p53 deficient backgrounds (Armit et al., 2002; Kawamura et al., 2006). There is also evidence showing that Kruppel-like transcription factor, KLF4, prevents centrosome overduplication following IR by transcriptionally suppressing expression of cyclinE (Yoon et al., 2005). Yet, how DNA damage causes an increase in centrosome number/multipolarity is not very clear.

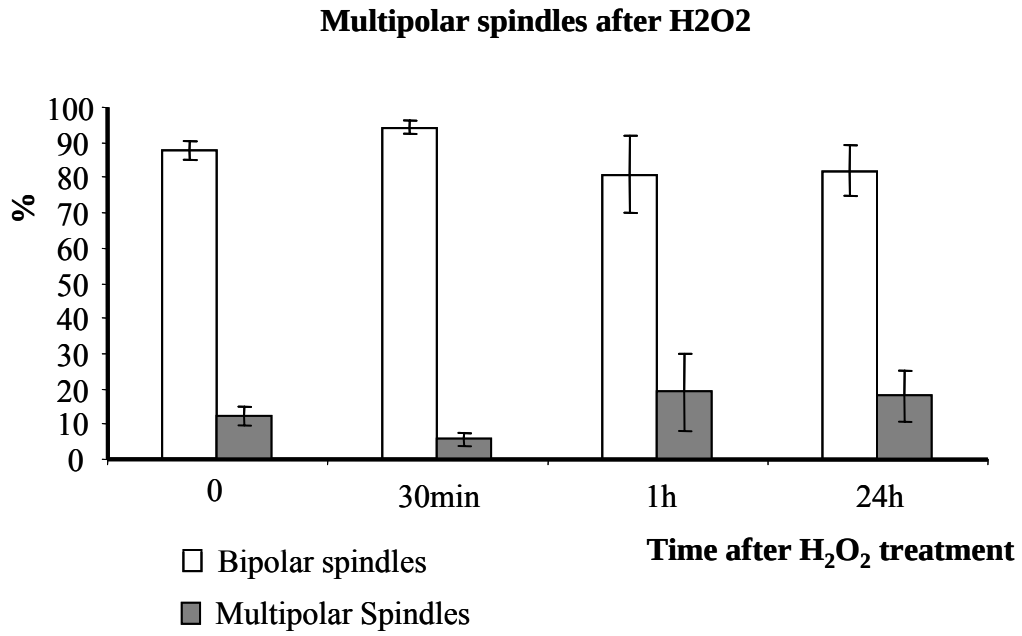


Figure 38 Scores for multipolar spindles in HEK-293 cells following H_2O_2 treatment. Cells were treated with 1.6mM of H_2O_2 for 10 minutes, changed to regular medium, fixed at indicated time points post-treatment, and scored for MPS by γ-tubulin staining and DNA shape.

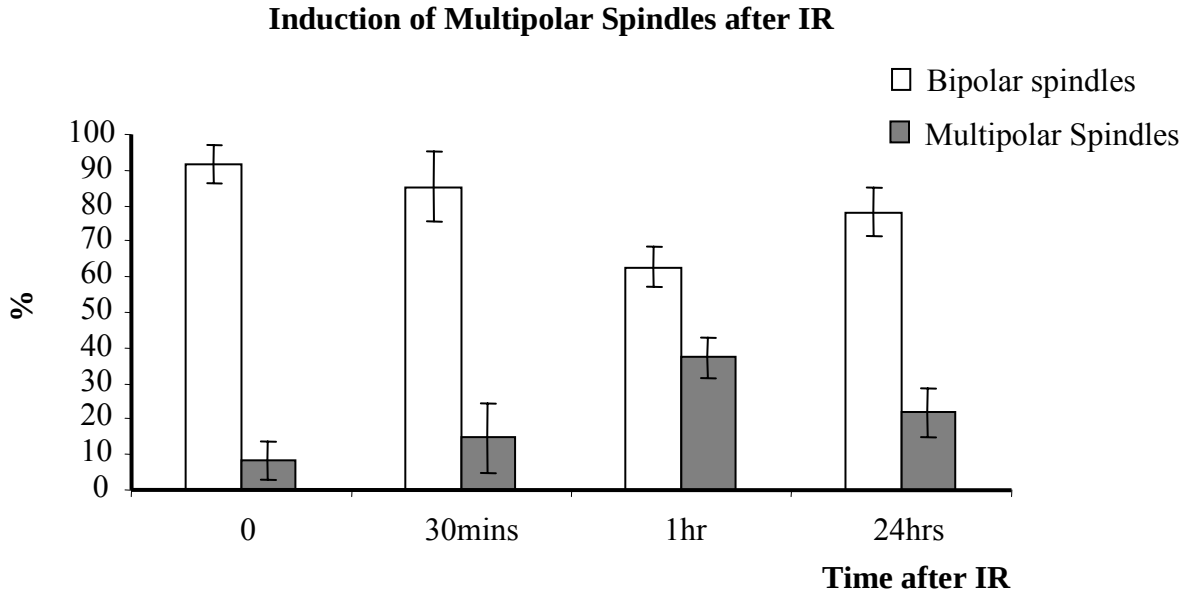


Figure 39 Multipolarity in HEK-293 cells following ionizing radiation. Cells were treated with 6Gys of IR, and fixed at indicated time points after treatment. MPS were scored by γ-tubulin staining and DNA shape.

5.2.2 MPS formation might be a result of centrosomal splitting after ionizing radiation

Interestingly, in our experiments the induction in multipolarity was detectable as early as 1h, but not 30min (Figure 39). The time frame for this increase suggests a cell cycle independent pathway, since MPS can be observed before the completion of one cell cycle (~24h). In order to further explore the mechanism of MPS formation in response to IR, cells were stained with common spindle pole markers, such as γ-tubulin and centrin-2, which are both centrosomal proteins found in PCM and on centrioles respectively (Rieder et al., 2001).

Two distinct γ-tubulin spots were observed at the spindle poles in untreated, control cells which represents the normal state (Figure 40). However, the staining appeared disperse and/or broken up upon IR treatment (Figure 40). Likewise, the majority of the cells (>60%) have two centrin-2 foci associated with each centriole at the poles in untreated cells (Figure 41, 42, blue bars (2+2)). Interestingly, cells with normal numbers of centrin foci were reduced (~40%) after ionizing radiation (Figure 41). The shift towards greater numbers of centrin foci occurred 30min following IR (Figure 42, red bars), but was reduced 1h after treatment, at the time when an increase in multipolar spindles was observed (Figure 42, yellow bars). Moreover, the size of these spots was irregular, which is consistent with centrosomal fragmentation (Figure 41, enlarged views).

Although this data is very preliminary, it suggests that ionizing radiation may cause centrosomes to fragment or split, as early as 30 minutes after treatment. Fragmented/split

centrosomes may behave like extra copies of centrosomes and perhaps nucleate new microtubules and eventually lead to abnormal spindles. Correspondingly, multipolar mitosis was seen at 1h, when there was a reduction in the centrin foci/pole. It can be speculated that this reduction was due the appearance of new multipolar spindles (Figure 43).

Other than centriolar fragmentation, this shift could also arise from displacement of centrin-2 from the centrioles, or *de novo* formation of centrin-2 at the poles. These possibilities will be covered further in the discussion.

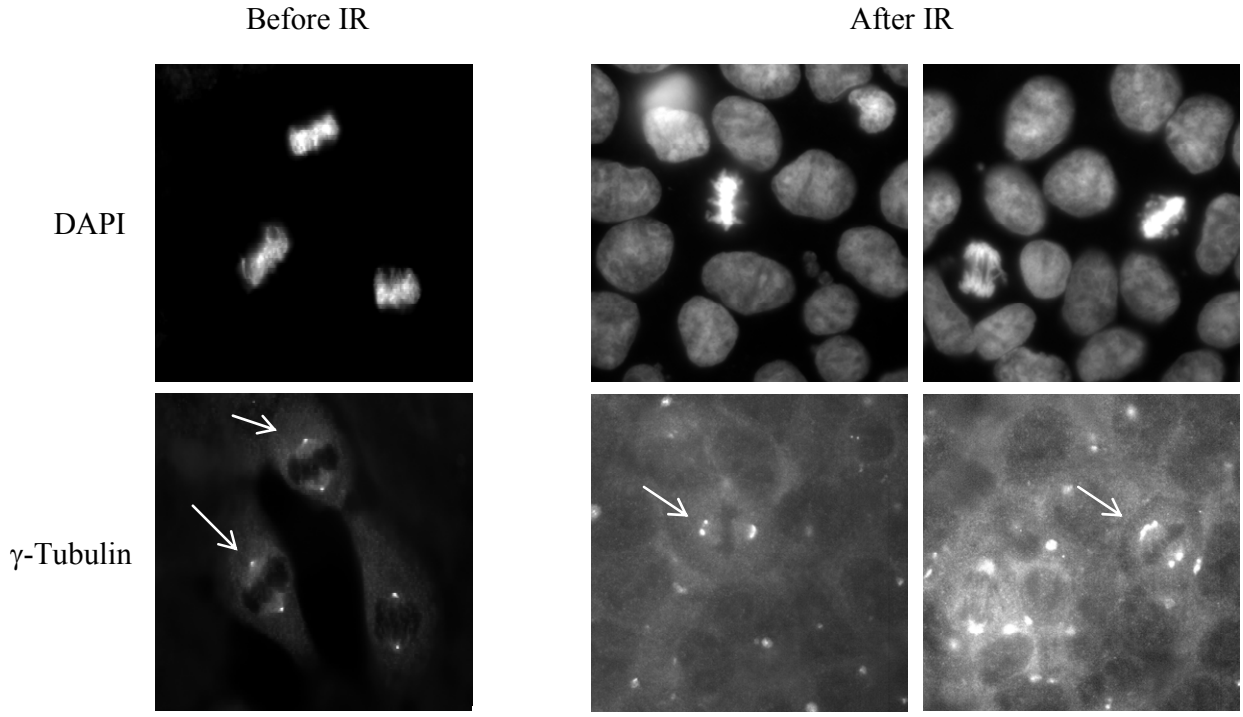


Figure 40 γ-tubulin staining in HEK-293 cells before and after IR. The arrows indicate the γ-tubulin at the poles. γ-tubulin staining becomes disperse or split at the spindle poles after ionizing radiation.

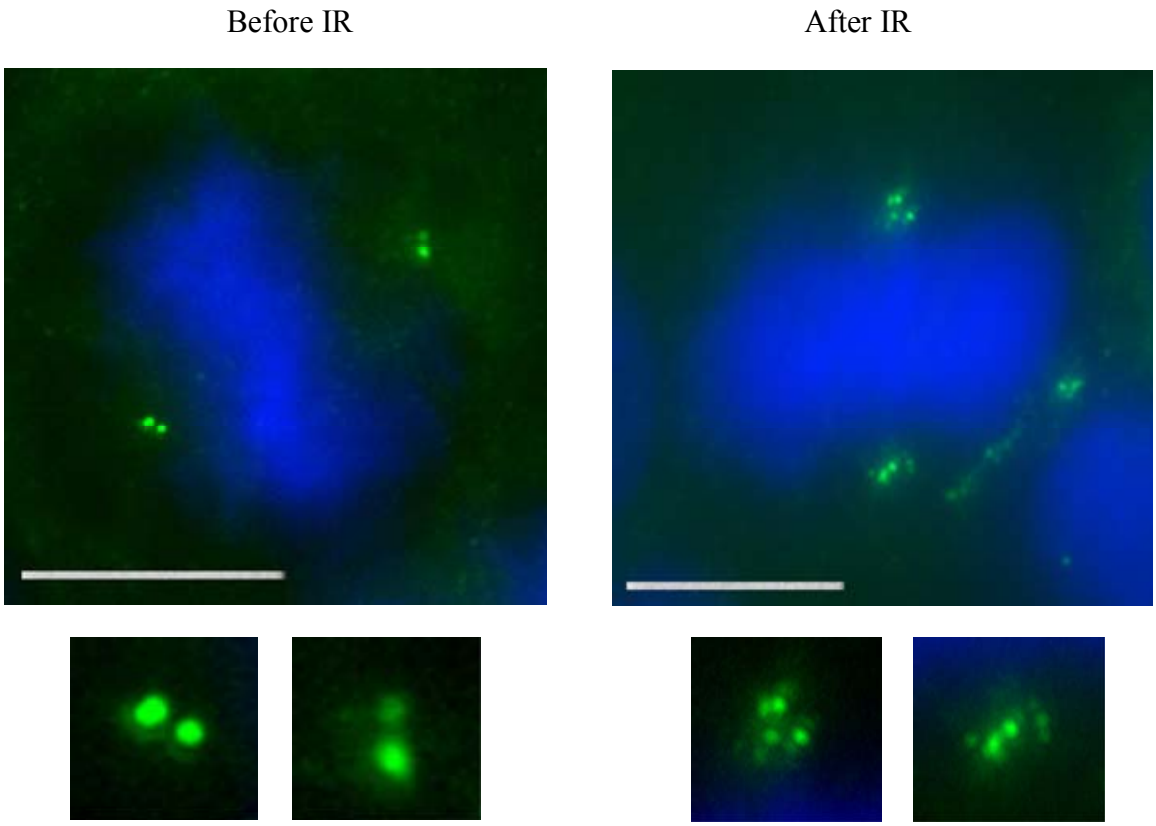


Figure 41 Centrin-2 staining at the spindle poles before and after IR in HEK-293 cells. Normally, cells have 2 centrin spots per pole in unradiated cells. Upon radiation treatment multiple dots can be observed. Scale bar represents 10 microns. Insets represent enlarged centrin-2 dots at the poles. Images are composed of the stack of focal planes, hence all foci are in focus.

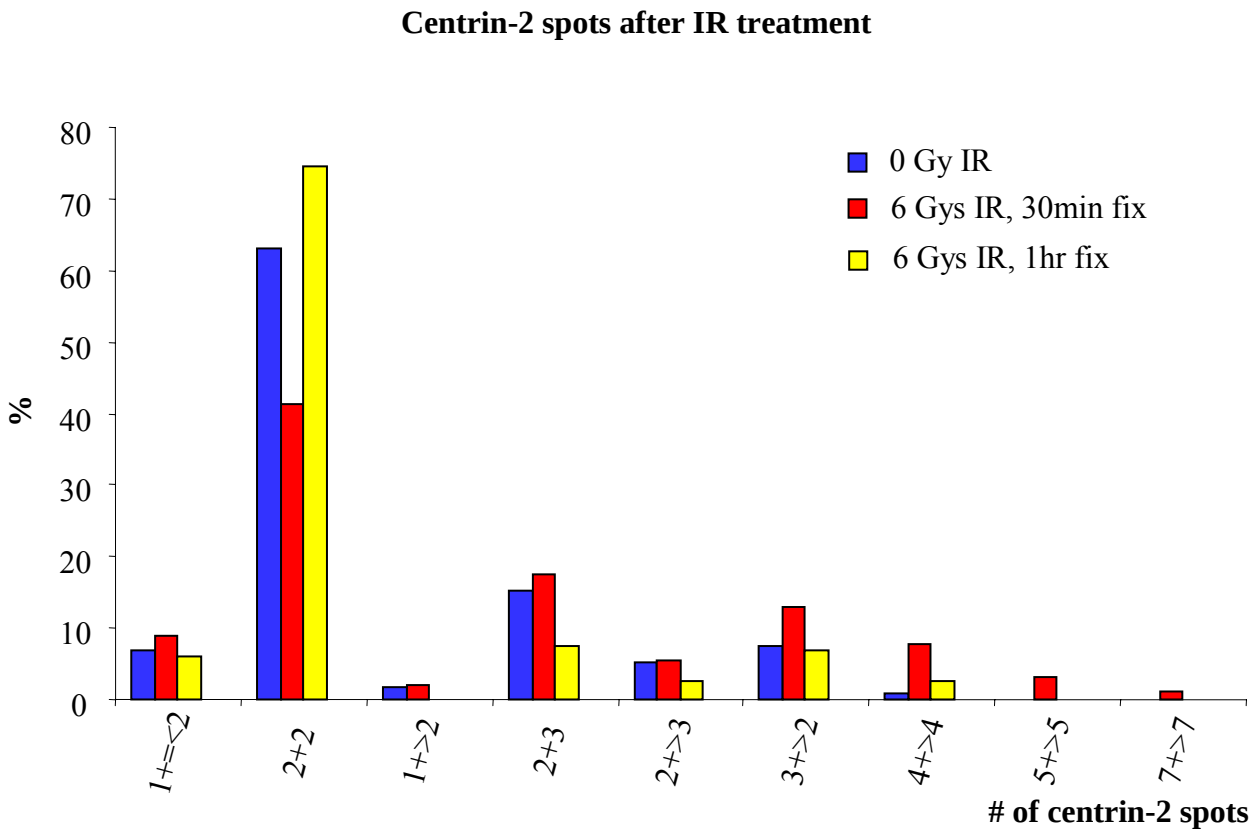


Figure 42 Number of centrin-2 spots/pole in HEK-293 cells following ionizing radiation treatment. Cells were treated with 6Gys of IR, fixed at the indicated time points, and stained for centrin-2 and DNA. Number of centrin-2 dots at each pole was counted for bipolar metaphases. (2+2) indicates 2 centrin dots at each pole, which is the normal state. (2+>3) indicates two dots at one pole and greater than 3 dots at the other pole. Likewise, (3+>2) indicates 3 dots at one pole and greater than 2 dots on the other. Ionizing radiation treatment can result in abnormal numbers of centrin-2 foci 30min following IR, which is reduced by 1h.

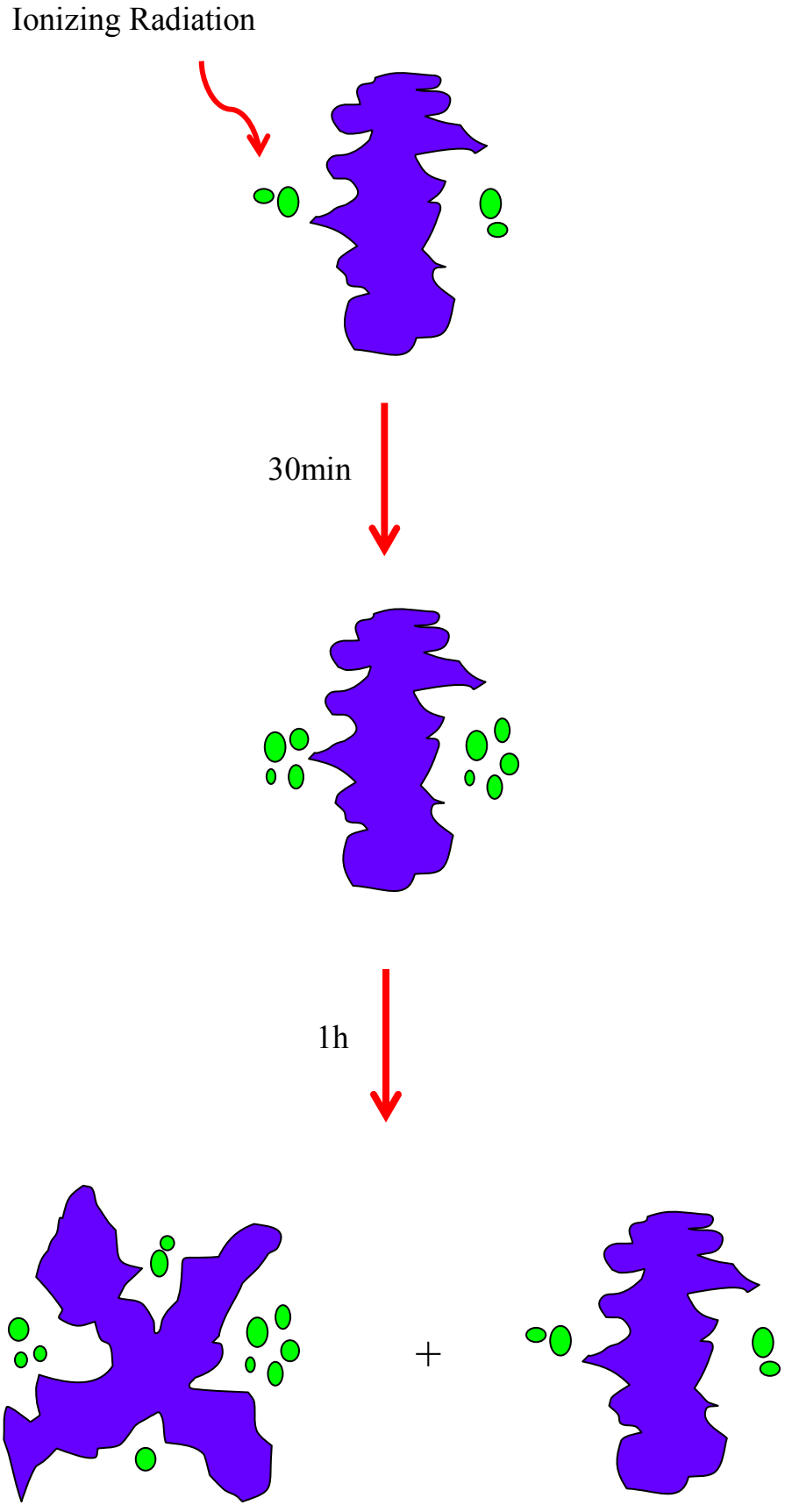

Ionizing Radiation
30min
1h
+

Figure 43 Normally, cells exhibit two centrin-2 foci at each pole at the metaphase plate. Upon irradiation, supernumerary centrin-2 foci is detected 30min following IR. On the other hand, an increase in multipolar spindles is observed 1h after treatment, which coincides with a drop in the number of centrin-2 spots.

5.3 DISCUSSION

This chapter summarizes the preliminary studies on how multipolar spindles form in response to ionizing radiation. Our initial data indicated that IR can cause splitting of centrosomal markers, namely γ-tubulin and centrin-2, as early as 30min after treatment and result in multipolarity at 1h. Yet, other DNA damaging reagents, such as H_2O_2, do not lead to MPS. It is important to note that while a link between IR and MPS was previously reported, these changes were known to occur 24h after IR and cell-cycle dependent mechanisms were under investigation (Sato et al., 2000). On the other hand, we detected an increase immediately after IR and our data are reminiscent for a role of IR in centrosome fragmentation or splitting rather than cell cycle changes or failure of cytokinesis. However, these results are not yet conclusive, and additional experiments need to be performed to study the mechanisms leading to multipolarity as a result of ionizing radiation.

Firstly, it has been demonstrated that IR can induce a transient change in cellular free [Ca^{2+}] concentration (Todd and Mikkelsen 1994) and that these rapid calcium fluxes can be detected at 30sec (Lyng et al., 2001). Moreover, self assembly of centrin-2 has been shown to be dependent on calcium (Tourbez et al., 2004; Yang et al., 2006) and at high [Ca^{2+}] levels, large centrin spots form at or near the center of the cell (Baron et al., 1994). Hence extra numbers of centrin foci could be explained by formation of new centrin satellites due to increased [Ca^{2+}] concentrations as a consequence of IR. Alternatively, centrin-2 could be displaced from the

centrioles or *de novo* centrioles might truly start emerging, which all would lead to an increase in centrin spots at metaphase.

In order to distinguish between these possibilities, other centrosomal markers could be tested for the togetherness of staining at the poles following IR. Among the possible candidates, dynein, dynactin, ninein, c-Nap1, Nek-2, EB1 or APC are all known to focus at the metaphase poles (Smith et al., 1994; Gaglio et al., 1996; Berrueta et al., 1998; Quintyne et al., 1999; Helps et al., 2000; Mayor et al., 2000; Mogensen et al., 2000) and alterations in the staining of these proteins could potentially argue against both calcium dependent formation of centrin foci or displacement of centrin from the centrioles, yet still would not be conclusive. On the other hand, *de novo* centriole formation could be tracked by live cell imaging using cells expressing centrin-GFP. While newly forming centrioles should emerge as a new spot, fragmentation of centrioles would appear as splitting of an already existing signal. However, there are some caveats associated with this experiment as well. First, it is not possible to irradiate cells under the microscope and centrosome splitting is observed soon after IR treatment. Hence events under observation may be missed by the time the experiment is set up. Second, centrin-GFP expression is usually low and the signal is weak. Moreover, centrin spots are rarely on the same focal plane and merging multiple planes increases the background making the results harder to interpret. To resolve these problems, electron microscopy could be used to analyze centrioles following radiation. While electron micrographs would be sufficient to distinguish among fragmentation, splitting or *de novo* formation of centrosomes, the sample size is usually a limiting factor. Therefore, a combination of these experiments will hopefully be able to enlighten our view about how supernumerary centrosome-like structures form upon IR.

Lastly, this experiment was performed in HEK-293 cells and normal number of centrin spots (2+2) was observed in only ~65% of the population (Figure 42, blue bars). This percentage is lower than the published literature (85-90%) (Salisbury et al., 2002), suggesting that there might be some intrinsic abnormalities in this cell line. It has been shown that in some cells IR treatment does not result in an increase in MPS, unless p53 levels are reduced using siRNA (Kawamura et al., 2006). Yet, we observe an elevation immediately after irradiation without any other treatments. Hence, it is important to repeat these experiments using normal cells, which do not exhibit any existing centrosomal defects.

6.0 CHAPTER VI: SUMMARY AND SPECULATIONS

Carcinogenesis requires multiple changes in the genome. It is estimated that at least 5-6 mutations should take place for a normal cell to become cancerous (Lengauer et al., 1997). Yet, the process of mutagenesis is slow and how it leads to cancer is determined by many factors. Less than 1.5% of the human genome encodes for proteins (Lander et al., 2001), and even when a DNA damaging event takes place, the majority of these mutations will occur in non-coding regions. Within the 1.5% coding region, only a minority of genes are involved in cell cycle regulation, and only mutations in these genes will actually be relevant to carcinogenesis. Furthermore, not all types of changes will lead to tumor formation, even if they occur in genes controlling the cell cycle. For instance, alterations cannot be silent mutations and should trigger the activity of oncogenes and often must deactivate both copies of the tumor suppressor genes. On the other hand, the cell also has defense mechanisms to correct these changes. Repair machinery actively removes lesions from the DNA. Cells either arrest or undergo apoptosis upon damaging events. Moreover, the immune system efficiently kills cancerous cells (Burnet 1965), as shown by the observation that organ transplant recipients with suppressed immune system carry a 65-fold increased risk of squamous and basal cell carcinoma (Jensen et al., 1999). Yet, despite the strong odds against it, cancer accounts for almost a quarter of all deaths in USA, exceeded by only heart diseases. As of 2005, the risk of an American man developing cancer over his lifetime is one in two, and one in three for women (results obtained from American

Cancer Society). So how is it possible that a single cell undergoes sufficient number of changes that lead to tumor formation? It is almost certain that mutations that allow further changes in the genome will speed up this process and lead to a hypermutable state (Nowell 1976). Among these alterations, defects in the DNA repair machinery and events that result in gross chromosomal changes could be expected to play a major role in tumorogenesis.

There are approximately 10,000 lesions that occur spontaneously in a mammalian cell per day (Lindahl 1993). While these lesions are efficiently removed, failure to do so can result in a catastrophic outcome. For instance, individuals with deficient nucleotide-excision repair (NER), which is only one of the several DNA repair pathways (xeroderma pigmentosum patients), are 5,000-fold more likely to have squamous and basal cell carcinoma and carry 2000-fold increased likelihood of melanomas (Kraemer et al., 1987). Higher incidences of malignancies are also observed in Fanconi anemia, Nijmegen breakage syndrome, or ataxia teleangiectasia, which are all associated with defects in repair pathways (Morrell et al., 1986; van der Burgt et al., 1996; Alter 2003). The enhanced risk of cancer in these patients provides strong evidence on how effectively DNA repair proteins protect the genome.

Chromosome segregation defects, such as anaphase bridges and multipolar spindles, can be considered as the other major mechanisms that may lead to cancer, hence present an important field of study. These defects are strongly associated with chromosomal instability (CIN) (Gisselsson et al., 2000; Lingle et al., 2002; Montgomery et al., 2003). CIN, the increased tendency for having abnormal chromosome number and structure, is a common feature observed in many cancer cells. As proposed in the classic paper titled “The Clonal Evolution of Tumor

Cell Populations" by Peter C. Nowell, CIN is one of the early events during tumorogenesis promoting other amplifications, losses or rearrangements (Nowell 1976). In his paper, Nowell also proposed the more aneuploid a cell becomes, the more aggressive the resulting tumor. Rationally, both bridges and MPS can result in aneuploidy, and once a cell becomes aneuploid, these changes are most likely irreversible. Besides, formation of a single bridge can drive BFB cycles triggering further instability and perhaps leads to a more aggressive tumor grade. Moreover, there is no evidence yet that these defects can be detected by cell-cycle checkpoints, which make them potentially more dangerous.

Interestingly, in our studies we found that these seemingly different mechanisms driving carcinogenesis are in fact related and that there is a statistically significant correlation between the efficiency of DNA repair and segregation defects, namely between double stranded break repair pathway Non-Homologous End Joining (NHEJ) and induction of anaphase bridges. Since cells that exhibit less NHEJ activity are more likely to form bridges, the effect could potentially be amplified by having both defects. Indeed, deficiencies in NHEJ proteins are associated with increased instability (Difilippantonio et al., 2000; Gao et al., 2000; Bassing et al., 2002; Rooney et al., 2003) and some of these abnormalities could actually be a result of anaphase bridge formation, not simply a failure of DNA repair.

The homologous recombination (HR) pathway has been generally considered as an error-free pathway, since it uses the undamaged homologous template to repair the break. Yet, it appears that this pathway can also lead to genomic imbalances, such as anaphase bridging, in the absence of NHEJ. The physiological nature of chromatin joint sites in anaphase bridges is not

currently known. While these may represent continuous covalently-linked DNA molecules, they may also be proteinous intermediate structures of the HR pathway. On the other hand, recent reports indicate the presence of γ-H2AX foci on anaphase bridges without fragmentation (as confirmed by metaphase spreads), and the authors have proposed that these "residual" foci represent abnormal chromosome structures as a result of illegitimate repair (Suzuki et al., 2006). Hence, it is entirely possible that HR repairs the break leading to continuous DNA strands in the bridges, but this repair is not equivalent to normal rejoining of the two broken ends, since γ-H2AX foci persist along these sites. In order to further characterize these joint sites, it will be important to know where exactly the breaks are formed. In an experimental system, where double stranded breaks are precisely induced by either the RAG recombinase or by the I-SceI endonuclease could provide insights in revealing the nature of these break sites in different repair deficient backgrounds. Moreover, fluorescence and PCR-based assays are described, which could be used to identify the individual contribution of NHEJ and HR (Weinstock et al., 2006).

Both results from our lab and others show that reduction of p53 or blocking the activity of either p53 or pRb by HPV encoded proteins E6 and E7 lead to enhanced levels of anaphase bridging (Luo et al., 2004; Wentzensen et al., 2004). Both of these proteins are involved in repair signaling pathways, but no direct involvement in the physical repair of DSBs has been described. Since we show that a reduction in HR alone is not sufficient to increase the frequency of radiation-induced bridges, enhanced levels of bridging does not appear to be a general phenomenon as a result of knockdown of any repair protein or pathway. p53 has been demonstrated to inhibit site-specific, but not radiation-induced NHEJ, suggesting a link between p53 and NHEJ, which may partly explain these findings (Tachibana 2004; Dahm-Daphi et al.,

2005). On the other hand, it can also be speculated that perhaps anaphase bridges are sensed by the cell by some mechanism such as tension on the DNA. p53 and pRb might be playing a role in elimination of these cells from the dividing population, which would reduce the observed frequency of bridges. Alternatively, the increase in the level of bridging could be a result of abnormalities in the pRb or p53 regulated signaling pathways that are involved in bridge formation. For instance, HR rates have been shown to increase in the absence of p53 (Linke et al., 2003), which could explain increased bridging in these cases similar to our findings where we demonstrated that HR is responsible for induction of bridges in the absence of NHEJ.

In this study, we also demonstrated preliminary evidence indicating that radiation treatment leads to not only an increase in anaphase bridges, but also in multipolar spindles. Surprisingly, these changes occur shortly after IR, suggesting cell-cycle independent mechanisms as a cause. IR can physically change the structure of DNA by introducing numerous types of lesions that include DSBs (Hutchinson 1985), but it will be really interesting to see if it can cause physical disassociation of proteins, such as centrin-2 from the centrioles or if it is strong enough to break bonds holding a centrosome together. Future lines of investigation will be required to answer these questions.

During the tumorogenesis process, we already know that multiple events should take place. However, while the potential contribution of segregation defects is not doubted, it is still not known whether these events, on their own, are powerful enough to lead to genomic instability or to cancer formation. Following clones from primary cells subsequent to an initial segregation defect could improve our current understanding of these abnormalities and disclose

their relevance in carcinogenesis. Our work has just put some pieces in the big puzzle of this process, revealing how these defects might be forming.

7.0 CHAPTER VII: MATERIALS AND METHODS

7.1.1 Cell Culturing

American Type Culture Collection (ATCC, Manassas, VA) cell lines, HCT116, SKHEP-1, U2OS, HEK-293, HeLa, MES-SA, A549 and CHO-K1, are cultured in the culture medium recommended by the supplier. Oral cancer cell lines, UPCI-SCC103 and UPCI-SCC70s, were gifts from Dr. S. Gollin (University of Pittsburgh, USA). They were maintained in MEM, supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, L-Glutamine, and gentamicin, and trypsinized in 0.05% trypsin, 0.053 mM EDTA. HCT116 RAD54B$^{-/-/-}$ and XRCC3$^{-/-}$ deletion cell lines (described in Miyagawa et. al. 2002, Yoshihara et. al., 2004) were generously donated by Dr. K. Miyagawa (Hiroshima University, Japan). They were maintained in 10% FBS, McCoy's 5A medium and trypsinized in 0.05% trypsin, 0.053 mM EDTA. All DT40 chicken cell lines (kindly provided by Dr. Y. Wang, Thomas Jefferson University, USA) were cultured in DMEM supplemented with 10% FBS, 5% chicken serum, and 10^{-5} M betamercaptoethanol. Ku80 deficient CHO-K1 cell line, XRS-6 (gift from Dr. P. Jeggo, University of Sussex, UK), and XRCC4 deficient XR-1 (gift from Dr. T. Stamato, Lankenau Institute for Medical Research, USA) were grown in Kaign's modification of F12 medium supplemented with 10% FBS. Irs3 (RAD51C-mutant), and V79 (wild-type parent) cells (kindly donated by Dr. J. Nickoloff, University of New Mexico School of Medicine, USA) were

maintained in MEMα with 10% FBS and with 1% penicillin/streptomycin (Gibco). KE $p53^{-/-}$, mouse primary epithelial cells were a gift from Dr. Fukasawa (University of Cincinnati College of Medicine, Cincinnati) and were derived from embryonic tissues from p53-null mice. These cells were cultured as described (Chiba, Okuda et al. 2000). All cells were incubated at 37°C, in 5% CO_2.

7.1.2 Transfections and ionizing radiation

HCT116 cells were grown on 22mm x 22mm sterile glass coverslips in six-well plates or 35mm dishes. Transfection with siKu80 (Dharmacon), siXRCC4 (Dharmacon), or the negative control siRNA, Alexa Fluor 488 (Qiagen) was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendations. 0.5μg of siRNA per dish was used for transfection. Cells were transfected 16-18 hours after being seeded on the coverslip, when they were around 50-60% confluent. Two days after transfection, cells were split 1:2. Three days after transfection, they were exposed to indicated doses of ionizing radiation (IR) using a gamma irradiator (^{137}Cs source, Manufactured by Atomic Energy of Canada Limited, Model CP-36-B-533, Serial number 1030), when the siRNA effect is determined to be maximum by immunoblotting. 24 hours after IR treatment, cells were fixed in -20°C methanol for 5 minutes and air dried. Coverslips were either immunostained immediately or stored at -20°C. HeLa and UPCI-SCC103 cells were transfected with 1μg p3XFlag-XRCC4 plasmid DNA using Fugene6 reagent (Roche) with a 1:3 ratio (w/v) according to manufacturers instructions (cells were kept in OPTI-MEM (Gibco) overnight during transfection, rather than regular growth medium). Transfections with two plasmids were performed similarly as single transfections except that 0.5μg of each plasmid DNA was mixed prior to incubation with transfection reagent.

These cells were treated with ionizing radiation 24 hours after plasmid transfection. Each experiment was performed at least 3 times.

7.1.3 H_2O_2 and wortmannin treatment

H_2O_2 was purchased from CVS or Giant Eagle and the bottles were not reused after opening. Cells were treated as a modification of (Kang et al., 2002). Briefly, H_2O_2 was diluted in regular cell culture medium and filter sterilized before putting on cells for 10min at 37^oC for both inductions of anaphase bridges and apoptosis. The medium was replaced with regular medium and cells were fixed at 1 day (for bridge formation) or collected at 3, 6 or 9 days (for apoptosis assays) after treatment. Cells were preincubated with 20μM wortmannin diluted in medium for 1.5h prior to H_2O_2 treatment, changed to medium containing H_2O_2 for induction of DNA damage, and switched back to medium with 20μM wortmannin until next day.

7.1.4 Isolation of genomic DNA for ladder formation

Cells grown on 100mm dishes were washed with 3mls of PBS and lysed with 2mls of 7M Guanidine hydrochloride. The lysate was mixed with 1ml of Wizard DNA purification resin (Promega) in 15ml Falcon tubes and incubated for 5min at room temperature. The tubes were centrifuged at 2000g for 3min, the pellet was collected and rinsed in 3mls of washing solution (90mM NaCl, 9mM Tris.Cl pH: 7.4, 2.25mM EDTA, 55% Ethanol). The pellet was then transferred to a 3cc syringe attached to a Wizard miniprep column and washed twice with 3mls of washing solution, and dried by centrifugation over a microfuge at 5000g for 5min. For eluting DNA, 50μls of TE buffer pH: 8 was incubated with the pellet for 5min. The mixture was

transferred to a new eppendorf tube, and centrifuged at 5000g for 5min. Then, the supernatant was incubated with RNAse A for 30min at 37^{o}C and run on 1% agarose gel, 400ng DNA/well, at 5V/cm.

7.1.5 Immunofluorescence staining

Cover slips were either processed immediately after fixation or stored at -20°C for methanol fixed cells or stored at 4^{o}C in blocking solution up to two weeks for paraformaldehyde fixed cells. Coverslips were rehydrated in PBS or 1.5% BSA/PBST. Staining for anaphase bridges was carried out as described in (Luo et al., 2004). Cells were immunostained with anti-XRCC4 (BD Biosciences, 1:300 dilution), anti-Ku80 (BD Biosciences, 1:300 dilution), anti-Flag (Sigma, 1:1000) antibodies for 1h following methanol fixation. Cells that were efficiently knocked down (no visible immunolabelling) for either XRCC4 or Ku80, or cells that had Flag signal were scored for bridge formation. For γH2AX, 53BP1 and phospho-ATM (P-ATM) staining, cells were fixed in 2% (γH2AX) or 4% paraformaldehyde (53BP1, P-ATM) in PBS at room temperature for 30min (γH2AX) or 10min (53BP1, P-ATM), rinsed in PBS and permeabilized in 1% Triton/PBS at 4°C (γH2AX) or room temperature (53BP1, P-ATM) for 5 min (γH2AX) or 30min (53BP1, P-ATM), and blocked in 4% goat serum/PBS in 37°C (γH2AX) or 1.5% BSA/PBST (53BP1, P-ATM) for 1 hr. Primary antibodies were diluted in the blocking solution and incubated on cells for 1h at room temperature. Cells were incubated with Alexa 488 goat anti-rabbit IgG and Alexa 568 goat anti-mouse IgG for 30min at room temperature and stained with DAPI. The coverslips were then mounted with anti-fade solution (1 mg/ml p-phenylenediamine, 0.1x PBS, pH: 9.0 with KOH, 90% glycerol, stored in the dark at -20°C).

Olympus BX60 epifluorescence microscope with 100x or 50x oil immersion objectives was used to view slides. Hamamatsu Argus-20 CCD camera was used to capture images.

7.1.6 Preparation of cell free extracts and Non-homologous end joining activity assay

Cell free extracts were prepared following the described protocol in Diggle et. al. (2003) with the following changes. 1-2 plates of (150 mm dish, 99% confluent) cells were resuspended in 1.5 volume of hypotonic buffer and homogenized with a tissue tearor (Biospec products, Model 985-370) Speed 2, 1min, 4^{o}C, rather than a dounce homogenizer. The same extracts were used for both immunoblotting and NHEJ activity reactions. DNA end joining assay was performed using pSilencer U6-Linker plasmid as template DNA, which was end labeled with ^{32}P after KpnI digestion. End joining reactions were carried out in 20µl with 40µg protein extract (determined by Bradford Assay) and 10ng radiolabeled DNA as described. The DNA from each reaction was purified using Eppendorf Perfectprep Cleanup kit prior to electrophoresis (0.7% agarose gel, 10V/cm, 1h). For DNA-PK inhibition reactions, cell lysates were preincubated with a final concentration of 30µM wortmannin, 50µM DNA-PK InhibitorII, or 50µM DNA-PK InhibitorIII (all Calbiochem) at 37^{o}C for 15-20min or XRCC4/Ku80 antibodies (1:3 dilution, BD Biosciences, in a total of 30µl) at room temperature for 45min prior to addition of DNA. Phosphorimage data was collected using FujiFilm BAS-2500 (serial number 2702264), and the data was quantified using ImageGuage software program (Fuji Film Science Lab).

7.1.7 Antibodies

rAb-XRCC3 (1:1000) and rAb-RAD54B (1:750) (gift from Dr. K. Miyagawa), mAb-Ku80, mAb-Ku70 and mAb-XRCC4 (BD BioSciences, 1:1000 dilution for immunoblotting and 1:300 for immunofluorescence), mAb-γTubulin (1:1000, Sigma), rAb-DNA Ligase IV (1:1000, GeneTex, Inc.), rAb- phosphorylated histone γH2AX (1:800, Upstate Cell Signaling Solutions, Lake Placid, NY), rAb-53BP1(1:500, Novus Biologicals), mAb-phospho ATM (1:200, gift from Dr. C. Bakkenist), mAb-Flag tag (1:1000, Sigma), mAb-p53 (DO-1, 1:50, Santa Cruz, for immunoblotting), mAb-p53 (1:100, Oncogene, for immunofluorescence), mAb-p21 (1:100, Santa Cruz), mAb-HA (Roche), rAb-actin (1:500, Sigma), mAb-centrin-2 (1:5000, gift from Dr. J. Salisbury), rAb-cyclinE (SantaCruz Biotechnology) were used as primary antibodies. Alexa Fluor488 and Alexa Fluor568 (1:250, Molecular Probes, Eugene, OR, for immunofluorescence), ECL anti-mouse IgG-HRP linked F(ab')α fragment and ECL anti-rabbit IgG-HRP linked F(ab')α fragment (1:5000, Amersham, GE Healthcare, UK) were used as secondary antibodies. 4', 6-diamidino-2-phenylindole (DAPI) at 1 μg/ml (Sigma, St. Louis, MO) was used to stain DNA. Antibodies were diluted in 1.5% BSA/PBST, DAPI in ddH_2O or PBS for immunofluorescence and the supernatant for 5% dry milk/TBST after centrifugation for 10 minutes, full speed on a tabletop centrifuge for immunoblotting.

7.1.8 Plasmids

pTSIGN-DNALigIV was generated by subcloning DNA ligase IV cDNA (ATCC, Cat # 5259632) into pTSIGN vector (gift from Dr. R.D. Wood) using the EcoRI and BamHI sites.

For pSilencerU6-Linker plasmid, two oligonucleotides (Invitrogen) 5'GATCCCGGGCCCGATATCGGTACCACGCGTTTCAAGAGAACGCGTGGTACCGATA TCGGGCCCTTTTTTGGAAA3'and 5'AGCTTTTCCAAAAAAGGGCCCGATATCGGTACCACGCGTTCTCTTGAA ACGCGTGGTACCGATATCGGGCCC GG 3' are annealed according to manufacturers instructions and cloned into pSilencerU6 plasmid (Ambion). p3XFlag-XRCC4 was a gift from Dr. MJ. Sadofsky.

7.1.9 Statistical methods

Correlation between XRCC4 level and NHEJ activity was assessed by a permutation test for which the test statistic was the sum of Spearman correlation coefficients for all possible combinations of replicated experiments; each coefficient was weighted by the number of XRCC4/NHEJ pairs that contributed. Tests of all other correlations were based on within-cell line medians of replicated experiments, and utilized Spearman's test. A t-test based on a pooled variance was used on the data for determining significance of the difference in the induction of bridges in NHEJ/HR mutant backgrounds.

For the assessment of correlation between NHEJ activity and induction of bridges, the measure of induction of bridges for a cell line was defined as difference in the median number of bridges before and after irradiation. Correlation of NHEJ activity and induction of bridges was assessed with a permutation test based on the weighted sum (over replicated NHEJ experiments) of Spearman correlation coefficients. Weights were proportional n-1, where n is the number of NHEJ-bridge induction pairs contributing to each coefficient, and the test was evaluated with

2000 replicates. A t-test, utilizing pooled variance, was used to assess significance of the cell-line differences in the induction of bridges.

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