CHAPTER II

REVIEW OF RELATED LITERATURE

Genomic Instability and Carcinogenesis

Genomic instability is believed to play a critical role in the malignant process. It has been proposed that genomic instability can be divided into two types including microsatellite instability (MIN), and chromosomal instability (CIN) (4). Cancers develop instability either at the sequence level, termed MIN or at the chromosomal level, termed CIN, but not generally at both levels. Since these instabilities are rarely found to coexist in tumors, it seems that one form of instability is sufficient to drive tumorigenesis (4).

I. Microsatellite Instability and Carcinogenesis

MIN is recognized as alterations at the nucleotide levels because of a deletion or insertion of DNA sequences, particularly in repetitive sequences. This instability is most easily detected at short sequences of DNA repeats scattering throughout the genome, called microsatellites (27, 28). It generates the characteristic of MIN in tumors. MIN is a direct consequence of defects in nucleotide mismatch repair (MMR). MMR has roles in genomic instability, including correction of chromosomal errors associated with DNA replication and recombination in both prokaryotes and eukaryotes. There are a lot of evidences that support the involvement of MMR deficiency in MIN. In bacteria, the defects of MMR genes, *mutS* or *mutL*, contribute to MIN. Similarly, yeast with mutations in *Msh2* or *Mlh1* gene, homologues of bacterial gene *mutS* or *mutL,* also induces MIN phenotype (29). Mammalian MMR genes are more closely related to yeast MMR genes. The first clue demonstrated that the role of MMR in cancer came from the discovery of MIN in sporadic colorectal tumors (30, 31). Although MIN is unusual in sporadic colorectal cancers, it occurs mostly in patients with hereditary non-polyposis colorectal cancer (HNPCC) (32). The basis of the MIN phenotype has been clarified by genetic analysis of HNPCC families. Linkage analyses originally led to the identification of *MLH1* and *MSH2* mutations in highly penetrant HNPCC pedigrees (33, 34). Moreover, targeted mutations in mouse homologues cause a spectrum of MIN and cancer susceptibility

phenotypes. *Msh2*$^{-/-}$ and *Mlh1*$^{-/-}$ mice have high levels of MIN in all somatic tissues tested and highly penetrant colon cancer susceptibility (35).

II. Chromosomal Instability and Carcinogenesis

CIN is the dominant phenotype of cancer cells and is recognized as numerical and structural aberrations of the genome. CIN has been related to genetic repair and mitotic control pathways, but the underlying mechanisms appear complicated and remain to be clarified (28). More than 100 genes are expected to cause CIN when mutated in eukaryotic cells, including genes that are involved in telomere metabolism, chromatid cohesion, spindle assembly and dynamics, cell-cycle regulation, DNA repair and checkpoint controls (36). For examples, mutations in the kinetochore binding proteins MAD, BUB, and the securins, key components of the large multi-protein cascade known as the anaphase-promoting complex (APC) in eukaryotes, increased chromosomal instability by different mechanisms (36, 37). *MAD2*$^{+/-}$ cells showed premature entry into anaphase and increased percentage of chromosome loss. Whereas, *hSecurin*$^{-/-}$ cells showed defect of sister chromatid separation at anaphase and increased nondisjunction (37). Targeted mutagenesis against the MAD2 gene in karyotypically stable, continuous cell lines led to CIN. The RecQ family of DNA helicases plays important roles in DNA repair, replication and recombination pathways (38). Defects of the RecQ family helicases encoded by the *BLM*, *WRN* and *RECQ4* genes in humans, give rise to Bloom's (BS), Werner's (WS) and Rothmund–Thomson (RTS) syndromes, respectively. These disorders are associated with cancer predisposition and premature aging. They also exhibited various types of chromosomal instability including elevated frequencies of sister chromatid exchanges (SCEs) (39, 40). Furthermore, the BRCA1 and BRCA2 proteins have been considered as caretakers of CIN because these proteins take part in DNA repair, cell-cycle checkpoint control, protein ubiquitination and chromatin remodeling. For example, the previous study demonstrated that murine lymphocytes carrying homozygous mutations of *Brca2*, frequently developed multiple cytogenetic lesions including chromosomal fragmentation, multi-radial formations, and random translocations (41, 42).

Genomic instability appears early in tumorigenesis and is believed to play a critical role in the malignant process (43). Oncogene activations or increasing number of copies of oncogenes and loss of tumor-suppressor genes are found in cancer cells with CIN. CIN can result in oncogene activations by translocation or gene amplification (9). Translocations can directly activate oncogenes by different mechanisms. First, the elevated expression of some oncogenes can result from proximity to strong transcriptional elements brought close to the target oncogene by translocation. Burkitt's lymphoma (BL) cells contain a t(8;14) reciprocal translocation that juxtaposes *c-myc* and strong transcriptional regulatory elements within the IgH locus on chromosome 14. This translocation results in potent transcriptional activation of *c-myc*, leading to overexpression and cellular transformation (44, 45). Second, Translocation can result in the creation of a fusion gene that encodes a novel oncogenic protein. The Philadelphia (Ph) chromosome, associated with chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL), represents this mode of oncogene activation through the generation of a BCR–ABL fusion protein with non-receptor tyrosine kinase hyperactivity that functions in the RAS-signaling pathway which drives cellular proliferation. The Ph chromosome is the product of a reciprocal translocation between the long arms chromosomes 9 and 22 (46, 47). Besides translocation, Amplification of proto-oncogenes is another common means of oncogene activation in numerous tumor types. Gene amplification is generally defined as an increase in the cellular copy number of a gene or genomic region relative to the rest of the genome. However, the mechanisms leading to amplification within a tumor have remained obscure (9). Gene amplification is an important process in human cancers. It is clearly associated with tumor progression, has prognostic significance and even provided a target for therapy in the case of breast cancers carrying HER2/neu amplification. In contrast, loss of large regions of a chromosome might promote tumorigenesis by the inactivation of tumor suppressor genes. Massive chromosomal deletion is frequently seen in myelodysplastic syndrome (MDS), a precursor of acute myeloid leukemia (AML). This deletion occurs frequently in chromosomes 5, 7 and 20. One candidate tumor suppressor is the ETF1 translation factor on chromosome 5, while candidate genes on chromosomes 7 and 20 remain to be elucidated (48, 49).

Global Hypomethylation

Genomic hypomethylation has been recognized as a common epigenetic change during cancer development (1, 2, 5, 50). Global losses of methylation in cancer may lead to alterations in the expression of proto-oncogenes critical to carcinogenesis and may facilitate chromosomal instability (5, 18). Genome-wide hypomethylation has been demonstrated by downregulation of methylated CpG dinucleotides, which disperse throughout the whole genomes both in noncoding repetitive sequences and genes (51, 52). Previous studies have described the hypomethylation of genomic repetitive sequences, a marker of the global genomic hypomethylation, in several malignancies including carcinoma of urinary bladder, liver, prostate, and colon (53-58). In those reports, Southern blotting and/or distinguishing PCR amplicons by methylation sensitive restriction enzymes were used to qualitatively evaluate the methylation status of LINE-1, a highly repeated and widely interspersed human retrotransposon, in isolated DNA from whole tissue samples. Additional studies described a quantitative method to analyze LINE-1 methylation by using COBRA (59). Chalitchagorn *et al.* (3) applied COBRA to evaluate the methylation status of LINE-1 repetitive sequences in genomic DNA derived from microdissected samples from several human normal and neoplastic tissues. They found that methylation of LINE-1 in leukocytes was independent of age and gender. In addition, normal tissues from different organs showed tissue-specific levels of methylated LINE-1. Globally, most carcinomas including breast, colon, lung, head and neck, bladder, esophagus, liver, prostate, and stomach, revealed a greater percentage of hypomethylation than their normal tissue counterparts. Furthermore, DNA derived from sera of patients with carcinoma displayed more LINE-1 hypomethylation than those of noncarcinoma individuals. Finally, in a colonic carcinogenesis model, they detected significantly greater hypomethylation in carcinoma than those of dysplastic polyp and histological normal colonic epithelium. Thus, the methylation status is a unique feature of a specific tissue type and the global hypomethylation is a common epigenetic process in cancer, which may progressively evolve during multistage carcinogenesis.

Global Hypomethylation and Carcinogenesis

Global hypomethylation may play crucial roles in carcinogenesis. Since genome-wide losses of DNA methylation has been found in many types of human cancer and associated with the degree of malignancy. Global hypomethylation potentially promotes cancer development through three possible mechanisms: oncogene activation, transposable element reactivation and chromosomal instability (18, 19).

I. Oncogene Activation

Loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality identified in cancer cells. It might be a mechanism of activation of oncogenes in addition to the mutation activation of oncogenes. The evidences supporting this notion came from the studies of hypomethylation of *CMYC* and *HRAS* oncogenes in bladder cancer and non-small cell lung cancer, respectively (60, 61). As mentioned above, one possible mechanism of global hypomethylation causing cancer may be the activation of oncogenes, which involve in tumor progression. A good example is the protease urokinase plasminogen activator (uPA), which is required for tumor invasion and metastasis. *uPA* gene is expressed in highly metastatic but not expressed in non-metastatic breast cancer cell lines. In non-metastatic cancer cell line, MCF-7 cells were treated with 5-aza-2-deoxycytidine, a DNA methylation inhibitor. The result showed activation of *uPA* and induction of metastasis in treated MCF-7 cells (62).

II. Reactivation of Transposable Elements

Hypomethylation in cancer cells may also lead to transcriptional activation of mobile genetic elements called retrotransposons. In normal cells, retrotransposons are highly methylated leading to inhibit of both gene expression and mobility of the inserted DNA. The function of methylation is to defense the genome from the deleterious effects of transposable elements. However, decreased methylation and consequent re-activation of expression of retrotransposons has been detected in a large number of human cancers. The most abundant retrotransposons in human genome are Long Interspersed Nuclear Element type 1 (LINE-1 or L1) which intersperse 17% throughout

the genome. Full length LINE-1s are 6 kb and have two open reading frames, ORF1 and ORF2. ORF1 encodes p40, an RNA-binding protein. ORF2 encodes a protein with endonuclease and reverse transcriptase activities, allowing their mobilization in genomes through an RNA intermediate (63). Loss of LINE-1 methylation can potentially lead to transcriptional activation, retrotransposition and integration at new site in the genome. Dangers resulting from LINE-1 reactivation in cancer cells comprise insertional mutagenesis and disturbance of transcriptional activity and gene regulation. Active LINE-1 transcripts have been detected in human testicular cancers (64), which intriguingly have the appearance of embryonal carcinoma or yolk sac tumor cells (65). Metastatic cells originating from these cancers also express LINE-1 transcripts. This study suggested that LINE-1 encoded proteins may function as oncoproteins in some tumors. The p40 product of LINE-1 ORF1 has also been found to be abundantly expressed in invasive malignant breast carcinoma compared with nonmalignant carcinoma or normal tissues (66). This observation proposed that LINE-1 expression was up-regulated during tumor progression. Additionally, LINE-1 insertional mutageneses have been found in sporadic cancers. LINE-1 insertions disrupt the *APC* gene and *CMYC* gene in a sporadic tumor of the colon and breast, respectively (19). In the disrupted *APC* gene, the nucleotide sequences in and around the insertion site exhibited the signature of retrotransposon integration (19). A role of genome hypomethylation in permitting transposition in cancer cells is not resolved, but there is substantial evidence for the unleashing of transcription of large numbers of retrotransposon sequences in a methylation dependent manner.

The association between Global Hypomethylation and Chromosal Instability

Chromosome aberrations are common finding in human cancers. It has been suggested that DNA methylation could be involved in the control of chromosome stability. In support of this notion, there is a study in ICF (Immunodeficiency, Centromeric region instability and Facial anomalies) syndrome, a rare genetic disorder in humans. This syndrome is caused by inherited mutations in the DNA methyltransferase *DNMT3B* (67). In all somatic cells of ICF patients, the pericentromeric heterochromatin of chromosomes 1 and 16 is abnormally hypomethylated. Mitogen stimulation of lymphocytes from ICF patients resulted in a high frequency of abnormalities involving chromosomes 1 and 16, and a lesser degree of chromosome 9 (68). Similarly, when Pro B cells are treated with the DNA methylation inhibitors 5-azadeoxycytidine and 5-azacytidine, the hypomethylated Pro B cells exhibited chromosomal anomalies in the pericentromeric region of chromosome 1 (69). From both studies, it inferred that global hypomethylation could induce CIN. Furthermore, DNA hypomethylation and CIN of the pericentromeric heterochromatin regions on chromosomes 1 and 16 had also been observed in ovarian epithelial tumors, breast adenocarcinomas and Wilms tumors (16). It implied that genomic hypomethylaion had a causal role in carcinogenesis by promoting CIN. Experimental studies from colorectal cancers by Vogelstein's group also supported this idea. They showed that colorectal tumors without MIN exhibited a striking defect in chromosome segregation, resulting in gains or losses of chromosomes. Furthermore, this group investigated the correlation between DNA methylation and genomic instability in colorectal cancer cell lines. They differentiated the methylation and consequently expression inhibition of exogenous retroviral genes in MMR-proficient (MMR^+) versus MMR-deficient (MMR^-) cell lines. MMR^- tumor cells inadequately expressed the foreign gene but MMR^+ tumor cells efficiently expressed. Therefore, MMR^- cell lines had higher potential of retroviral vector methylation than MMR^+ cell lines. Finally, they proposed that hypomethylation was positively associated with CIN, but not MIN (4). To further explore the link between DNA hypomethylation and CIN, Jaenish and colleagues showed that murine embryonic stem cells carrying defect of *DNMT1* gene (*DNMT1*$^{-/-}$) exhibited significantly elevated mutation

rates at both the endogenous hypoxanthine phosphoribosyltransferase (*Hprt*) gene and an integrated viral thymidine kinase (*tk*) transgene. Gene deletions were the predominant mutations at both loci (6). Moreover, this group also generated mice carrying mutant *DNMT1* allele, which reduced DNMT1 expression. They found that mutant mice developed aggressive T cell lymphomas at 4 to 8 months of age and cancer cells displayed chromosomal aberrations (5). In the final experiment, they studied the effect of DNA hypomethylation on tumor-prone mice carrying mutations in both the Neurofibromatosis 1 (*Nf1*) and *p53* tumor suppressor genes. They found that $Nf1^{+/-}$/ $p53^{+/-}$ (NPcis) mice showed a significant increase in loss of heterozygosity (LOH) rate when a hypomorphic *Dnmt1* allele was introduced (5). All studies of Jaenish's group indicated that mammalian DNA methylation played an important role in maintaining genome stability and a causal role in tumor formation by promoting CIN. Another potential connection between global hypomethylation and CIN, the evidence demonstrates indirect mechanisms by which retrotransposons could promote CIN in human cancer. Hypomethylation of the satellite sequences was thought to be the cause of decondensation of pericentromeric chromatin and an increased propensity for chromosomal breaks and rearrangements in this region. In a similar fashion, hypomethylation of transposable elements dispersed in the genome could facilitate illegitimate recombination. In favor of this idea, LINE-1 sequences are enriched at the ends of 3p14.1 and 9p21 deletions in carcinomas and homozygous deletions arise preferentially in chromosomal regions with high LINE-1 content. It has also been suggested that LINE-1 sequences were involved in the formation of double-minute circular chromosomes in cancer cells.

DNA Double-strand Break Repair

In order to maintain genomic stability, cells have to deal with a variety of DNA lesions. As mentioned in chapter I, the DNA double-strand break (DSB) is regarded as one of the particularly dangerous form of DNA damage induced by intrinsic sources such as the by products of cellular metabolism or by extrinsic sources such as ionizing radiation (IR). DSBs can easily lead to gross chromosomal aberrations if not rejoined quickly. Even if repaired quickly, the repair process may be error-prone, and may eventually be detrimental to the organism. Therefore, mammalian cells have mechanisms for rapidly transmitting the damage signal to the cell cycle arrest or apoptotic machineries and DNA repair mechanisms. Cell cycle arrest is necessary in order to give the cell enough time for repair. In some cases, it may be more prudent for the cell to undergo apoptosis when faced with excessive or unrepairable DNA damage. Both these processes are as effective barriers to carcinogenesis. Another important barrier to genomic instability and carcinogenesis is DSB repair. Mammalian cells employ two mechanisms for DSB repair, non-homologous end joining (NHEJ) and homologous recombination (HR). HR operates only in the S/G2 phases of the cell cycle when a sister chromatid is available. NHEJ, which simply pieces together the broken DNA ends, can function in all phases of the cell cycle and is the predominant repair pathway in mammalian cells (70).

I. Non-homologous end joining

NHEJ uses little or no homology to couple DNA ends. This pathway is not only used to repair DSBs generated by exogenous DNA-damaging agents, such as IR, but also required to process the DSB intermediates that are generated during V(D)J recombination. The possible three steps have been suggested for the repair of DSBs *via* NHEJ: (i) end binding, (ii) end processing, and (iii) ligation (Fig. 1). In the initial step, the Ku heterodimer, which consists of Ku70 (70 kDa) and Ku80 (86 kDa), binds the DNA ends. The end-binding activity of Ku indicates that it has an early role in the NHEJ process. Ku-bound DNA end attracts the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), a 470-kDa polypeptide with a protein kinase domain near its

carboxyl terminus. DNA-PKcs can subsequently phosphorylate several cellular target proteins as well as itself. At present, it is unclear which phosphorylation targets of DNA-PKcs are relevant *in vivo*. The second step, end processing is particularly important for repairing of more complex non-complementary ends. They require their terminal processing *via* nucleolysis and polymerization before the final ligation step. Several enzymes have been implicated to function in this step of NHEJ including RAD50–MRE11–NBS1 (MRN) complex, Artemis and WRN (Werner syndrome helicase). The MRE11 subunit of MRN complex is 3'-5' exonuclease. Thus, the role of the MRN complex involves in the unwinding and/or nucleolytic processing of the ends. Artemis, a 5'-3' exonuclease, has also been identified to interact with DNA-PKcs and is also phosphorylated by this kinase. Upon complex formation and DNA-PKcs-mediated phosphorylation, Artemis acquires endonucleolytic activity capable of opening the hairpin loop during V(D)J recombination as well as removing both 5' and 3' protruded ends for NHEJ repair. WRN is 3'-5' exonuclease with a preference for recessed 3' ends, and stimulated by Ku but inhibited by DNA-PKcs. WRN can displace DNA-PKcs from DNA-PK holoenzyme bound to a DNA end. In the final step, the coordinated assembly of Ku and DNA-PKcs on DNA ends is followed by recruitment of the DNA Ligase IV–XRCC4 complex that is responsible for the rejoining step. This complex lies at the center of the NHEJ pathway. It is present in all eukaryotes including yeast, which lacks DNA-PKcs but not Ku. Ligase IV is stabilized by forming a tight complex with XRCC4 and then stimulates its DNA ligation activity (10, 71, 72).

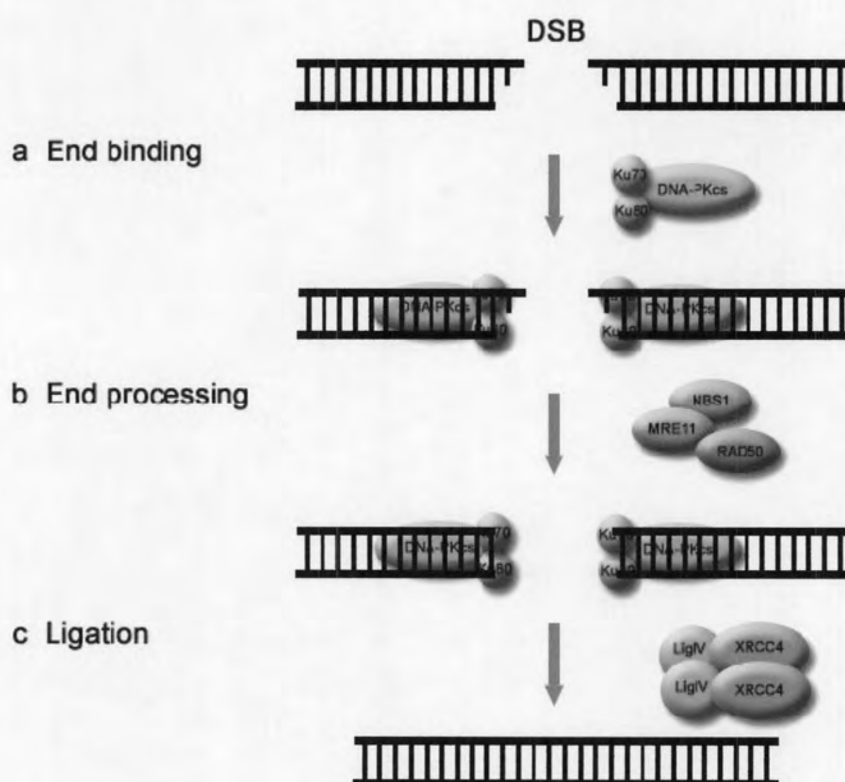

Figure 1 DSB repair via non homologous end-joining. (a) After induction of a DSB, the Ku proteins bind DNA end of DSB and recruit DNA-PKcs to this end leading to activation of DNA-PKcs. (b) The RAD50–MRE11–NBS1 (MRN) complex might be required to process broken end making it available for ligation. (c) This is followed by end-to-end ligation by the Ligase IV–XRCC4 complex. (MRE11, meiotic recombination 11; NBS1, Nijmegen breakage syndrome 1; XRCC4, X-ray-repair-crosscomplementing defective repair in Chinese hamster mutant 4)

II. Homologous recombination

In contrast to NHEJ, HR can repair DSBs by using undamaged sister chromatid as a template. Therefore, HR generally results in the accurate repair of the DSB. HR appears to involve a large number of proteins, including RAD51, RAD52, RAD54, BRCA1, BRCA2, the RAD51 paralogs (RAD51B, C, D), XRCC2 and XRCC3, and the MRN complex. A schematic outline of HR with its variations is shown in Figure 2. The first event is DNA recognition and resection to yield single-strand overhangs. This step could be controlled by ATM (ataxia telangiectasia mutated protein) since NBS1 is part of the MRN complex and is a direct substrate for ATM phosphorylation. The NBS1 subunit appears to be important for nuclear transport and for transmitting signals from DNA damage sensors to MRN. The RAD50 subunit of MRN has ATPase activity that is believed to facilitate DNA unwinding, whereas MRE-11 subunit has 3'-5' exonuclease activity. Next event, the RAD51 forms nucleoprotein complexes on single-strand DNA (ss DNA) tails coated by RPA (replication protein A) to initiate strand exchange. The RAD51 paralogs act as accessory proteins and are believed to facilitate the action of RAD51. In this step, the proteins mutated in inherited forms of breast cancer, BRCA1 and BRCA2, are also involved at an early point of DSB repair. Recently, it was demonstrated that BRCA2 interacts with RAD51 and RPA at the DSB and helps load RAD51 onto the DNA or to organize RAD51 filaments. Immunoprecipitation experiments suggested that BRCA1 and BRCA2 associated with RAD51 *in vivo*. BRCA1 and BRCA2 are physically associated with DSB repair foci, together with RAD51 and PCNA. Altogether, BRCA1 and BRCA2 are believed to be important at early points of HR. Then, the RAD52 helps RAD51 form DNA exchange intermediates. *In vitro* studies demonstrated that RAD52 forms ring structures consisting of seven RAD52 monomers and a hole that interacts with both ss and dsDNA. The RAD54 protein has ATPase activity and is related to DNA helicases. Thus, it is possible that RAD54 helps RAD51 and RAD52 unwind the DNA at the DSB to facilitate access of other repair factors. Following sister-chromatid pairing, the RAD51 nucleoprotein filament searches for the homologous duplex DNA. After the search has been successfully completed, DNA strand exchange generates a joint molecule between the homologous damaged and undamaged duplex DNAs. Finally, the gaps are filled by DNA polymerase and the

breaks are sealed by ligase. However, the DNA polymerase and ligase necessary for this step have not been identified (10, 73).

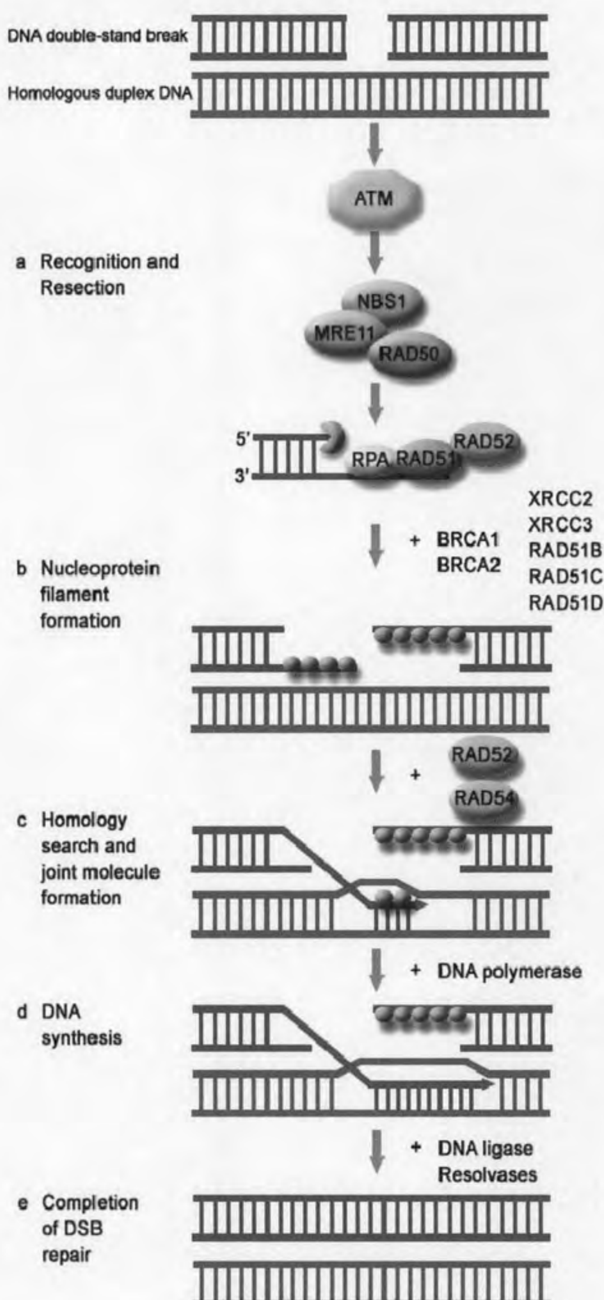

Figure 2 DSB repair via homologous recombination. (a) DSB formation triggers ATM sensor and NBS1 in MRN complex is phosphorylated by ATM. Processing of the ends occurs by the MRX complex and results in the formation of 3′ single stranded (ss) DNA overhangs. (b) The ssDNA-binding protein replication protein A (RPA) binds to the ssDNA overhangs, and RAD51 and RAD52 are recruited to the DSB. Both RPA and RAD2 help to load Rad51 onto ssDNA to form ssDNA–RAD51 nucleoprotein filaments. Other proteins implicated in the orchestration of a proper RAD51 response include BRCA1, BRCA2 and the Rad51 paralogues, XRCC2, XRCC3, RAD51B, RAD51C and RAD51D. (c) This nucleoprotein filament searches for the homologous duplex DNA in the undamaged sister chromatid. A successful search results in strand invasion, strand exchange and joint molecule formation. A reaction is stimulated by the RAD52 and RAD54 proteins. (d) DNA synthesis by DNA polymerases generates the genetic information that is required to seal the break. (e) Ligation and the resolution of the two double helices joined by strand exchange complete this error-free repair event.

Histone H2AX and DNA Double-strand Breaks Repair

The DSB repair must occur in the context of chromatin. The basic unit of chromatin is nucleosome, which comprises 147 bp of DNA wrapped around eight histones, two of each of H2A, H2B, H3 and H4. Linker histones, termed H1, associate with the DNA between individual nucleosomes establishing a higher level of organization (74). Thus, chromatin modifications are important for all cellular processes that involve DNA, including transcription, replication and DNA repair. Therefore, it is reasonable to consider that one of the first steps in the DNA repair process is reorganization of chromatin structure to allow access and action of the repair machinery. From genetic and biochemical studies, transcription is assisted by two classes of enzymes which alter the structure of chromatin. The first family functions through covalent modifications on the histone tails. Covalent modifications, including Phosphorylation, acetylation, methylation and ubiquitination, are known to be present on histones and to influence subsequent action of DNA- or histone-interacting factors. Members of the second family are characterized by their ability to disrupt histone-DNA contacts in the nucleosomal core using the energy of ATP hydrolysis. Similar to transcription, the process of DSB repair is also influenced by histone modifications. A modification that occurs specifically at sites of DSBs is rapid phosphorylation of histone H2AX on serine 139. This phosphorylated histone is often referred as γ-H2AX (74, 75).

The H2A family includes three subfamilies which are H2A1-H2A2, H2AZ, and H2AX. In mammals, the H2AZ represents about 10% of total cellular H2A, the H2AX represents 2–25% depending on the cell line or tissue type, and the H2A1- H2A2 represents the balance. H2AX variants contain a conserved SQ(E/D) motif in the C-terminus, and this motif also exists on the major replication linked H2A species in many lower eukaryotes (Fig. 3) (74-76). In mammalian cells, members of the PI-3 kinase-like family (PIKK) can phosphorylate the serine residue in the SQ motif of H2AX including mammalian DNA-PKcs, ATM and ATR (ataxia telangiectasia related protein). The SQ motif of H2AX is rapidly phosphorylated upon DNA damage at sites of DSBs, suggestive of a direct role in detection, signaling or repair of the lesion itself. Time-dependent phosphorylation of H2AX in response to DSB damage induced by extrinsic agents has

been studied extensively because of the availability of reagents and controllable experimental conditions. These studies have allowed the examination of the role of γ-H2AX in DSB repair. It has been shown that γ-H2AX is generated within 1 minute and forms foci within 3 minutes after irradiation. The phosphorylation of H2AX rapidly increases and peaks at around 30 minutes after irradiation. At this point, γ-H2AX starts being dephosphorylated, with a half-life of approximately 2 hours. Moreover, this phosphorylation was found to extend from the DSB in cis up to 2×10^6 base pairs (2 Mbp) (23, 77).

Since H2AX phosphorylation of the SQ motif is highly conserved, it might play an important role in DNA damage responses. To determine the physiological role of H2AX in mammalian cells, two groups produced targeted disruptions of mouse H2AX ($H2AX^{-/-}$). $H2AX^{-/-}$ deficient embryonic stem cells were hypersensitive to IR, but H2AX was not essential for survival. $H2AX^{-/-}$ mice were growth retarded and embryo fibroblasts from them proliferated poorly in vitro owing to premature senescence. Interestingly, these phenotypes resembled the ones of mice deficient for Ku80, Ku70 or ATM. As with Ku or ATM deficient murine fibroblasts, $H2AX^{-/-}$ cells exhibited elevated levels of both spontaneous- and IR-induced genomic instability (78, 79). The absence of H2AX impaired DNA repair caused by IR, probably accounting for an increased radiation sensitivity of $H2AX^{-/-}$ mice. Further studies, these two groups investigated the role of H2AX in mammalian DSB responses by examining the phenotypes of the offsprings of $H2AX^{-/+}$ and $p53^{-/+}$ mice. While $H2AX^{-/-}$ mice exhibited only a modest predisposition to lymphomas, either haploid or diploid mutations in H2AX in combination with the absence of the tumor suppressor p53, severely predisposed mice to various forms of cancer. $H2AX^{-/+}/p53^{-/-}$ and $H2AX^{-/-}/p53^{-/-}$ exhibited dramatic genomic instability, which led to clonal translocations. The finding that haploid insufficiency also results in increased genomic instability and cancer incidence in the absence of p53 is particularly intriguing, and indicates that H2AX expression levels are crucial for its functions. Thus, H2AX may impinge on DSB repair pathway (80, 81).

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         H2A Saccharomyces cerevisiae KLLGNVTIAQGGVLPNIHQNLLPKKS............AKATKASQEL
      H2A Schizosaccharomyces pombe KLLGHVTIAQGGVVPNINAHLLPKKSESHH........GRTGKPSQEL
        H2A Drosophila melanogaster SLI.KATIAGGGVIPHIHKSLIGKKEETVQDPQRKGN....VILSQAY
                  H2AX Mus musculus KLLGGVTIAQGGVLPNIQAVLLPKKSSATVGPKAPAVGKKASQASQEY
                   H2AX Homo sapien KLLGGVTIAQGGVLPNIQAVLLPKKTSATVGPKAPSGGKKATQASQEY
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Figure 3 Alignment of H2A and H2AX C-terminal sequences downstream of the histone fold from the indicated organisms. Budding yeast H2AS122 and analogous residues from other organisms are highlighted in green. Budding yeast H2AT126 and the H2AX upstream SQ / TQ motifs, which may or may not be functioning analogously in DNA-damage responses, are highlighted in blue. The major DNA-damage phosphorylation SQ motif present in the budding yeast core H2A (S129), mammalian H2AX (S139), and Drosophila H2Av (S137) is highlighted in red.