CHROMOSOMAL STABILITY AND THE DNA DOUBLE-STRANDED BREAK CONNECTION

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Genome stability is of primary importance for the survival and proper functioning of all organisms. Double-stranded breaks in DNA are important threats to genome integrity because they can result in chromosomal aberrations that can affect, simultaneously, many genes, and lead to cell malfunctioning and cell death. These detrimental consequences are counteracted by two mechanistically distinct pathways of double-stranded break repair: homologous recombination and non-homologous end-joining. Recently, unexpected links between these double-stranded break-repair systems, and several human genome instability and cancer predisposition syndromes, have emerged. Now, interactions between both double-stranded break-repair pathways and other cellular processes, such as cell-cycle regulation and replication, are being unveiled.

NUCLEOTIDE EXCISION REPAIR (NER). A DNA-repair pathway that removes ultraviolet-light-induced DNA damage (such as thymidine dimers) and bulky DNA adducts by excising the oligonucleotide that contains the damaged base(s). The single-stranded gap is filled in by using the intact strand as a template.

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The faithful maintenance and replication of the genetic instructions that are encrypted in DNA are of primary importance for living organisms. However, damage to DNA is intrinsic to life because its integrity is under constant attack from numerous exogenous agents, including radiation and chemicals, and from endogenous sources, such as free radicals generated during essential metabolic processes. The accumulation of DNA damage can induce permanent changes that lead to cancer or it can lead to severely impaired cellular functioning, which might eventually cause cell death by triggering apoptosis or irreversible cell growth arrest (FIG. 1). To counteract the deleterious effects of DNA damage, all organisms are equipped with an intricate network of DNA-repair mechanisms that each correct a subset of different lesions. This review focuses on the repair of a particularly genotoxic form of DNA damage — the DNA double-stranded break (DSB). These differ from many other types of DNA lesion in that both DNA strands of the double helix are damaged, which prevents the use of the complementary DNA strand as a template for repair. As a result, DSBs can be potent inducers of chromosomal aberrations.

DNA-repair pathways that do use the complementary strand as a template for repair include NUCLEOTIDE EXCISION REPAIR (NER) and MISMATCH REPAIR (MMR). The NER pathway repairs helix-distorting damage to the bases, whereas MMR corrects mispaired bases. The biological significance of DNA-repair mechanisms is underscored by their conservation throughout evolution and by the phenotypes of patients with inherited defects in DNA-repair genes. For example, NER deficiency in humans causes xeroderma pigmentosum, which is characterized by sun-sensitive skin and a predisposition to skin cancer[1]. Patients with defects in the complex cellular response to DSBs, including ataxia telangiectasia (AT) and Nijmegen breakage syndrome (NBS) patients, are also predisposed to cancer[2].

Two main forms of genomic instability are associated with tumour cells[3]. The mutational instability (MIN) phenotype is characterized by point mutations or small deletions. This type of instability is usually connected to MMR defects. By contrast, the chromosomal instability (CIN) phenotype is characterized by the gross rearrangement of chromosomes. Common chromosomal aberrations include the loss or gain of whole chromosomes or

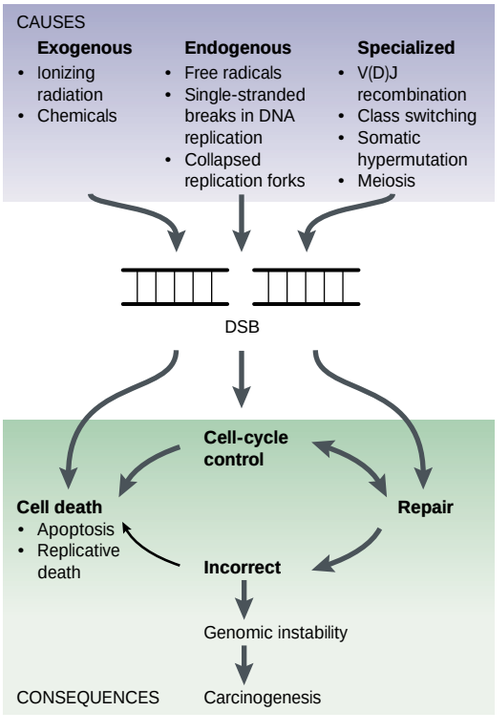


Figure 1 | **Causes, cellular responses and consequences of DNA double-stranded breaks.** Double-stranded breaks (DSBs) result not only from exogenous insults but also from cellular processes and during certain specialized recombination reactions. Cells have evolved several protective responses to counteract the harmful effects of DNA damage. Extensive DNA damage can result in cell death, which can occur by different routes. Cells can enter a state of irreversible growth arrest (replicative death), or they can trigger apoptosis, a highly regulated process in multicellular organisms. An alternative protection mechanism is provided by the combination of cell-cycle checkpoints and DNA-damage repair. After DNA damage, dividing cells can activate cell-cycle checkpoints to arrest the cell cycle before replication of the genome, during replication or before chromosome segregation into daughter cells. DNA-repair pathways can restore the integrity of the DNA during cell-cycle arrest. The fidelity of repair is of great importance to the fate of the cell. Inaccurate repair can lead to mutations and/or chromosomal aberrations (genome instability) that can contribute to carcinogenesis.

chromosome fragments, and the amplification of chromosome segments. (For more on different types of chromosomal rearrangements, see the link to Tokyo Medical University's animations of chromosomal structural abnormalities.) Loss of large regions of a chromosome can lead to the inactivation of tumour suppressor genes (for example, by loss of heterozygosity)[3], whereas amplification of chromosomal regions might promote tumorigenesis by the activation of proto-oncogenes[3], or by the induction of multidrug resistance after cytostatic drug treatment[4]. A different type of gross chromosomal aberration that is often observed in tumours is the (balanced) translocation, in which chromosome arms are exchanged. These rearrangements can be associated with the deregulation of gene expression or the fusion of two genes that then acquire oncogenic potential. A famous example is the fusion of the breakpoint cluster region (*BCR*) gene to the v-abl Abelson murine leukaemia viral oncogene homologue 1 (*ABL1*) gene on the PHILADELPHIA CHROMOSOME, which is found in the cancer cells of chronic myelocytic leukaemia patients[5]. The mechanism by which these translocations are produced has remained obscure for many years, although the prevailing idea is that DSBs on the two participating chromosomes might be the initiating lesions. Recent identification of several DSB-repair genes, the development of mouse models and new techniques, such as SPECTRAL KARYOTYPING (SKY), have provided insights into the processes that prevent chromosomal instability. In this review, we survey recent evidence that DSBs are among the lesions that cause chromosomal instability and discuss how DSB-repair factors counteract this instability.

DSBs and chromosomal aberrations

Double-stranded breaks can be caused by many different factors (FIG. 1). They can be induced by ionizing radiation, certain chemicals or free radicals that are produced during normal cellular metabolic reactions. In addition, they can also arise during DNA replication; when a replication fork passes through a template that contains a single-stranded break, the break will be converted into a DSB on one of the sister chromatids[6,7]. Finally, DSBs are also naturally occurring intermediates in several essential cellular processes. Most notably, they are intermediates during recombination in meiosis[8], which is necessary for germ-cell development during the assembly of mature IMMUNOGLOBULIN (Ig) or T-CELL RECEPTOR (Tcr) genes by V(D)J recombination and during Ig heavy chain (*IgH*) class switching[9] (BOX 1). Recent evidence also indicates that DSBs in the Ig loci are generated during somatic hypermutation[10,11].

Chromosomal aberrations have been observed in many types of tumour, with a bias towards tumours of lymphoid origin from which METAPHASE SPREADS can be easily obtained[12]. In many well-characterized cases, chromosomal translocations with one of the breakpoints in either an Ig or a Tcr locus have been found. The location of these breakpoints indicates that there might be a link between the chromosomal rearrangement and V(D)J recombination (in the Ig or Tcr loci), class-switch recombination (in the *IgH* locus) or somatic hypermutation (in the Ig loci). So, DSBs are implicated in the generation of translocations in lymphoid tumours, at least on chromosomes 2, 7, 14 and 22 that carry the Ig and Tcr loci[12]. However, numerous translocations that involve other loci have also been documented.

Other evidence for the involvement of DSBs in chromosomal aberrations comes from studies in which cells or animals have been exposed to ionizing radiation. Although this treatment generates a spectrum of DNA damage, including DNA single-stranded breaks, the most genotoxic lesions caused by ionizing radiation are DSBs. At relatively low doses, ionizing radiation does not cause extensive cell death, but it does contribute to chromosomal instability. Recent experiments revealed that this effect is particularly marked in cells that are

MISMATCH REPAIR
(MMR). A DNA-repair pathway that removes mismatched bases and corrects the insertion or deletion of short stretches of (repeated) DNA.

PHILADELPHIA CHROMOSOME
Chromosome 22, from which the tip of the q arm has been exchanged for a small region of the q arm of chromosome 9, fusing together the *BCR* and *ABL1* genes.

SPECTRAL KARYOTYPING
(SKY). A karyotyping method that allows all the chromosomes of an organism to be identified in a single metaphase spread. Each chromosome is labelled with chromosome-specific probes that can be visualized as different colours. This technique is useful for identifying certain chromosomal abnormalities.

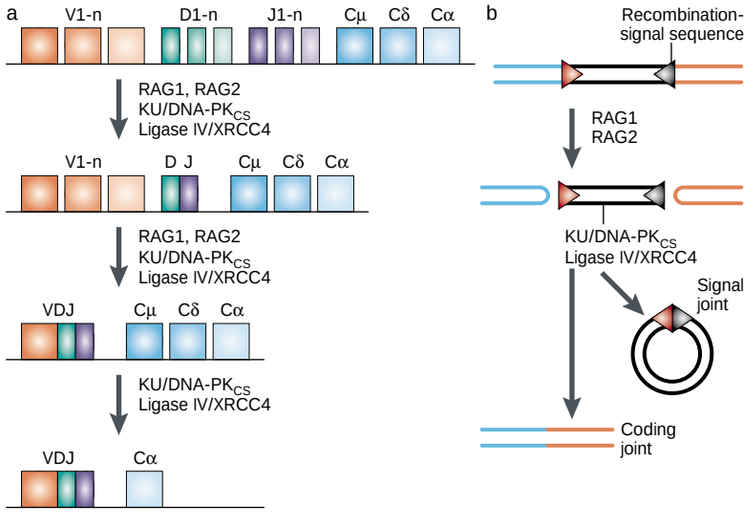
IMMUNOGLOBULIN
(Ig). Antigen-receptor molecules produced by B cells that consist of two heavy chains and two light chains.

T-CELL RECEPTOR
(Tcr). Antigen-receptor molecules produced by T cells that consist of either α and β, or γ and δ chains.

METAPHASE SPREAD
A collection of chromosomes arrested at the prophase of mitosis. Because the chromosomes are highly condensed at this stage of cell division, they are visible under a light microscope.

Box 1 | DNA rearrangements in the immune system

Genes that encode active immunoglobulins (Ig) and T-cell receptors (Tcr) are not present in the germ line. Instead, they occur as separate gene fragments that are assembled into active genes in specialized cells by a process called V(D)J recombination. Ig and Tcr genes are assembled in B and T cells, respectively. **a** | V(D)J recombination at the Ig heavy chain locus. Some of the proteins involved are indicated. First, one of the diversity (D) segments is coupled to a joining (J) segment, which is followed by coupling of a variable (V) segment to the DJ assembly, to give rise to a mature Ig heavy chain gene. After V(D)J recombination, Ig heavy chain class switching can delete several constant (C) segments, which results in the expression of a different ISOTYPE (for example, μ, δ, α) of the immunoglobulin. This process is mechanistically not well understood. However, it might proceed through a double-stranded break (DSB) intermediate because it requires the KU70, KU80 and DNA-dependent protein kinase (DNA-PK_{CS}) proteins. A third process that is important for the functioning of the immune system is somatic hypermutation, which introduces point mutations into the assembled Ig genes in response to antigen recognition by immature B cells. It was recently discovered that this process also correlates with the introduction of DSBs[10,11]. However, how these breaks contribute to mutagenesis is still an unresolved question. **b** | The basic mechanism of V(D)J recombination. The RAG1 (recombination-activating gene 1) and RAG2 proteins initiate V(D)J recombination by inducing a DSB at the border of the recombination signal sequence and of the gene segment. This produces a blunt end on the signal-sequence side and a DNA hairpin (in which the top strand is covalently coupled to the bottom strand) on the coding-sequence side. Subsequently, the DSBs are repaired by the general non-homologous end-joining (NHEJ) machinery (KU70/80, DNA-PK_{CS}, DNA ligase IV and XRCC4). The combinatorial repertoire of segments that can be joined allows for a large diversity of potential Ig and Tcr genes. In addition, the inaccuracy of DSB repair through NHEJ further enhances this diversity. (For more on V(D)J recombination, see the link to the V(D)J recombination animation.)



ISOTYPE
Class of immunoglobulin (Ig) protein that is determined by the constant region of the *IgH* gene that is placed nearest to the joining (J) segments. The isotype can switch during development of the B cell.

deficient in DSB repair (see below). These experiments have greatly benefited from technical breakthroughs in karyotyping that allow a genome-wide analysis of chromosomal aberrations. For example, with the use of SKY, DNA from each individual chromosome can be distinguished in a single metaphase spread[13].

Finally, elegant experiments that involve the introduction of site-specific DSBs, rather than randomly introduced DNA damage, have revealed that DSBs are potent inducers of chromosomal translocations. The direct introduction of a site-specific DSB has been accomplished by the expression of the I-*Sce*I restriction endonuclease in cells that harbour the 18-base-pair I-*Sce*I recognition site. If two such restriction sites are engineered into mouse embryonic stem (ES) cells, expression of the endonuclease results in a marked increase in interchromosomal recombination, which shows that DSBs can initiate chromosomal translocations[14]. In conclusion, there is much evidence that DSBs can give rise to chromosomal instability, although it has not been excluded that other lesions might also result in chromosomal translocations.

Cell-cycle checkpoints

DSBs are particularly dangerous lesions if they occur during the replication of the genome and during the segregation of duplicated chromosomes into daughter cells. Proper genome duplication is hampered by DSBs, and if broken chromosomes are carried through mitosis, the acentric chromosome fragments will not partition evenly between daughter cells. Therefore, eukaryotes have developed several checkpoints to prevent cells from starting DNA replication (the G1/S checkpoint), from progressing with replication (the intra S checkpoint) or from going into mitosis (the G2/M checkpoint), if they contain damaged DNA[15,16].

The marked biological consequences of the failure to enforce such checkpoints are exemplified by the human syndrome AT. This disorder is characterized by a high incidence of chromosomal translocations and frequent malignancies in lymphoid cells[2]. Furthermore, AT cells cannot enforce DSB-induced cell-cycle checkpoints (FIG. 2) and, as a result, AT patients are extremely sensitive to ionizing radiation. The gene responsible for AT (AT mutated or *ATM*) encodes an enzyme with protein kinase activity — a member of the phophatidylinositol-3-OH kinase (PI(3)K) family. Homozygous *Atm* knockout mice show a high incidence of lymphoid tumours, which frequently contain translocations at Tcr loci, indicating that the DSBs produced by the V(D)J recombination machinery might be responsible for these chromosomal aberrations[17–20]. Indeed, the prevention of DSB formation by inactivating one of the recombination-activating genes (*Rag1* or *Rag2*) strongly reduces tumorigenesis. The tumours that are observed arise later and do not contain Tcr rearrangements[21,22].

The actions initiated by the ATM kinase on activation by DSBs are multiple and complex[2]. Part of the signal-transduction cascade is mediated by the p53 protein, which is phosphorylated in response to ionizing radiation in an ATM-dependent fashion. In humans and mice, deletion of the gene that encodes p53 results in a defective G1/S cell cycle checkpoint and a reduced apoptotic response upon irradiation. Mice harbouring a homozygous *Trp53* deletion develop T-cell-derived tumours at a young age[23]. Furthermore, most human tumours also contain somatically acquired mutations in the *TP53* gene, which encodes p53, and the Li-Fraumeni syndrome (which is characterized by a high tumour incidence) can be caused by a heterozygous *TP53* mutation, underlining the importance of this gene for tumour prevention[15]. Another phosphorylation target of ATM is the chromatin component H2AX. H2AX is a variant of histone H2A and its phosphorylation is an

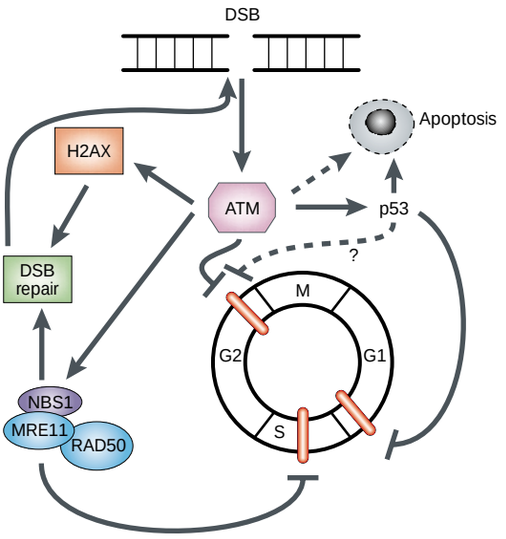


Figure 2 | **Ataxia-telangiectasia-mutated protein kinase and the cell-cycle checkpoint response to double-stranded breaks.** The presence of double-stranded breaks (DSBs) leads to activation of the ataxia-telangiectasia-mutated (ATM) protein kinase. Downstream targets of ATM phosphorylation include p53 and Nijmegen breakage syndrome 1 (NBS1) and probably the histone variant H2AX. In this way, ATM can influence cell-cycle progression and DSB repair, in addition to cell death through apoptosis. Because the ATM protein is one of most widely studied components in the cellular response to DNA damage, it has been placed in the centre of this figure. However, numerous other factors, such as the ataxia telangiectasia and Rad3-related (ATR) protein kinase, are essential for cell-cycle modulation after the induction of DNA damage[110] and some of its functions might be redundant with ATM. The red bars indicate the three main cell-cycle checkpoints; dashed lines show that the effect is not a principal pathway; and the question mark shows that whether the effect exists remains controversial.

early reponse to DSBs[24]. Phosphorylated H2AX is found in foci at which other DSB-repair proteins, including RAD50, accumulate over time[25]. At least one other PI(3)K family member, DNA-dependent protein kinase (DNA-PK, also known as PRKDC; see below), has been implicated in H2AX phosphorylation[25]. This redundancy suggests that H2AX phosphorylation might be crucial for the cellular response to DSBs.

Mechanisms of double-stranded break repair

All eukaryotes have evolved several mechanisms to deal with DSBs, which indicates the importance and difficulty of repairing this type of DNA injury. The two main pathways are homologous recombination (HR) and non-homologous end-joining (NHEJ). These two repair modes differ in their requirement for a homologous template DNA and in the fidelity of DSB repair. Whereas HR ensures accurate DSB repair, NHEJ does not. The relative contribution of these two DSB-repair pathways is likely to differ depending on the stage of the cell cycle[26,27]. However, the pathways are not mutually exclusive because repair events that involve both pathways can be detected[28]. HR is most efficient in the S and G2 phases of the cell cycle because of the availability of sister chromatids as repair templates[29]. In the absence of a sister chromatid, DSB repair in G1 phase could still efficiently occur through NHEJ. Below, we briefly discuss the salient features of DSB repair through HR and NHEJ.

Homologous recombination. Homologous recombination can repair DSBs by using the undamaged sister chromatid as a template (FIG. 3). Therefore, HR generally results in the accurate repair of the DSB. In organisms that range from yeasts to mammals[30,31], HR is mediated through the so-called RAD52 group of proteins[32,33], which includes RAD50, RAD51, RAD52, RAD54 and meiotic recombination 11 (MRE11). A brief summary of HR and the roles that these proteins have during this process is shown in FIG. 3. The initial cellular response to DSBs is mediated through ATM and NBS1. The latter is part of a protein complex together with RAD50 and MRE11. Subsequent steps of DSB repair through HR include DNA-end recognition, possibly by RAD52[34], and nucleolytic processing of the broken ends of DNA into 3′-end single-stranded DNA. This single-stranded DNA is bound by the RAD51 protein[35], which mediates crucial steps in the reaction — the search for a homologous duplex template DNA and the formation of joint molecules between the broken DNA ends and the repair template. Other proteins, including replication protein A (RPA), RAD52, RAD54[30] and several RAD51 PARALOGUES (such as RAD51B, RAD51C, RAD51D, XRCC2, XRCC3 and DMC1)[36,37], are thought to function as accessory proteins for RAD51 at various stages of HR. The later steps of the process include polymerization of nucleotides to restore degraded DNA strands and resolution of the recombination intermediates. Although the breast-cancer-susceptibility proteins BRCA1 and BRCA2 are clearly implicated in HR (see below), their role is not well understood. Compared with the RAD52 group proteins, the BRCA proteins are, from an evolutionary point of view, a more recent addition to HR because they have no obvious homologues in yeast.

Repair of a DSB by HR involves a reaction between three DNA molecules: the two DNA ends and the template DNA (FIG. 3). The RAD52 group of proteins can only accomplish this complicated task by close coordination and cooperation at the molecular level, which is reflected in numerous protein–protein interactions in this group[33,38]. This coordination is also found at the cellular level. Upon induction of DNA damage, many of the RAD52 group proteins undergo a dynamic relocalization into nuclear foci[39] (FIG. 4). Although it is not clear at present what processes occur in these foci, it has been established that many HR proteins co-localize to them[40,41]. Furthermore, RAD51-containing foci form at or near the site of DNA damage[42], and their DNA-damage-induced formation depends on RAD51 paralogues[36,37] and on BRCA1[43] and BRCA2[44].

Non-homologous end-joining. In contrast to HR, NHEJ uses little or no homology to couple DNA ends (FIG. 5). This pathway is not only used to repair DSBs generated by exogenous DNA-damaging agents, such as ionizing radiation, but also required to process the

PARALOGUE
A locus that is homologous to another in the same genome.

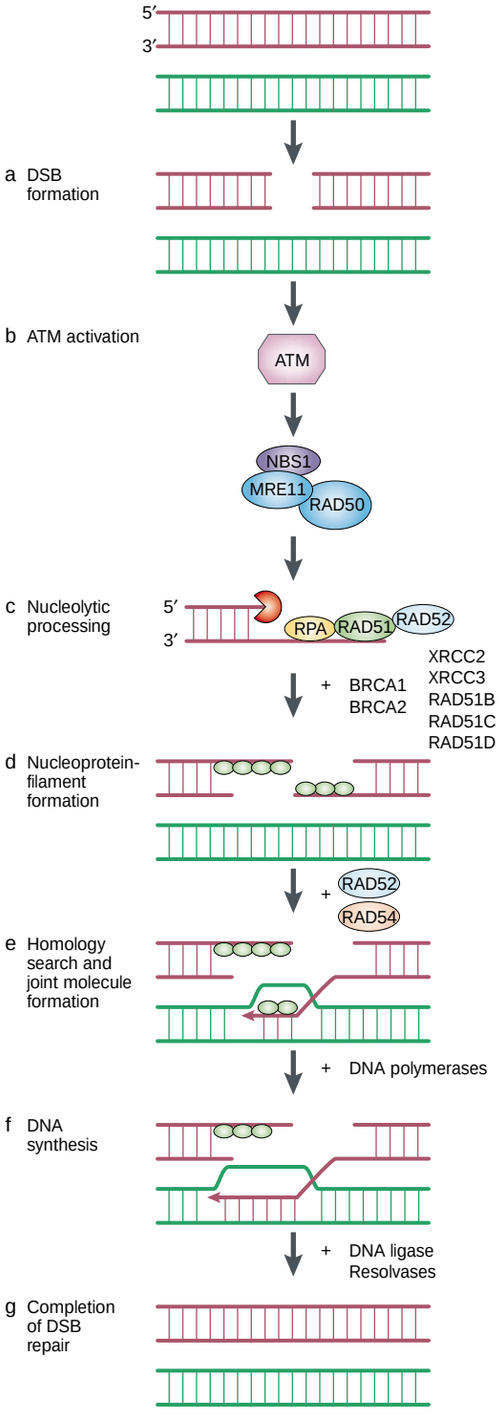


Figure 3 | **Double-stranded break repair through homologous recombination.** The differently shaded double-stranded DNAs represent homologous sequences (sister chromatids). **a** | Double-stranded break (DSB) formation triggers cell-cycle checkpoints through **b** | the activation of the ATM kinase. **c** | Subsequent events include the recognition of DNA ends, possibly by the RAD52 protein, and the nucleolytic processing of the DNA ends, which might require the activity of the RAD50–MRE11–NBS1 complex. NBS1 is one of the downstream substrates of ATM. The eventual result of this processing is the generation of a single-stranded region with a 3′ overhang. **d** | RAD51 polymerizes onto the single-stranded DNA to form a nucleoprotein filament, with the aid of the single-stranded DNA-binding protein, replication protein A (RPA) and RAD52. Other proteins implicated in the orchestration of a proper RAD51 response include BRCA1, BRCA2, and the RAD51 paralogues, XRCC2, XRCC3, RAD51B, RAD51C and RAD51D. **e** | 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 in a reaction that is stimulated by the RAD52 and RAD54 proteins. **f** | DNA synthesis, which requires DNA polymerases and their accessory factors, fills in the break in the strand. **g** | Ligation and resolution of recombination intermediates results in accurate repair of the DSB. (ATM, ataxia telangiectasia mutated; MRE11, meiotic recombination 11; NBS1, Nijmegen breakage syndrome 1; BRCA, breast cancer susceptibility; XRCC, X-ray-repair-cross-complementing defective repair in Chinese hamster mutant.)

RAD51 NUCLEOPROTEIN FILAMENT
A helical filament of RAD51 protein that covers single-stranded DNA. It contains approximately three bases for every RAD51 monomer and six monomers per helical turn. The nucleoprotein filament can pair with homologous double-stranded DNA.

DSB intermediates that are generated during V(D)J recombination[45] (BOX 1). Several proteins that are involved in NHEJ have been identified. The KU heterodimer, which consists of KU70 and KU80, has a high affinity for DNA ends, which indicates that it has an early role in the NHEJ process. KU bound to a DNA end attracts the catalytic subunit of the DNA-dependent protein kinase (DNA-PK_{CS}), a 470-kDa polypeptide with a protein kinase domain near its carboxyl terminus[46]. DNA-PK_{CS} can subsequently phosphorylate several cellular target proteins, including p53, the KU polypeptides and itself. At present, it is unclear which phosphorylation targets of DNA-PK_{CS} are relevant *in vivo*. A complex that consists of DNA ligase IV and XRCC4 (X-ray-repair-cross-complementing defective repair in Chinese hamster mutant 4) accomplishes the final ligation step. Cell lines or animals that lack either of the genes encoding these proteins do not carry out V(D)J recombination and are sensitive to ionizing radiation[47–50]. In addition to the involvement of the RAD50–MRE11-containing complex in HR, genetic experiments with yeast indicate that this complex also has a role in NHEJ[51,52].

Cellular models of chromosomal instability

The correlation between the formation of DSBs and the generation of chromosomal aberrations, as described above, indicates that the DSB-repair machinery is involved in the prevention and/or formation of such aberrant junctions. We first discuss studies at the cellular level, followed by an overview of recently developed animal models.

To study the effects of the loss of specific gene products on chromosomal instability at the cellular level, it is convenient to have cells that can be genetically modified with high efficiency. The development of efficient gene knockout and knock-in technology for *Saccharomyces cerevisiae* and the ability to combine different mutations by using genetic crosses have made this fungus a model for the dissection of DSB-repair mechanisms[33]. More recently, it has become possible to carry out gene disruption very efficiently in a vertebrate cell line. Most

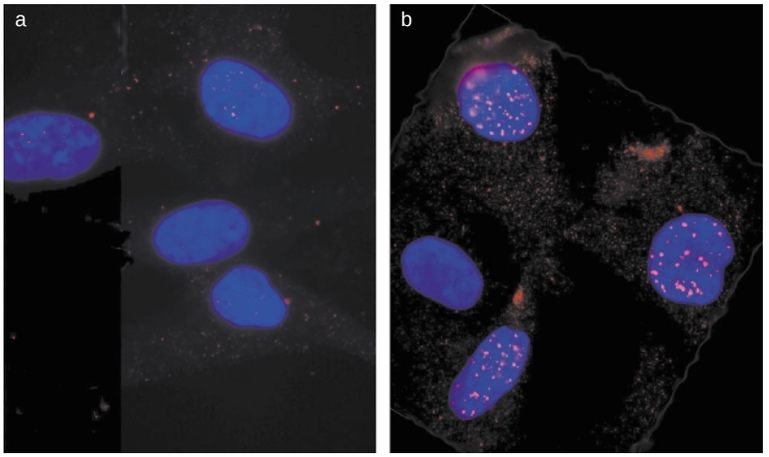


Figure 4 | **Foci induced by DNA damage.** Exposing cells to ionizing radiation causes several proteins that are involved in double-stranded break repair to relocalize to nuclear foci. **a** | Primary human fibroblasts that have not been irradiated. **b** | Primary human fibroblasts after exposure to 12 gray (Gy) of ionizing radiation. The resulting nuclear foci have been detected using a fluorescently tagged antibody against the RAD51 protein.
Courtesy of L. R. van Veelen, Department of Cell Biology and Genetics, Erasmus University, Rotterdam, the Netherlands.

mammalian cell lines integrate gene-targeting constructs at random locations in the DNA, with a minor fraction targeted to the homologous locus. By contrast, the chicken B-cell line DT40 shows a homologous targeting efficiency of more than 50% at virtually all loci, which allows the creation of many disruption mutants and combinations of more than one mutation in the same genetic background[53]. Chromosomal aberrations, which are infrequent in wild-type DT40 cells, can be scored by observing the 11 MACROCHROMOSOMES. Although these advantages make this cell line an attractive model system, DT40 cells can behave very differently to, for example, mammalian fibroblasts, as discussed at the end of this section.

DT40 cells without functional Ku70 and/or Rad54 have been generated as model systems for NHEJ and HR, respectively[26]. Disruption of the *RAD54* gene, which encodes a component of the HR pathway, causes radiosensitivity, whereas inactivation of *KU70*, which encodes a component of the NHEJ pathway, has no detectable effect on survival after γ-irradiation. However, *KU70*$^{-/-}$/*RAD54*$^{-/-}$ cells show higher radiosensitivity than *RAD54*$^{-/-}$ cells, revealing the additional effect of a NHEJ deficiency when combined with a HR defect. Disruption of *RAD54* also increases the rates of chromosomal aberration, mainly in the form of chromatid-type breaks. A low dose of γ-irradiation markedly increases the number of breaks in this mutant. The *KU70* mutation does not significantly affect chromosomal instability in DT40 cells, which suggests that HR is the main pathway to repair DSBs in these cells. However, a *KU70* mutation does increase the number of chromosomal aberrations in a *RAD54*$^{-/-}$ background, which shows that NHEJ can function as a back-up mechanism if HR does not function properly.

Deletion of *RAD51*, which is a main component of the HR pathway, results in non-viable chicken[54] and mammalian cells[55,56]. *RAD51*$^{-/-}$ DT40 cells that are rescued by an ectopic copy of the human *RAD51* gene under the control of a repressible promoter, can be studied after *RAD51* gene expression has been turned off. In these circumstances, the cells die within one or two cell divisions. They show a very high incidence of chromosome-type breaks in mitosis, which indicates a fundamental role for HR during replication. One plausible explanation for this is that HR promotes replication past DNA lesions. For example, replication of a chromosomal region that contains a single-stranded break converts the single-stranded break to a DSB on one of the sister chromatids that leads to the collapse of the replication fork. Repair of the DSB and the restart of replication are assisted by HR[57–60].

The RAD51 paralogues, RAD51B, XRCC2 and XRCC3 are also involved in HR and the maintenance of chromosomal stability. *RAD51B*-deficient DT40 cells show reduced levels of HR and a high percentage of the cells have chromosomal aberrations[37]. Similarly, murine cells in which the *Xrcc2* or *Xrcc3* gene has been mutated have low levels of HR[61,62] and high levels of chromosomal aberrations and/or chromosomal mis-segregation at mitosis[63,64], indicating that these Rad51 paralogues might all be required for the HR-mediated maintenance of genome integrity.

In yeast, the protein complex that contains Rad50 and Mre11 functions in HR, as well as in NHEJ. Although the exact role of this complex is still unclear, it is essential for the viability of vertebrate cells[65–67]. Similarly to the results with the conditional *RAD51* knockout, a DT40 cell line that expresses a repressible chicken *MRE11* gene loses viability upon repression of *MRE11* expression, probably as a result of chromosomal instability[65].

DT40 cells have a very short cell cycle, which is mostly taken up by the S phase. As several cell-cycle checkpoints do not function because the cells are *TP53* deficient, the precise effects of mutations in additional checkpoint genes might be difficult to assess. Because the ATM protein probably exerts its cell-cycle regulatory effects mainly through the p53 protein, inactivation of the *ATM* gene in these cells would not be expected to have a large effect on cell-cycle regulation. Nevertheless, *ATM*$^{-/-}$ DT40 cells are radiosensitive and show increased radiation-induced chromosomal instability, indicating that there might be a direct role for ATM in DSB repair in addition to its effects on cell-cycle regulation. Interestingly, a combination of *ATM* and *KU70* disruption enhances the chromosomal instability phenotype of the *ATM* mutant[68]. By contrast, the combination of *ATM* and *RAD54* mutations does not alter chromosomal instability relative to either of the single mutants, indicating that ATM might function in the HR pathway of DSB repair. It remains to be resolved whether this is due to the effect of ATM on cell-cycle checkpoints or more directly on DNA repair.

Most of the genes discussed above have also been studied in mammalian fibroblasts or ES cells. In some instances, the results obtained in these systems differ from the DT40 data discussed above; the relative impor-

MACROCHROMOSOME
The chicken genome is divided into macro- and minichromosomes. Only macrochromosomes are large enough to be easily observable under a light microscope.

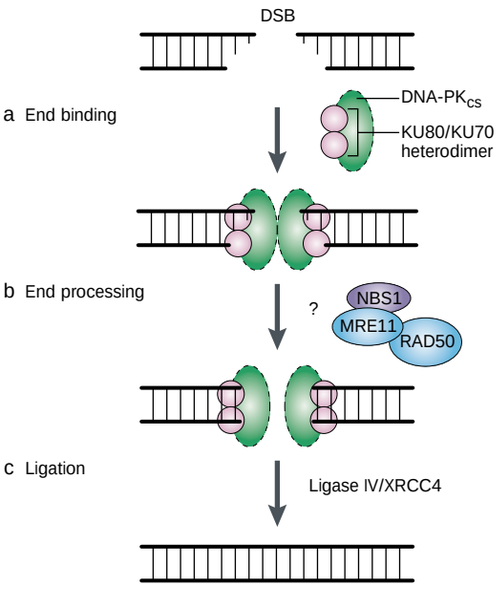


Figure 5 | **Double-stranded break repair by non-homologous end-joining. a** | After double-stranded break (DSB) formation, the KU–DNA-PK$_{CS}$ complex is probably involved in the initial recognition of the DSB and in the juxtaposition of the DNA ends. **b** | The ends might be processed, which results in the removal or addition of a few base pairs. **c** | This is followed by end-to-end ligation by the DNA ligase IV–XRCC4 complex. The role of the RAD50–MRE11–NBS1 complex is not yet clear. It might be involved in the unwinding and/or nucleolytic processing of the ends. Non-homologous end-joining does not make use of a template for repair and, therefore, this DSB-repair pathway is intrinsically error prone. (DNA-PK$_{CS}$,catalytic subunit DNA-dependent protein kinase; XRCC4, X-ray-repair-cross-complementing defective repair in Chinese hamster mutant 4; MRE11, meiotic recombination 11; NBS1, Nijmegen breakage syndrome 1.)

tance of HR and NHEJ can vary between different cell lines. For example, *Ku70*$^{-/-}$ DT40 cells are not radiosensitive, whereas disruption of the same gene in mouse ES cells results in a severely increased sensitivity to ionizing radiation[69]. Differences in cellular responses can even be found between different mammalian cell types. For example, DNA-PK$_{CS}$-deficient mouse ES cells are not radiosensitive, whereas DNA-PK$_{CS}$-deficient embryonic fibroblasts are[70]. However, although the relative importance of genes and pathways can differ between organisms and between cell types, both DSB-repair pathways can contribute to ionizing-radiation resistance and chromosomal stability.

Mouse models and genomic instability

Although much can be learned from cellular studies, the effects of DSBs on various differentiated cell types (with, for example, differences in cell-cycle checkpoint systems) and on the biologically relevant process of carcinogenesis can only be investigated in animal models. The recent development of several mouse models with defective NHEJ and HR has allowed the detailed analyses of the phenotypes that result from the disruption of several of the genes involved in either pathway or in both (TABLE 1).

The first known DSB-repair-defective mouse mutant was the SCID (severe combined immunodeficiency) mouse, which carries a spontaneous mutation that prevents the production of mature B and T cells, owing to a defect in joining the DSB intermediate in V(D)J recombination. These mice not only have a defect in the development of their immune system, but also are hypersensitive to ionizing radiation[46]. This phenotype is caused by a mutation in *Prkdc*, which results in a deletion of the 83 carboxy-terminal amino acids of the encoded protein (DNA-PK$_{cs}$)[71]. Subsequent experiments showed that deletion of the complete kinase domain and the complete knockout of the gene give rise to a similar phenotype[70,72,73]. The mutant mice develop lymphoid tumours of T-cell origin at high frequencies. The DNA-binding component of DNA-PK, encoded by the *Ku70* and *Ku80* genes, is also required for V(D)J recombination. However, in addition to the phenotypes described for the *Prkdc* mutant, *Ku70*$^{-/-}$ and *Ku80*$^{-/-}$ mice show reduced body weight at birth, slow growth and reduced lifespan[74–78]. Fibroblasts derived from these animals show early senescence, indicating a general proliferative defect. The more severe phenotype of the Ku compared with the *Prkdc* mutants might be related to a DNA-PK$_{CS}$-independent function of the Ku heterodimer, for example at telomeres[79,80].

The complex that contains DNA ligase IV and Xrcc4 accomplishes the ligation step of NHEJ. As expected, disruption of either gene results in an inability to complete V(D)J recombination and, consequently, the blockage of B- and T-cell development[47–49]. However, null mouse mutants have a different phenotype to DNA-PK mutants. Unexpectedly, mouse embryos that are deficient for either DNA ligase IV or Xrcc4 have postmitotic neurons that undergo widespread apoptotic death in the developing brain. This neuronal defect can be rescued by disrupting either the *Trp53* or *Atm* genes. The inactivation of these genes allows the animals to develop past birth, but does not rescue normal V(D)J recombination or the development of their immune system[81–83]. It is possible that p53 and Atm are required to promote apoptosis of neural cells that have accumulated DNA damage.

In mutant mice that lack components of DNA-PK or the DNA ligase IV–Xrcc4 complex (which we will collectively call NHEJ mutants), DSB repair is severely disturbed in all cells. Specifically in lymphoid cells, the Rag-induced DSB could be expected to cause a high incidence of tumours with a relatively short LATENCY TIME. However, NHEJ mutant mice exhibit a relatively long latency or even the absence of tumorigenesis. This can probably be explained by very efficient apoptosis in lymphoid cells, because the inhibition of apoptosis by a homozygous *Trp53* mutation, in addition to the NHEJ gene mutation, causes the very fast development of tumours[81,82,84]. Interestingly, these tumours are of B-cell origin, whereas the *Trp53* or NHEJ single-mutant mice develop T-cell tumours (with a much longer latency), indicating that each mutation might counteract the deleterious effects of the other much more efficiently in B cells than in T cells. Subsequent analysis of the rearrangements in these

LATENCY TIME
The time required to develop visible signs of disease, for example, a tumour.

Table 1 | Consequences of mutations in double-stranded break-repair genes

Gene	Cellular phenotype*	Mouse-knockout phenotype	Phenotype when combined with *Trp53* knockout	References
Non-homologous end-joining (NHEJ)				
PRKDC (encoding DNA-PK_{CS})	Radiosensitive, low V(D)J recombination	Radiosensitive SCID, T-cell tumours	Early B-cell tumours, chromosomal instability	70,72,73 110,111
KU70	Radiosensitive, low V(D)J recombination	Radiosensitive SCID, T-cell tumours, growth retardation		26,77,78 112
KU80	Radiosensitive, low V(D)J recombination	Radiosensitive SCID, T-cell tumours, growth retardation	Early B-cell tumours, chromosomal instability	74–76,84
LIG4 (encoding DNA ligase IV)	Radiosensitive, low V(D)J recombination	Embryonic lethal, apoptosis of postmitotic neurons	Rescues lethality, early B-cell tumours	47,48,50 81
XRCC4	Radiosensitive, low V(D)J recombination	Embryonic lethal, apoptosis of postmitotic neurons	Rescues lethality, early B-cell tumours, chromosomal instability	49,82
Both pathways				
MRE11	Not viable, chromosomal aberrations	ND		65, 66
RAD50	Not viable	Embryonic lethal		67
Homologous recombination (HR)				
ATM	Radiosensitive, chromosomal aberrations, radioresistant DNA synthesis	Radiosensitive, T-cell tumours, neurological dysfunction, infertility, growth retardation		17–19
RAD51	Not viable, chromosomal aberrations	Embryonic lethal	Delays embryonic lethality by a few days	54–56
XRCC2	Radiosensitive	Embryonic lethal, chromosomal aberrations	ND	63
RAD52	Slightly decreased HR	Wild type	ND	87,113
RAD54	Radiosensitive, MMC sensitive, decreased HR	MMC sensitive, increased radiosensitivity when combined with SCID mutation	ND	26,27,88
BRCA1	Not viable	Embryonic lethal	Delays embryonic lethality	114
BRCA2	Not viable	Embryonic lethal	Delays embryonic lethality in some embryos	114,115

*Phenotypes of mouse or chicken cells defective in DSB-repair genes. In some cases, the phenotype of the same mutation in different cell types can vary markedly. For example, whereas *DNA-PK_{CS}*-deficient embryonic fibroblasts are radiosensitive, *DNA-PK_{CS}*-deficient embryonic stem cells are not[70]. (*DNA-PK_{cs}*, catalytic subunit of DNA-dependent protein kinase; *XRCC,* X-ray-repair-cross-complementing defective repair in Chinese hamster mutant; *MRE11,* meiotic recombination 11; *ATM*, AT mutated; *BRCA,* breast cancer susceptibility; MMC, mitomycin C; SCID, severe combined immunodeficiency; ND, not determined.)

tumours by fluorescence *in situ* hybridization (FISH) and SKY analysis revealed translocations that involve Tcr or *IgH* loci, which emphasizes the importance of a DSB as the initiating lesion[85]. Chromosomal instability of NHEJ mutants is not limited to lymphoid cells: primary fibroblasts from *Ku80*$^{-/-}$ mice were also found to acquire more chromosomal aberrations than their wild-type counterparts[86]. These experiments were carried out with cultured cells or cells that were directly isolated from animals, in contrast to cells that were amplified and selected in tumours, as used in most other studies.

Mouse models in which genes that are involved in HR have been disrupted seem to fall into two classes. One class, which includes the *Rad52* and *Rad54* knockouts, contains viable mice that show no increase in spontaneous tumorigenesis[87,88]. The other class, which includes *Rad51* and *Xrcc2*, contains animals with an embryonic-lethal phenotype[55,56,63]. Given these phenotypes, the role of HR, through the Rad52 group of proteins, in the prevention of carcinogenesis is less clear than for the NHEJ pathway. Clues that implicate HR in the maintenance of genomic stability and in the prevention of carcinogenesis have come from cells and animals that are deficient in the *Brca1* and *Brca2* genes[38]. The products of *Brca1* and *Brca2* localize together with DNA-damage-induced Rad51 foci and seem to be required for the formation of such foci in response to specific insults to the DNA[43,44]. Furthermore, mouse cells that contain HYPOMORPHIC *Brca2* alleles show increased numbers of chromosomal rearrangements[89,90], and mouse ES cells carrying hypomorphic alleles of the *Brca1* gene show a reduced efficiency of HR[91,92]. The few mice that reach adulthood, which are homozygous for hypomorphic *Brca2*

HYPOMORPHIC MUTATION
A mutation that does not completely eliminate the wild-type function of a gene and gives a less severe phenotype than a loss-of-function mutation.

alleles, all succumb to thymic lymphomas[93,94]. The effects of Brca mutations and the lack of evolutionary conservation of the Brca genes compared with the Rad52 group of genes indicate that the Brca proteins are not directly involved in HR. It is possible that the Brca proteins have a regulatory role in HR that is required in mammals because of their greater complexity. Given that the Brca1 and Brca2 proteins differ greatly from each other in amino-acid sequence, it is likely that their roles in HR will also be different. Finally, the tumorigenic effects of mutations in the Brca genes might be attributable to other functions of these proteins, such as the regulation of cell-cycle responses or transcriptional control[38].

Insight from human syndromes

Defects in DNA-repair pathways can result in cancer predisposition syndromes. As mentioned above, defective NER causes xeroderma pigmentosum. However, for human syndromes, the involvement of proteins that are directly implicated in DSB repair, through an (enzymatic) action on the DNA substrate, is less clear. Only a single leukaemia patient has been identified as carrying a DNA ligase IV mutation, which resulted in a severely increased sensitivity towards ionizing radiation[95].

A classical human disorder with a defect in the response towards DSBs is AT. Patients with AT are radiosensitive and prone to cancer, with a particular predisposition to lymphoid malignancies. Cells from these patients show spontaneous chromosomal instability and fail to suppress DNA synthesis in response to ionizing radiation (radioresistant DNA synthesis). The gene that is mutated in these patients (*ATM*) encodes a member of the PI(3)K family, which also includes DNA-PK_{CS} (REF. 2). Cells from AT patients have defective cell-cycle checkpoints and might also be directly defective in DNA repair[96].

The cellular phenotypes of AT cells are very similar to those of cells from NBS patients. Cells from these radiosensitive patients are defective in the NBS1 protein. Recently, a satisfying molecular explanation was provided for the similarity in phenotypes of AT and NBS cells. It was shown that the ATM kinase phosphorylates the polypeptide that is derived from the *NBS1* gene, which is mutated in these patients[97–100]. The ATM-catalysed phosphorylation of NBS1 might regulate the activity of the RAD50–MRE11–NBS1 complex and indicates that ATM and the RAD50–MRE11–NBS1 complex function in the same pathway. Further support for this idea has come from the recent discovery that a human cancer predisposition syndrome with features very similar to AT, known as ataxia telangiectasia-like disorder (ATLD), is caused by mutations in the *MRE11* gene[101].

Several other chromosomal instability and cancer predisposition syndromes have links to DSB-repair pathways. Examples include Bloom syndrome and Werner syndrome, which are caused by mutations in members of a class of DNA-unwinding enzymes from the so-called RecQ helicase family. Cells from Bloom syndrome patients are defective in the *BLM* gene and show abnormally high levels of sister chromatid exchanges through the HR pathway[102,103]. The protein responsible for Werner syndrome (WRN) interacts with the KU heterodimer. This interaction results in increased WRN exonuclease activity and therefore indicates a possible link between the WRN protein and DNA end-joining[104,105]. Finally, the breast-cancer-susceptibility gene products BRCA1 and BRCA2, and their connection with HR, were mentioned in the preceding section. Interestingly, like NBS1, BRCA1 is also phosphorylated by the ATM kinase[106], which indicates that there might be an intricate complex of regulatory networks tied in with the actual DNA-repair machinery to coordinate cell-cycle checkpoints and repair pathways.

Conclusion and future directions

DSB-repair pathways have pivotal roles in the maintenance of chromosomal stability. In addition to processing DSBs that are induced by DNA-damaging agents or errors in replication, HR and NHEJ are essential for the repair of programmed DSBs in meiosis and immunoglobulin gene rearrangements, respectively. So, both DSB-repair pathways have overlapping as well as specialized roles. Furthermore, the relative contribution of the pathways in mammals might differ depending on developmental stage and cell type, and on the specific type of DNA damage[27].

An important intriguing, but unsolved, question is why the type and location of tumours differ among the different DSB-repair-associated mutants. Mutations in *BRCA1*, for instance, cause breast cancer, whereas *ATM* mutations are mainly associated with lymphoid malignancies. The reason for this specificity could be related to differences in cell-cycle regulation, requirements for repair pathways, or the cellular environment of different cell types from which the tumours arise. Therefore, it will be interesting to find out more about the relative contributions of these various factors to DSB repair, as well as chromosomal stability in various cell types and tissues. Generation of new mouse models, which combine different mutations in mice, and a more detailed analysis of existing mouse models, will provide valuable information about the intricate interactions of the various DNA-repair pathways and cell-cycle checkpoints. The availability of the complete mouse and human genomes in the near future, and the development of new techniques to study networks of protein–protein interactions, will rapidly expand our insight into this complex process[107,108].

Links

DATABASE LINKS xeroderma pigmentosum | ataxia telangiectasia | Nijmegen breakage syndrome | *BCR* | *ABL1* | chronic myelocytic leukaemia | Tcr | *IgH* | *ATM* | *Rag1* | *Rag2* | *Trp53* | Li-Fraumeni | PI(3)K | DNA-PK | RAD52 | RAD50 | RAD51 | RAD52 | RAD54 | MRE11 | NBS1 | RPA | RAD51B | RAD51C | RAD51D | XRCC2 | XRCC3 | DMC1 | BRCA1 | BRCA2 | KU70 | KU80 | DNA-PK_{CS} | DNA ligase IV | XRCC4 | SCID | ataxia telangiectasia-like disorder | Bloom syndrome | Werner syndrome | *BLM* | WRN

FURTHER INFORMATION Tokyo Medical University's animations of chromosomal structural abnormalities | V(D)J recombination animation

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