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Recombinational DNA repair and human disease

Larry H. Thompson [a,*], David Schild [b]

[a] *Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory L-441, P.O. Box 808, Livermore, CA 94551-0808, USA*
[b] *Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA*

Abstract

We review the genes and proteins related to the homologous recombinational repair (HRR) pathway that are implicated in cancer through either genetic disorders that predispose to cancer through chromosome instability or the occurrence of somatic mutations that contribute to carcinogenesis. Ataxia telangiectasia (AT), Nijmegen breakage syndrome (NBS), and an ataxia-like disorder (ATLD), are chromosome instability disorders that are defective in the ataxia telangiectasia mutated (*ATM*), *NBS*, and *Mre11* genes, respectively. These genes are critical in maintaining cellular resistance to ionizing radiation (IR), which kills largely by the production of double-strand breaks (DSBs). Bloom syndrome involves a defect in the BLM helicase, which seems to play a role in restarting DNA replication forks that are blocked at lesions, thereby promoting chromosome stability. The Werner syndrome gene (WRN) helicase, another member of the RecQ family like BLM, has very recently been found to help mediate homologous recombination. Fanconi anemia (FA) is a genetically complex chromosomal instability disorder involving seven or more genes, one of which is *BRCA2*. FA may be at least partially caused by the aberrant production of reactive oxidative species. The breast cancer-associated BRCA1 and BRCA2 proteins are strongly implicated in HRR; BRCA2 associates with Rad51 and appears to regulate its activity. We discuss in detail the phenotypes of the various mutant cell lines and the signaling pathways mediated by the ATM kinase. ATM's phosphorylation targets can be grouped into oxidative stress-mediated transcriptional changes, cell cycle checkpoints, and recombinational repair. We present the DNA damage response pathways by using the DSB as the prototype lesion, whose incorrect repair can initiate and augment karyotypic abnormalities.
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1. Introduction

One of the hallmarks of tumor cells is their highly rearranged karyotypes with respect to both chromosome number and the structural integrity of each homologous pair. Numerous rearrangements can be visualized in vivid detail using spectral karyotyping, which specifically colors each chromosome, e.g. [1]. These changes presumably reflect the selective outgrowth of outlaw cells whose rearrangements have conferred growth advantages during the evolution of the tumor. Chromosomal rearrangements are a consequence of the loss of fidelity in repairing double-strand breaks (DSBs). These dangerous lesions are corrected by the two primary pathways of nonhomologous end-joining (NHEJ) and homologous recombinational repair (HRR) (reviewed in [2–7]). Recent studies employing mouse models have shown that the absence of either pathway leads to genomic instability, including potentially oncogenic translocations [8]. In humans, inherited genetic defects in these pathways are often manifest as increased radiosensitivity [9] since DSBs

* Corresponding author. Tel.: +1-925-422-5658; fax: +1-925-422-2099.
E-mail address: thompson14@llnl.gov (L.H. Thompson).

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are the major contributor to cell lethality and mutation produced by ionizing radiation (IR).

The NHEJ pathway involves a "duct-tape" approach to mending a DSB that can result in the gain or loss of nucleotide sequence [10]. DNA-dependent protein kinase (DNA-PK, composed of DNA-PKcs catalytic subunit and the Ku70–Ku86 heterodimer), along with the ligation factors LIG4 and XRCC4, are five critical components of this repair process. Besides their role in DSB repair, Ku and DNA-PKcs proteins provide another level of chromosome stability by participating in capping chromosome ends, or telomeres, thereby protecting them from end-to-end fusions [11–14]. However, in the absence of one of the NHEJ proteins in mutant cells, residual end-joining reactions still operate as evidenced by chromosomal translocations [15–17]. During normal V(D)J recombination in B and T lymphocytes, DSBs created by the site-specific cleavage by the RAG proteins are subsequently processed and rejoined by components of the nonhomologous DNA end-joining pathway [18].

A prominent role for the HRR pathway in maintaining chromosome stability has become apparent from biochemical [10] and genetic studies on a variety of mutant cell lines (reviewed by [5,19,20]). An emergent concept is that the formation of DSBs is a normal by-product of DNA replication, especially in higher eukaryotes, which have much larger chromosomes than yeast. Replication forks encountering single-strand breaks (SSBs) or other lesions may undergo collapse with the formation of a "one-sided" DSB. Restoration of the fork can be accomplished by break-induced replication to re-establish strand continuity. In addition, replication-blocking lesions such as bulky adducts may result in gaps in one of the nascent strands. A free single-stranded end associated with the gap may pair with the intact sister DNA duplex and undergo recombination to achieve an error-free elimination of the gap with the adduct still in place.

2. Cancer-prone genetic disorders involving proteins associated with homologous recombination

2.1. Ataxia telangiectasia (AT)

AT is a rare human genetic disorder that is characterized by progressive neuronal degeneration with loss of cerebellar function, immunodeficiency, sterility, cancer predisposition, and clinical radiation sensitivity [21]. Along with the cerebellar ataxia, telangiectasis (dilation of small blood vessels and capillaries) of the sclera, face, and ears are a characteristic feature of AT. The increased risk of cancer is estimated to be 60–180-fold higher than the general population, with ~70% of malignancies being lymphomas and T cell leukemias; a wide variety of solid tumors make up the remainder [21]. Studies with ataxia telangiectasia mutated (*ATM*) mouse models support the concept that phenotypic heterogeneity may be caused in part by the nature of the mutations [22].

The cellular phenotype of AT is characterized by chromosomal breakage, telomere instability, radiosensitivity, radioresistant DNA synthesis, defective cell cycle checkpoints, dysfunctional apoptosis, and a reduced p53 response (reviewed in [21,23–26]). AT fibroblasts and other cell types are typically killed at radiation doses 3–5-fold lower than those that kill normal cells [27,28], and similar sensitivity is manifest as chromosomal aberrations. Levels of spontaneous mutations at the GPA and *hprt* loci are elevated in AT cells, supporting a causal link between susceptibility to somatic mutation and cancer [29,30]. Several studies have also shown that AT cells have higher levels of residual unrepaired DSB after irradiation [31–33].

The gene that is defective in AT cells is designated ataxia telangiectasia mutated [34] and encodes a protein of 3056 a.a. [34] having sequence similarity with several checkpoint genes in yeast (e.g. $Mecl^{Sc}$, $Rad3^{Sp}$, and $Tel1^{Sp}$) that encode members of the phosphoinositol 3-kinase family of proteins [24,35]. Although ATM appears to have cytoplasmic functions in certain cell types [24,36,37], it is predominantly nuclear in localization in fibroblasts and is not induced by DNA damage [38]. As a kinase with preference for the S/TQ motif, ATM is the master regulator in a signaling network responsible for coordinating repair of DSBs, checkpoint functions, and other signaling processes that promote cellular recovery and survival [23,24,39,40]. As illustrated in Fig. 1, the ATM kinase has numerous substrates that are involved in the activation of cellular functions in response to spontaneous and IR-induced DSBs. Whereas some of these target proteins are directly involved in HRR (e.g. NBS1 and RPA), most of them participate in other signaling

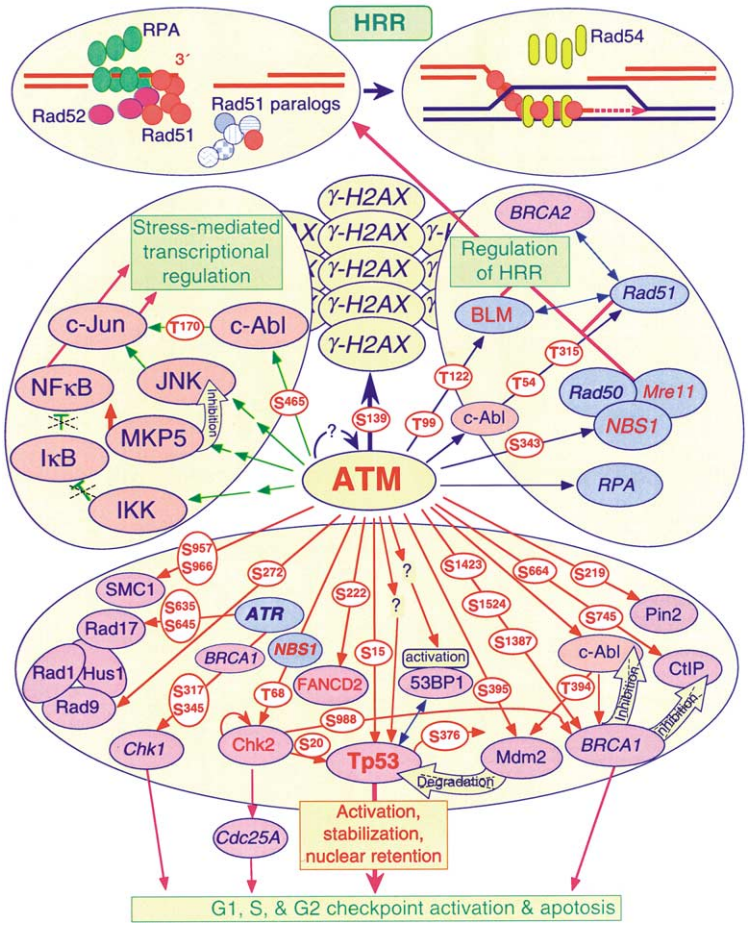

Fig. 1. DSB-induced phosphorylation reactions mediated by the ATM kinase that lead to transcriptional changes, implementation of cell cycle checkpoints, and execution of DNA repair processes. Names of proteins that are involved in human genetic disorders are shown in red, and proteins that are known to be essential for cell viability are shown in italics. Blue lines with arrowheads at both ends designate important interactions that do not involve phosphorylation. Many of the ATM substrates are involved in the cascade of events representing recognition and signaling of the presence of a DSB. These reactions recruit NHEJ proteins (not shown), or the HRR machinery when replication forks or sister chromatids are present. The "×" marks designate the overriding effect in response to DSBs that occurs to reverse the baseline function of the protein. Most of the specific phosphorylations shown have demonstrated biological importance. ATM's target proteins include: SMC1 [346,347], Rad9 [348], Chk2 [349–352] (this phosphorylated form of Chk2 specifically localizes at sites of DSBs [353]), Tp53 [354,355], Mdm2 [356], BRCA1 [203,206,357], CtIP [207], c-Ab1 [358,359] (which also phosphorylates Mdm2, BLM [123], and Pin2 [360]. Activated Chk2 also phosphorylates Tp53 [361–363]. BRCA1 is essential for activating the Chkl kinase in the G2 checkpoint [210]. It is not established whether the ATM-dependent phosphorylation of 53PB1 results from a direct interaction [364,365]. The phosphorylation of other key proteins in checkpoint responses is mediated by ATR, i.e. Radl7 [366,367] and Chkl [366,368,369]. Chk2 [370], and likely ATM as well, undergoes autophosphorylation in response to IR damage.

processes and checkpoint functions (e.g. H2AX, Tp53, Mdm2, BRCA1, Chkl, Chk2), or other regulatory responses promoting apoptosis or cell proliferation (e.g. c-Ab1 and E4-BP1). Most ATM-mediated signaling events involve phosphorylation, but some proteins in these networks, such as MKP5 [41,42] and p53 [43], undergo dephosphorylation at specific sites.

Acting in parallel and cooperatively with ATM is the closely related ATR (ATM and Rad3 related) kinase [44]. A human disorder has not been associated with the *ATR* gene, which is associated with early

embryonic lethality when knocked out in the mouse [45,46], unlike the ATM knockout which is viable (discussed in the subsequent sections). ATR appears to overlap with ATM in implementing checkpoints functions but is the main kinase mediating signaling responses to damage affecting DNA replication, such as photoproducts from UV radiation or inhibition of DNA replication (e.g. hydroxyurea). ATR acts on many of the same substrates as ATM (e.g. H2AX, Tp53, BRCA1, Chk1, and Chk2). The roles of ATM and ATR as checkpoint mediators were recently reviewed extensively by Abraham [47].

One of the earliest phosphorylation events detected at sites of DSBs is the abundant modification of histone H2AX at Ser^{139} (referred to as γ-H2AX) [48–50]. The formation of γ-H2AX appears to be absolutely dependent on ATM [51] although an earlier report mentioned conflicting results [48]. Moreover, a fraction of the ATM pool becomes resistant to extraction and is detected in nuclear aggregates [52] that co-localize with γ-H2AX and with foci of the Nbs1 protein. These findings suggest a major role for ATM in the early detection of DSBs and subsequent induction of cellular responses. The specific activity of ATM increases in response to DSBs but the basis for this "activation" is not well understood but may involve self-phosphorylation [53,54]. There is evidence that ATM molecules can interact with each other [55].

Although *H2AX* appears dispensable in knockout mice for IR-induced cell cycle checkpoints, $H2AX^{-/-}$ mice are radiation sensitive, growth retarded, and immune deficient [56]. These mice seem to have normal NHEJ but show high levels of chromosomal aberrations (20–25% abnormal metaphases) [57]. Defective DNA repair is further indicated by the findings of chromosomal instability, mild MMC sensitivity, reduced DSB rejoining, reduced gene targeting efficiency, and notably impaired recruitment of NBS1, 53Bp1, BRCA1 (but not Rad51) into IR-induced nuclear foci. These findings show that γ-H2AX is critical for mediating the assembly of specific DNA repair complexes on damaged DNA and preserving chromosome stability. This study suggests that the signals for implementation of checkpoints in response to IR damage are generated independent of γ-H2AX.

The trimeric RPA complex binds and protects single-stranded DNA and is involved in numerous DNA transactions. In the presynaptic stage of HRR (Fig. 1, top), RPA must be displaced from single-stranded DNA to allow formation of the Rad51 nucleoprotein filament, which initiates homologous pairing. It is not known whether ATM-mediated phosphorylation directly influences the proteins in this exchange process, e.g. phosphorylation of Rad51.

Genetic evidence supporting a major contribution of the ATM protein in the HRR pathway of DSB repair is provided by the analysis of single and double mutants in chicken DT40 cells [58]. An *atm* knockout mutation combined with a *ku70* null showed very high radiation sensitivity whereas the *atm rad54* double mutant was more similar in sensitivity to each of the single mutants, suggesting that *ATM* and *RAD54* act in the same repair pathway.

Mice carrying one or two disrupted alleles of *ATM* mimic AT in most respects [59,60]. Heterozygous ATM deficiency in mice confers shortened life span and premature greying after sub-lethal exposure to 4 Gy radiation [61]. Using cataractogenesis in the lens as an assay for late effects in irradiated tissue, Hall and workers found that cataracts developed earlier in ATM heterozygotes than in normal mice [62]. *Atm* homozygous null mice develop thymic lymphomas at an early age, and *atm* fibroblasts in culture exhibit premature growth arrest and elevated constitutive p21/CDKN1A [60,63]. In contrast, fibroblasts from double-null *atm p53* mice, as well as *atm p21* mice, show no p21 protein and do not exhibit the premature growth arrest seen with *atm* null MEFs [63,64]. These results imply that p53 and p21 are responsible for the proliferation defects in *atm* cells. Tumor formation in *atm p53* double-null mice is dramatically accelerated relative to single null mice [63], indicating that the two genes collaborate to prevent tumorigenesis. In contrast, *atm p21* double null mice show a delay in thymic lymphomagenesis and increased IR sensitivity [65].

The contribution of ATM-mediated stress-signaling pathways (Fig. 1, left) to cellular recovery from IR damage is poorly understood. NFκB is important in protecting cells against proapoptotic stimuli such as DSBs. The activation of IκB kinase(s) (IKK), which in turn lead to initiation of transcriptional activation of NFκB, is mediated by ATM [66]. The phosphorylation of c-Ab1 by ATM has been suggested to regulate HRR [67,68], but analysis of a c-Ab1 null mutant of DT40 cells showed that c-Ab1 was not required for ATM's chromosome stability functions

[69]. Besides activating stress kinases, ATM also mediates increased expression of the phosphatase MKP5, which acts in a feedback loop to down-regulate JNK and p38 stress-activated kinases [42].

The issue of whether AT heterozygotes are at increased risk of breast cancer has engendered controversy [70,71]. Recent biochemical studies show that several ATM missense mutations, as well as a truncation mutation, behave in a dominant negative manner [55,72]. This effect may be explained through the demonstrated multimerization of ATM protein [55,73]. These missense mutations, when expressed as transgenes, abolished the radiation-induced ATM kinase activity, caused chromosome instability, and reduced cell viability after irradiation. In contrast, four of five low frequency (1/384) breast-tumor variants showed no such dysfunction and, therefore, may not confer increased susceptibility. These findings provide an explanation for the observation that heterozygotes of both *ATM* and *NBS1* have abnormally high levels of chromosomal breaks after IR exposure [74], reinforcing the idea that the *ATM* and *NBS1* genes occupy a position of central importance in cancer biology (see Fig. 1).

2.2. Nijmegen breakage syndrome (NBS)

NBS is a genetic disorder, first described in 1981 [75] and closely related to AT, that involves a distinct gene that was identified in 1998 [76–78]. NBS patients resemble AT patients with respect to immunodeficiency, radiosensitivity, and predisposition to malignancy, but they lack both ataxia and telangiectasis (reviewed in [79,80]). The cellular phenotype of NBS cells is remarkably similar to that of AT cells [80]. NBS cells grow poorly and exhibit premature senescence [81,82]. Some NBS cells exhibit a partial defect in the Gl checkpoint response to IR damage [83–87], and a partial defect in the S-phase checkpoint, manifest as radioresistant DNA synthesis, is characteristic of NBS cells [88]. The G2 checkpoint after IR exposure has been variously described as deficient [89], normal [81,85], or abnormally prolonged [82,84,90]. However, one nonimmortalized NBS fibroblast line having essentially normal checkpoint responses [81] led to the conclusion that the sensitivity to IR is caused by a DSB repair defect and not faulty checkpoints. A DNA repair defect likely explains the defective class switching of immunoglobulin genes in NBS that impairs immune system development [91]. A defect in *NBS1* is also associated with premature telomere shortening [92].

The 754 a.a. NBS1 protein contains FHA and BRCT domains in the C-terminal region and has a low level of similarity with the *S. cerevisiae* Xrs2 protein (28% identity over an 87-residue region). NBS1 is a component of a stable complex containing mammalian homologs of the yeast Mre11 and Rad50 proteins [76,93]. The Rad50–Mre11–NBS1 (R–M–N) complex associates with sites of DSBs within 30 min after IR exposure [94] and forms nuclear foci that are maximal at ∼8 h [95], after most DSB rejoining has occurred. Likewise, in developing thymocytes, NBS1 and γ-H2AX co-localize in foci at the T cell receptor alpha locus in response to recombination activating gene (RAG) protein-mediated V(D)J cleavage. Direct interaction of NBS1, through its C-terminal region, with Mre11 is required for nuclear localization and normal radiation resistance [96]. Recently, the kinetics of R–M–N focus formation was shown to be more rapid under conditions of in situ fractionation with detergent to remove most nucleoplasmic protein; granular foci formed within 10 min after IR treatment and may more accurately correspond to sites of DSB repair [97]. Phosphorylation of NBS1 at Ser^{343} and other sites by ATM (Fig. 1) is necessary for resistance to IR [98–101]. Furthermore, phosphorylation and activation of Chk2 kinase by ATM requires the phosphorylation of NBS1 at S^{343} [89].

Although the NBS1 protein was often not detected in western blots of NBS cells [76,81], the highly predominant 657de15 allele [77] results in a 70 kDa truncated protein [102] that likely retains partial function. In mice, an exon 2–3 deletion in *NBS1* [82] or an exon 4–5 deletion is compatible with embryonic development [103], but a null mutation disrupts early embryogenesis [104] and is likely incompatible with cell viability. These findings are consistent with the observation that the occurrence of deletion mutations in the *NBS1* gene are not the cause of tumorigenesis in human B and T cell lymphomas [105].

2.3. Connection between NBS and Mre11-Rad50

Recently mutations in the *Mre11* gene were identified in four patients from two families, who were

described as having an "ataxia telangiectasia-like disorder" (ATLD) [106]. The phenotype of these mutant cells resembles that of AT cells, but ATLD fibroblasts show mild radiation sensitivity (~1.5-fold). ATLD lymphoblasts, like those of AT, are defective in IR-induced JNK activity [106].

Much evidence supports the idea that an intact Rad50–Mre11–NBS1 (R–M–N) complex is essential for a normal cellular response to DSBs (Fig. 1) [107,108]. In ATLD cells, the stability of Mre11's interactions with Rad50 and NBS1 is reduced, and IR-induced nuclear focus formation for NBS1 and Mre11 is greatly diminished [106]. Since loss of Mre11 protein is incompatible with viability in mouse ES cells [109], a conditionally lethal knockout of Mre11 was constructed in chicken DT40 cells [110]. Transient Mre11 deficiency causes increased radiosensitivity and strongly reduced targeted integration frequencies, which underscores the importance of Mre11 in homologous recombination and chromosome stability. Similarly, Rad50 protein is also indispensable for cell viability [111]. Analysis of a *ku70 mre11* double mutant suggests that Mre11 acts primarily in the HRR pathway and not NHEJ [110].

In contrast to NBS1, both Mre11 and Rad50 have enzymatic activities that are likely essential in HRR. The R–M–N complex purified from human Raji lymphoma cells has manganese-dependent single-stranded DNA endonuclease and 3′–5′ exonuclease activities [112]. Human Mre11 alone has endonuclease activity, but in the presence of Rad50 and NBS1 there is activity for partial unwinding of duplex DNA, which is dependent on Rad50's ATPase activity [113,114]. Recently, in a reconstituted DSB rejoining assay employing DNA with cohesive ends, evidence was presented that the R–M–N complex stimulated the end-joining activity mediated by Ku86-70 and LIG4-XRCC4, suggesting that R–M–N may also provide an alignment function in addition to its nuclease activity [115].

2.4. Bloom syndrome gene (BLM)

Like AT and NBS, Bloom syndrome is a rare human genetic disorder that is a recessive and autosomally inherited. BS is characterized by a wide variety of abnormalities, including stunted growth, immunodeficiency, fertility defects (males are sterile, but females are partially fertile), sun sensitivity, increased frequency of diabetes, and greatly increased frequency of various types of cancer [116]. The types of cancers broadly reflect those seen in the general population, and include leukemia, various types of carcinomas and lymphomas, and cancers that are rare in the general population, such as Wilms tumor and osteosarcoma. The mean age of cancer diagnosis is 24 years.

The most obvious change seen in cells from BS individuals is a greatly increased frequency of sister-chromatid exchange (SCE) (reviewed by [117,118]). An increase in exchanges between homologous chromosomes is also seen, as indicated by the presence of symmetrical quadriradial configurations at metaphase [116,119]. Cultured BS cells also show a greatly increased number of chromatid breaks, gaps, and rearranged chromosomes. BS cells grow slowly in culture and S-phase is protracted. BS cells also exhibit greatly elevated mutation rates (≥10-fold) [120,121], but are only mildly sensitive to some DNA damaging agents [117]. Lymphoblastoid cell lines derived from BS individuals have variously been reported to have both increased or decreased killing by γ-irradiation [122,123]; increased resistance was attributed to their reduced p53-mediated apoptosis [122].

The gene defective in BS has been identified and named *BLM*. The *BLM* gene encodes a protein of 1417 a.a. that is a member of the RecQ family of DNA helicases [124]. This family of 3′–5′ helicases also includes the Werner syndrome protein (WRN) and RecQL4, the protein defective in Rothmund–Thomson syndrome (RTS; reviewed in [117,125]). The *blm* mutations in BS individuals frequently result in truncated proteins that are degraded, suggesting that BS results from the absence of the entire protein and that BLM is not an essential protein in humans [124,126]. However, the *BLM* gene is essential for embryonic development in mice since blm knockout embryos die by E13.5 [126].

Considerable evidence suggests that the BLM protein plays a role in HRR and is involved in repairing damage at stalled replication forks. BLM has been shown to directly interact with the Rad51 strand-transfer protein, and to partially co-localize with both Rad51 and RPA in untreated cells, with the fraction of BLM foci and Rad51 foci that co-localize increasing following IR damage [127,128]. Formation of Rad51 foci does not require BLM, and the number of Rad51 foci is actually elevated in untreated BS

cell lines, as compared to other cell lines [127]. Following irradiation, BLM is phosphorylated by ATM [122,123,129] and, like Rad51, associates with sites of ssDNA, consistent with BLM playing a role in repairing damage by HRR [128]. In response to hydroxyurea, but not IR, BLM's function is specifically required to properly relocalize the R–M–N complex at sites of replication arrest [122]. (In contrast, the WRN helicase discussed below does not share this property.) This BLM-dependent re-localization of R–M–N requires phosphorylation of BLM by the ATR kinase. HU-mediated cell killing is greatly elevated in BS cells and manifest as apoptosis and micronucleated cells [122].

BLM also directly interacts with MLH1, a protein involved in both mismatch repair and recombination. Since BS cells are not deficient in mismatch repair, the MLH1 interaction with BLM may play a role in HRR [130,131]. The enzymatic activity of the BLM helicase is also consistent with a role in recombination. BLM can perform branch migration of Holliday junctions (a recombination intermediate) and other types of structures that might be found at stalled replication forks, although it cannot unwind duplex DNA from a blunt end or from a nick [132].

The chicken *BLM* gene has been partially deleted in DT40 cells, resulting in high sensitivity to methyl methanesulfonate, greatly elevated levels of SCE, and increased gene targeting efficiency [133]. In a DT40 *blm rad54* double mutant, the SCE and gene targeting frequencies were nearly normal. This result strongly suggests that the increased SCEs seen in BS cells are due to events processed by the HRR pathway.

Although the above evidence implies that BLM is involved in HRR, its precise role is unclear. One model suggests that the BLM helicase plays a role prior to HRR, by decreasing the fraction of stalled replication forks that are repaired by recombination, thus, explaining the higher SCE seen in BS cells. Another model suggests that the BLM helicase acts during recombination, probably by increasing the branch migration of Holliday junctions. In this case, the increased SCE in BS cells might result from the atypical resolution of recombinational events (i.e. gene conversion events) that do not normally result in SCE. Gene conversion, and not crossing-over, appears to be the predominant mode of HRR in mammalian cells [134].

BLM may also play a role in apoptosis, since BLM directly interacts with p53 and helps regulate its transcriptional activity [135,136]. Although probably unrelated to its proposed role in regulating apoptosis, BLM is cleaved (by caspase-3) during apoptosis [137,138]. Many other DNA repair proteins such as ATM, Rad51, and DNA-PKcs are also inactivated by caspases during apoptosis. Although BLM cleavage does not abolish helicase activity, it does block focus formation. Further studies are needed to determine which aspects of the BS phenotype relate to the function of BLM in repair, as opposed to its suggested apoptotic function.

3. Cancer-prone disorders that may have defects linked to homologous recombination

3.1. Werner syndrome gene (WRN)

Like Bloom syndrome, Werner syndrome is a rare recessive genetic disorder associated with a greatly increased risk of cancers. Although WS shares a number of similarities with BS, including having a defect in a RecQ-related helicase, there are many differences. WS individuals are short in stature like BS individuals. However, unlike BS, WS is an adult progeria syndrome that includes a wide range of age-related traits having greatly accelerated onset although generally occurring after puberty. These features include osteoporosis, calcification of soft tissue, atherosclerosis, cataracts, diabetes mellitus, and premature graying and loss of hair (reviewed in [139]). The most prevalent types of cancer are soft tissue sarcomas, but thyroid carcinomas, meningiomas, melanomas, and osteosarcomas are also seen. WS patients die at an average age of 47, usually either from cancer or cardiovascular disease.

Cells from WS patients show greatly increased spontaneous genomic instability, including extensive deletions, reciprocal translocations, and inversions (reviewed in [117,139]). Unlike BS cells, WS cells have normal SCE frequencies [140,141]. WS cells are modestly sensitive to camptothecin (an inhibitor of type I DNA topoisomerases), 4-NQO, and some DNA crosslinking agents [142–144]. WS cells are slightly sensitive to IR (∼1.2-fold), and this sensitivity is reversed by complementation with *WRN*, the *WS* gene [145]. A knockout mutant of *WRN* in chicken DT40

cells showed sensitivities to 4-nitroquinoline-1-oxide, camptothecin, and methyl methanesulfonate, and had a 2.5-fold increase in targeted integration rate of exogenous DNAs [141]. Fibroblasts from WS individuals show a prolonged S-phase and limited replicative potential (reviewed in [139]). Like BS cells, WS cells are highly sensitive to hydroxyurea-induced apoptotic killing, and this occurs only during S-phase [146]. WS cells have a defect in the repair of both spontaneous and camptothecin induced DNA breakage as measured by the alkaline single-cell comet assay [146,147]. A special class of unrepaired spontaneous damage that occurs during DNA replication is likely responsible for many aspects of the phenotype of WS cells.

The *WRN* gene encodes a 1432 a.a. protein that is a member of the RecQ family of DNA helicases [148]. In addition, the WRN protein possesses a 3′–5′ exonuclease activity, which is not found in any other RecQ-family member [149,150]. WS individuals normally lack any detectable WRN protein or immune-precipitable helicase activity [151]. Unlike *BLM*, mice with knockouts of the *WRN* helicase domains are viable, but do not show obvious signs of premature aging [139,152]. Mice lacking the helicase domains exhibit only a slightly increased frequency of cancer, if any increase, but this frequency is greatly elevated in a $Tp53^{-/-}$ background [153]. The WRN protein directly interacts with several proteins, including PCNA (proliferating cell nuclear antigen), RPA, the Ku complex, DNA topoisomerase I, Tp53, and DNA polymerase δ (reviewed in [139,154]). In addition, WRN is phosphorylated by DNA-PKcs [145,155]. WRN also interacts with FEN 1, the flap endonuclease involved in DNA replication, recombination and repair, and stimulates the cleavage activity of FEN1 [156]. The interactions of WRN with DNA-PKcs and the Ku complex, combined with evidence that WS cells produce extensive deletions at the sites of nonhomologous joining ends in transfected plasmids [157], suggests a role for this protein in NHEJ.

There is increasing evidence that WRN participates in HRR. WS cells showed >20-fold reduction of intrachromosomal recombination using a *neo* gene selection assay, and also a defect in a *LacZ* recombination-dependent expression assay [158]. Remarkably, the defects in WS cell growth and production of viable *neo* recombinants were complemented by introducing the bacterial RusA resolvase [159]. Partial restoration of WS cells toward normal growth was also achieved by expressing a dominant-negative chimeric Rad51 protein to suppress recombination [159]. These findings suggest a defect in WRN cells in resolving recombination intermediate structures.

In addition, the WRN helicase activity, like that of BLM, functions on specific DNA structures such as Holliday junctions [132,160], which also suggests a role in HRR. Evidence for an association of WRN with BLM reinforces this idea. Following replication arrest (hydroxyurea) or DNA damage (camptothecin, etoposide, 4-NQO and bleomycin), WRN is found in nuclear foci that partially co-localize with both RPA and Rad51 [160,161]. Like BS, WS cells show an elevated level of Rad51 foci in untreated cells, but unlike BS, WS cells show reduced induction of Rad51 foci by camptothecin (possibly because of elimination of damaged cells by apoptosis [146]). No direct interaction between the WRN and Rad51 proteins has been reported. It has also been suggested that WRN may “prevent aberrant recombination events at sites of stalled replication forks by dissociating recombination intermediates” [160]. However, the exact functions of WRN’s helicase and exonuclease activities in HRR and NHEJ have yet to be determined.

3.2. Rothmund–Thomson syndrome (RTS)

Rothmund–Thomson syndrome is also a rare autosomal recessive disorder that shares similarities with both BS and WS. RTS individuals show stunted growth and increased rates of cancer, particularly osteogenic sarcomas [162]. RTS cells show genomic instability, including trisomy, aneuploidy and chromosomal rearrangements. Mutations in the *RECQL4* gene [163] have been shown to be responsible for at least some cases of RTS [117,162,164]. The RECQL4 protein, as its name implies, is a member of the RECQ family of helicases. Little is known about this protein, and the proteins with which it interacts, so it is not clear yet whether this protein plays a role in HRR, NHEJ, or both.

3.3. Fanconi anemia (FA)

Fanconi anemia (FA) is another chromosomal instability disorder that is genetically much more complex and less understood than most of the single-gene

disorders discussed above (AT, NBS, ATLD, BS, WS) (reviewed in [165–168]). FA patients have a predisposition to cancer, especially acute myeloid leukemia (15,000-fold elevated risk) and squamous cell carcinoma, and they suffer progressive aplastic anemia caused by progressive loss of bone marrow stem cells [21]. About 70% of patients have diverse developmental abnormalities including reduced fertility and deformities of the upper limbs, skeleton, GI tract, skin, kidney, heart and central nervous system [21]. Diagnosis is most reliably made by testing lymphocytes for chromosomal sensitivity to the crosslinking agents diepoxybutane or mitomycin C [169]. The knockout mouse models constructed for *FancA* [170,171], *FancC* [172,173], and *FancG* [174,175] have consistently shown neither developmental defects nor progressive anemia.

FA lymphoblast cell lines have been classified into at least eight genetic complementation groups, and at least some FA proteins appear to have both cytoplasmic and nuclear functions. The FA nuclear complex, composed of the FA proteins A, C, E, F, and G is essential for protection against chromosome breakage by MMC [176–179]. Following MMC or IR treatment, the FA A, C, E, F, and G proteins are all required for the activation of a portion of FANCD2 protein by converting it to a monoubiquitinated isoform [180]. Monoubiquitinated FANCD2 is localized to nuclear foci that contain the breast cancer susceptibility protein, BRCA1 [180]. (As discussed below, BRCA1 is implicated as an important player in HRR.) FANCD2 also coimmunoprecipitates with BRCA1 in extracts from irradiated cells. Interestingly, in BRCA1 mutant cells FANCD2 focus formation is diminished, and the damage-induced monoubiquitination of FANCD2 does not occur in BRCA1 mutant cells or in FA cells from groups A, C, and G [180]. The relevance of these findings to HRR remains open to interpretation since lethal levels of DNA damage (10–20 Gy) are generally used in these experiments, and the foci are visualized after most DSBs have already been repaired.

A connection between FA proteins and ATM was established with the discovery that FANCD2 is phosphorylated on serine 222 by the ATM kinase [181]. Phosphorylation of FANCD2 is required for activation of an S-phase checkpoint, i.e. inhibition of the initiation of DNA replication, a feature of AT cells. However, phosphorylation is not required for MMC resistance or for FANCD2 focus formation.

Most recently, the B and D1 complementation groups were surprisingly [182] identified as having biallelic (homozygous) mutations in *BRCA2* [183], the breast cancer susceptibility gene that plays an important role in HRR (see Section 4.2). *FANCD1* mutant cells showed restoration of MMC resistance upon transfection with *BRCA2* cDNA. These observations suggest that FANCB and FANCD1 are synonymous with BRCA2. These results, along with the BRCA1 results discussed above, link the six cloned FA genes with both BRCA1 and BRCA2 in a hypothetical pathway that plays a role in mitigating DNA damage [183].

Although FA cells are consistently sensitive to a variety of DNA crosslinking agents, a general deficiency in crosslink repair has not been demonstrated (see review [165]), and the precise function of the FA protein pathway in genomic stability remains unknown. The genetic data from various CHO hamster mutant cell lines that are hypersensitive to mitomycin C (and other crosslinking agents) does not clearly support a role for the *FANCG* gene in a recombination pathway that repairs DNA crosslinks. Whereas the hamster UV40 and NM3 *FancG* mutant lines are typically 3–10-fold sensitive to DNA crosslinking agents [184], the mutants that are defective in HRR (*XRCC2/3* mutants) and the ERCC1/XPF incision function are much more sensitive (10–100-fold). These phenotype comparisons suggest some other role(s) for FA proteins, e.g. DNA replication, transcription, chromatin remodeling, or maintaining redox status.

4. Genes involved in homologous recombination that were identified as cancer suppressor genes

4.1. *BRCA1* cancer suppressor gene

Mutations in the *BRCA1* tumor suppressor gene are found in ∼70% of all of the families with inherited breast and ovarian cancers and ∼20% of the families with only breast cancer [185]. Considerable evidence supports the idea that BRCA1 and BRCA2 proteins have multiple, complex roles in cellular responses to DNA damage. They participate in the processes that implement cell cycle checkpoints in response to DSB,

help coordinate the repair of those breaks, facilitate transcription coupled repair of oxidative base damage [186,187], and also act a transcriptional modifiers. Although BRCA1 and BRCA2 physically interact [188] and are often reviewed together [189–195], the two proteins have distinct interacting partner proteins and different functions. BRCA1 was originally seen to co-localize with Rad51 in S-phase nuclear foci, and to associate with BRCA2 and Rad51 in immunoprecipitates [188]. There is conflicting evidence concerning a requirement for normal BRCA1 in Rad51 focus formation [196,197]. These links to Rad51 suggested a role for BRCA1 in HRR, and this function was explicitly confirmed in BRCA1-defective mouse ES cells carrying an integrated substrate for the I-*Sce*I endonuclease [198,199]. These mutant cells exhibit some mitomycin C sensitivity (∼4.5-fold) as well as IR sensitivity.

BRCA1 localization to the sites of DSBs (represented by abundant γ-H2AX formation) produced by IR precedes the appearance of Rad50 and Rad51 [50]. The finding that purified BRCA1 strongly binds DNA, with a preference for branched structures, suggests that BRCA1 could act as a damage sensor that helps mediate repair [200]. Recent studies implicate BRCA1 in promoting NHEJ of DSBs both in vivo [201] and in cell extracts [202]. BRCA1-defective MEFs (having a 5′ exon 11 truncation) exhibit a 50–100-fold reduction in micro-homology mediated end-joining activity for defined chromosomal DSBs generated by endonuclease I-*Sce*I [201], which differs from the conclusions of a previous study [198].

Phosphorylation of BRCA1 in response to IR damage is performed primarily by ATM [203,204] and Chk2 [205], and secondarily by ATR [206] (see Fig. 1). ATM also phosphorylates the BRCA1 inhibitor, CtIP, and this event helps activate BRCA1 [207]. These phosphorylation events suggest that BRCA1 acts as a signaling factor between damage sensing/recognition and the DNA repair machinery. One role of BRCA1 may be to route the repair of DSBs through the HRR pathway in cells that are in the appropriate phases of the cell cycle (S and G2). A second major function of BRCA1 is its contribution to the checkpoints in both S and G2 phases after ionizing radiation [208,209]. It helps regulate the G2 checkpoint by activating Chk1 kinase upon DNA damage [210]. Moreover, in BRCA1 mutant cells, abnormal centrosome duplication/amplification occurs and leads to aneuploidy through unequal chromosome segregation [208], and the G2/M sister-chromatid dec atenation checkpoint is also defective (but not in AT cells) [211]. During mitosis BRCA1 is associated with the centrosome [212], and γ-tubulin associates preferentially with a hypophosphorylated form of BRCA1 [213]. These observations imply that BRCA1 plays an important role in centrosome regulation and chromosome segregation.

Clues about BRCA1's specific biochemical functions stem from its interactions with numerous, diverse proteins (Fig. 2) and its presence in several multimeric complexes that are dynamically altered after DNA replication blockage [214–219]. The interaction of BRCA1 with the Rad50–Mre11–NBS1 (R–M–N) complex [214] could direct the repair of DSBs through HRR by mediating the resectioning of broken termini to generate the long single-stranded tails required for assembly of Rad51 nucleoprotein filaments. During this end processing, BRCA2 may participate by regulating the assembly of Rad51. Although BRCA1 was reported to be necessary for the organization of R–M–N focus formation induced by IR [214], this finding has been disputed [220]. This discrepancy is not yet resolved.

BRCA1's N-terrninal RING finger domain interacts with BARD1 to form a RING-finger dimeric complex [218,221,222] that contains E3 ubiquitin ligase activity [223,224] (discussed in [193]). Within this RING domain of BRCA1, several cancer-predisposing mutations that alter BRCA1's self association or its ability to interact with ubiquitin-conjugating enzymes are identified [225,226]. Some breast cancer-associated BRCA1 mutant proteins that lack ubiquitin ligase activity result in sensitivity to IR [227]. These findings suggest that BRCA1 may have a direct role in DNA repair that is mediated by its ubiquitin ligase activity, which may be relevant to the function of the FANCD2 protein mentioned above in Section 3.1. Through its association with BARD1, BRCA1 could also have a role in down regulating mRNA 3′ processing in response to DNA damage [228]. BAP1, another interactor with the RING finger domain, is a nuclear ubiquitin carboxy-terminal hydrolase that may play a role in BRCA1 function [229].

BRCA1 is associated with RNA polymerase II holoenzyme [218,230,231] and appears to function in transcriptional control in several ways: (a) by

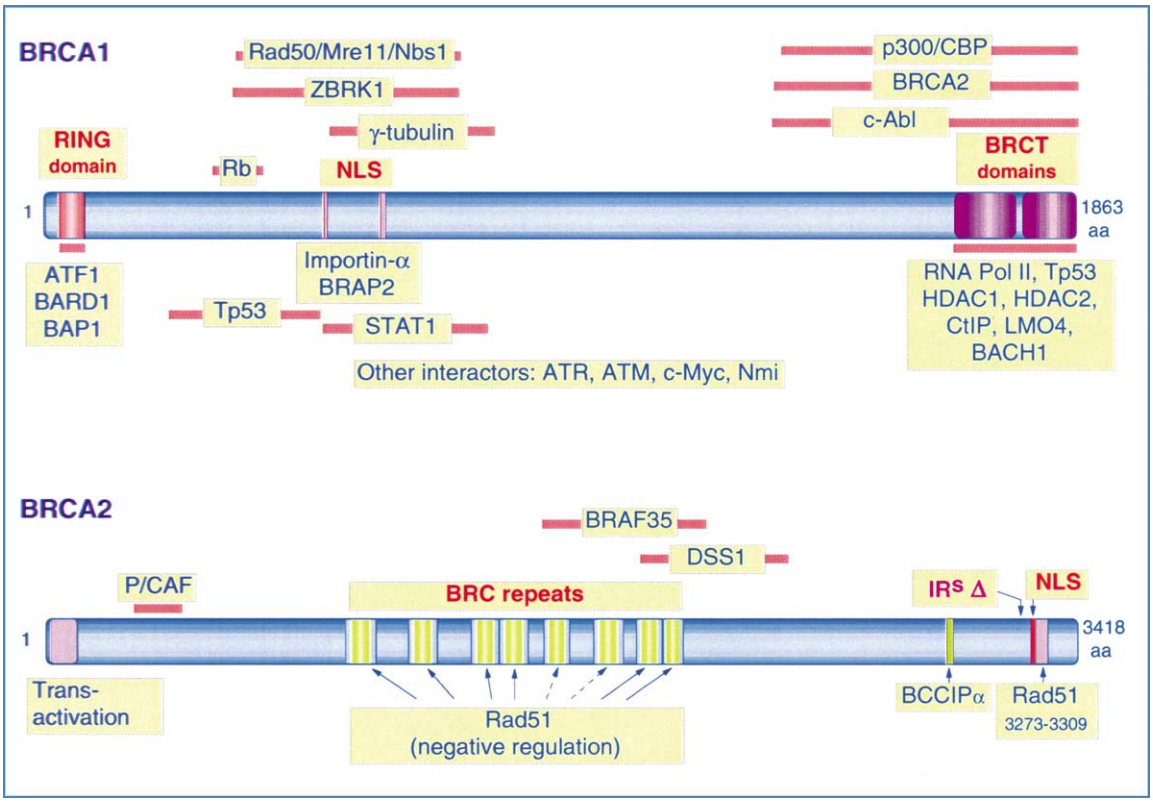

Fig. 2. Domains and interaction regions of the BRCA1 and BRCA2 proteins. BRCA1 has been found to interact with more than 20 proteins, many of which interact through the N-terminal RING finger domain (ATF1 [236], BARD1 [218,221–223], and BAP1 [229]) or the C-terminal BRCT domains (RNA Pol 11 [230], Tp53 [233], HDAC1 [245], HDAC2 [245], CtIP [237,238], LMO4 [240], and BACH1 [246]. Other BRCA1 interactions include Rb [371], a second Tp53 interaction [372,373], Rad50 of the R–M–N complex [214], ZBRK1 [241], γ-tubulin [213], STAT1 [374], p300/CBP [247], BRCA2 [188], c-Ab1 [375], and c-Myc [376], and Nmi (N-myc interacting protein) [377]. Importin-α and BRAP2 interact through the NLS region [378,379]. The very large BRCA2 protein has so far been found to interact with a much more limited number of proteins than BRCA1, and these are discussed in the text.

interacting with proteins that remodel chromatin structure (e.g. SWI/SNF) [232]; (b) by transactivating genes (such as *p21/CDKN1A* and *GADD45*, which are induced by DNA damage) through interaction with Tp53 [233–235], ATF1 [236], and CtIP [207,237–239]; (c) by interacting with LMO4 [240], thereby repressing BRCA1's transcriptional activation function (see Fig. 2); and (d) by acting as a co-repressor of transcription [241].

The two BRCT domains of BRCA1 [242–244] associate with multiple proteins including RNA polymerase holoenzyme [230], Tp53 [233], and the histone deacetylases HDAC1 and HDAC2 [245]. CtIP was identified by its interaction with CtBP, a transcriptional co-repressor. LMO4 interacts with both BRCA1 and CtIP, and a stable complex comprising LMO4, BRCA1, and CtIP was demonstrated in vivo [240]. Tumor-derived BRCA1 mutations in the BRCT region abolish interaction with CtIP [238]. Interestingly, BRCA1 interacts in vivo with a novel protein, BACH1, a member of the DEAH helicase family, which contains XPD [246]. Importantly, in S-G2 phase cells BACH1 forms nuclear foci that co-localize with BRCA1, and BRCA1-deficient cells are deficient in BACH1 foci. A BACH1 K52R mutant protein interfered with normal DSB repair in a manner that was dependent on its interaction with BRCA1. The C-terminal portion of BRCA1

also interacts with BRCA2 and the transcriptional co-activators/acetyltransferases p300 and CBP [247]. Some of the chromatin-associated proteins that interact with BRCA1, such as BACH1 and HDAC1/2, may alter DNA topology in a way that facilitates access of repair proteins.

Given the myriad BRCA1 interactions, it is not too surprising that truncations and deletions cause early embryonic lethality in mice [248–251]. Mice having a genotype of exon 11 deletion (*BRCA1*$^{\Delta 11/\Delta 11}$) exhibit embryonic lethality associated with widespread apoptosis, which, remarkably, is rescued in a *Tp53*$^{+/-}$ background [252]. The resulting female mice develop mammary tumors associated with loss of the remaining *Trp53* allele within 6–12 months. Cells from *BRCA1*$^{\Delta 11/\Delta 11}$ *Tp53*$^{+/-}$ embryos had below normal Tp53 levels, which resulted in attenuated G1 checkpoint control, less apoptosis, and more cells continuing to proliferate.

4.2. BRCA2 cancer suppressor gene

Germline mutations in one allele of *BRCA2* are associated with a very high risk (up to 85%) of breast cancer in women and ∼15% risk of ovarian cancer [185]. BRCA2 mutations are also the most common inherited genetic alteration so far identified in familial pancreatic cancer [253]. The very large (3418 a.a.) BRCA2 protein was first linked to homologous recombination by showing direct interactions with Rad51 [254–258] (see Fig. 2), as well as co-localization of BRCA2 with Rad5l (and BRCA1) in the nuclear foci of undamaged replicating cells [188,259].

Moreover, BRCA2 mutant cells are defective in Rad51 nuclear focus formation in response to IR damage [197,260]. Consistent with BRCA2 having a role in HRR, cells carrying mutations in both alleles of *BRCA2* show moderate sensitivity to killing by ionizing radiation, which is corrected upon transfection of a complementing cDNA or genomic sequence [261–263]. Overexpression of a BRC repeat element [264,265] (see Fig. 2) in normal cells also confers radiation sensitivity through a dominant negative mechanism [266]. This can be understood from studies demonstrating that several of the BRC repeats prevent the self-association of Rad51 and inhibit the formation of Rad51 nucleoprotein filaments on single-stranded DNA [258].

In BRCA2 mutant cells, the genotype/phenotype relationship is highly complex. Like BRCA1 and Rad51, complete loss of BRCA2 protein is incompatible with cell viability in dividing populations. Many of the BRCA2-defective human-tumor or mouse cell lines have internal deletions or truncations ranging from termination points within exon 11 (containing the BRC repeats) [267,268] to more benign ones in exons 26 or 27 at the very C-terminus (which contains the NLS and a Rad51 interaction region [261,269]. Even the exon 27 deletion confers increased tumor incidence and decreased survival compared with heterozygous litter mates [269]. The widely used Capan-1 pancreatic cancer cell line has a BRCA2 truncation at amino acid 1981 in BRC repeat 7 [270]. An attempt to correct these cells by stable expression with BRCA2 cDNA resulted only in a few clones that expressed the protein at a low level and showed marked suppression of growth [271]. The reason for this inhibition is unclear, but it is possible that constitutive expression by the viral CMV promoter is deleterious. Since BRCA2 is normally regulated during the cell cycle [272–276], constitutive expression may inhibit cell growth.

In the mouse models, truncations of Brca2 result in MEF cultures that show arrest of proliferation and overexpression of CDKN1A/p21 [261,268,277], which may seem at odds with *Brca2* being a cancer suppressor gene. However, in MEF cultures carrying *Brca2*$^{1492Tr/Tr}$ alleles, the expression of dominant-negative mutants of *Tp53* or *Bub1* kinetic-core kinase confer even more robust growth than seen in *Brca2*$^{+/+}$ control cultures [278]. The thymic lymphomas in these *Brca2*$^{1492Tr/Tr}$ animals acquired mutations in *Tp53* and/or mitotic spindle checkpoint genes (*Bub1* or *Mad3L*), and in culture the lymphoma cells were defective in the mitotic checkpoint. The findings strongly suggest that mutations inactivating the mitotic checkpoint interact with Brca2 deficiency to promote the proliferation of transformed cells that develop into malignancies.

In several respects, BRCA2 mutant cells are phenotypically similar to mutants of the five Rad51 paralogs (XRCC2, XRCC3, Rad51B, Rad51C, & Rad51D), which are thought to act as Rad51 accessory factors (discussed below). These common properties, which we now review, are based on comparing human, hamster, and mouse BRCA2 mutant lines with the mutants

of *Rad51* paralogs in hamster cells [279–283] and chicken cells [284,285].

4.2.1. Increased spontaneous chromosomal instability

Very high levels of chromosome breaks and exchanges are seen in BRCA2 mutant cultures or embryos [260,263,268]. As expected, increased levels of micronuclei are also seen in *BRCA2* mutant cells [277,286]. In BRCA2-defective hamster cells, most spontaneous *hprt* mutations are deletions, which is indicative of a failure to repair DSBs in an error-free manner [263].

4.2.2. Reduced resistance to diverse DNA-damaging agents

BRCA2 mutants show ∼1.5–2-fold increased sensitivity to killing by IR [261–263] and higher levels of sensitivity to crosslinking agents (e.g. MMC, cisplatin) [263]. As discussed in Section 3.3, the FA groups B and D1 are now identified as having causative *BRCA2* mutations [183]. In one study [287], FA group D1 diploid fibroblasts did not show IR sensitivity when compared with a control cell line although a comparison to gene-complemented cells was not available (groups A and C also showed normal sensitivity). It is noteworthy that the hamster Brca2 mutant V-C8 also has high sensitivity to methyl methanesulfonate (∼10-fold) [263], and human Capan-l cells are hypersensitive to this mutagen [257]. This sensitivity may be caused by the production of unrepaired DSBs that arise when DNA replication forks encounter single-strand breaks that arise as intermediates during base excision repair of methylated bases.

4.2.3. Missegregation of chromosomes

In addition to chromosomal aberrations, *BRCA2* mutant cells exhibit excess aneuploidy, which appears to be caused by abnormalities in the centrosome replication cycle. The centrosomes appear fragmented or present in excess numbers, which likely results in excess mitotic spindles [263,277]. A similar abnormality is seen in BRCA1 mutant cells [208,288].

4.2.4. Reduced homologous recombination

BRCA2 mutant cells have gross defects in homologous recombination (intrachromosomal) shown by measuring repair events at DSBs introduced by the I-*Sce*I endonuclease into chromosomally integrated substrates [289,290]. Similarly, the repair of an integrated mutant gene by transfected homologous sequences is also reduced [262]. Nonhomologous end-joining of DSBs remains intact in BRCA2 mutant cells [262].

4.2.5. Reduced sister-chromatid exchange

SCEs are a manifestation of HRR events in which crossing-over occurs between sister chromatids [291]. A deficiency in Rad51 causes a reduction in SCEs. Both spontaneous SCEs, as well as exchanges induced by MMC, are reduced in *Brca2* mutant cells [263,290]. These findings are in accord with the idea that HRR is required for the error-free repair of interstrand crosslinks and that a deficiency in HRR reduces SCE [291].

4.2.6. Reduced efficiency of gene targeting

Gene targeting in *Brca2*-defective mouse ES cells showed a deficiency, but the magnitude was only ∼2-fold [289], which is less the ∼20-fold reduction seen in *BRCA1* mutant cells [198].

4.2.7. Reduced numbers of Rad51 foci after IR treatment

As mentioned earlier, *BRCA2* mutant cells show greatly reduced numbers of Rad51 nuclear foci, implying that BRCA2 is required for efficient assembly of Rad51 into nucleoprotein filaments, which initiate strand exchange between sister chromatids. These observations, together with the biochemical studies addressed above, imply that BRCA2 acts “downstream” of BRCA1 and has a more immediate role in the recombination process. So far there is no evidence that BRCA2 interacts directly with any of the Rad51 paralogs, or other components of the HRR machinery (i.e. RPA, Rad52, Rad54, Rad54B).

4.2.8. Proteins interacting with BRCA2

As an essential protein for efficient HRR, BRCA2 is only present in higher eukaryotes. Chicken BRCA2 is ∼40% identical to human BRCA2 [270,292]. The spectrum of proteins with which BRCA2 interacts is much more restricted in comparison with BRCA1, and there appears to be little if any overlap between the interacting proteins (Fig. 2). Although an association

of BRCA1 and BRCA2 has been reported [188], this was not found in another study [216], suggesting only a transitory interaction that might reflect a step during the repair of DSBs.

The N-terminus of BRCA2 contains two regions that may involve transcriptional activation [293] (see Fig. 2). The transcriptional co-activator protein, P/CAF (p300/CBP associated factor), possesses histone acetyltransferase activity. Thus, the BRCA2 interaction with P/CAF may regulate transcription through the recruitment of histone-modifying activity of the P/CAF co-activator [294]. As mentioned, Rad51 interacts with BRCA2 through the BRC repeats in exon 11 [264] as well as with a short region at the C-terminus next to the nuclear localization signal. The BRC3 and BRC4 polypeptides interfere with Rad51 nucleoprotein filament formation, and, importantly, cancer-associated mutations in these repeats lack the ability to interfere with Rad51's function [258]. BRCA2 also has a role in controlling the nuclear transport of Rad51 since Rad5l is predominantly in the cytoplasm in Capan-1 cells [258]. This defect is also clearly seen in the V-C8 hamster cell mutant [263]. Thus, BRCA2 may exert both positive and negative regulatory influences on Rad51. Notably, mouse cells having a truncation mutation in exon 26, which removes the NLS and Rad51 interaction region, show IR sensitivity and premature senescence [261].

Other proteins that interact with BRCA2 are BCCIPα, a candidate tumor suppressor protein for breast and brain cancer [295], and BRAF35, a structure-specific (cruciform) DNA binding protein that binds to chromatin during mitotic prophase and influences cell cycle progression [296]. BRCA2 also interacts the putative cell cycle protein DSS1 [297], which has apparent orthologs in yeasts. Finally, BRCA2 was reported to interact with Tp53 and inhibit its transcriptional activity [256].

5. Other recombinational repair genes showing mutations in tumors

5.1. *RAD51, RAD52, RAD54, and RAD54B*

As a homolog of RecA, Rad51 is the major strand-transfer protein in eukaryotic cells. It is assisted in recombination by the Rad52, Rad54, and Rad54B proteins, as well as the Rad51 paralogs discussed below. Rad51 has been extensively characterized and found to interact with many proteins, including c-Ab1, BRCA2, RPA, BLM, Rad52, Rad54, and XRCC3 [5]. The enzymatic activities of the Rad51, Rad52, and Rad54 proteins, as well as their interactions with other proteins, were recently reviewed [5] but some additional important findings with these proteins have since been reported. Although Rad51 had been shown to have ATPase activity in vitro, it was unclear whether this activity was necessary for recombination in vivo. A recent study found that this ATPase activity is necessary for HRR in mouse embryonic stem cells [298].

In in vitro experiments, Rad52 was shown to assist Rad51 in displacing RPA from ssDNA during an early stage in HRR [299–301], similar to the role postulated for the human Rad51 paralogs. Consistent with their similar in vitro activities, Rad52 and the Rad51 paralogs have now been shown to have partially overlapping functions in vivo. The *rad52 xrcc3* double knockout in DT40 cells is inviable, but each mutation by itself is viable [302]. Rad54 and Rad54B are DNA-dependent ATPases and members of the Snf2 family of proteins, related to DNA helicases. These two proteins may play a role in unwinding DNA during strand invasion, or in making the chromatin of the donor strands more accessible to the invading strand and/or other recombination proteins [5,303]. A recent study has demonstrated that the Rad54B protein also plays an important role in HRR. The *RAD54B* gene was knocked out in a human colon cancer cell line, and these cells show greatly reduced gene targeting efficiency although they are not sensitive to DNA damaging agents [304]. A role for *RAD54B* in HRR is consistent with its association with Rad51 [305] and with a previous report of RAD54B mutations in some tumors [306].

Although no human genetic syndrome has been associated with *RAD51*, *RAD52*, *RAD54*, or *RAD54B*, tumor cell lines occasionally contain mutations in one of them (as reviewed in [5,307]). This finding suggests a normal role for these genes in cancer prevention. Recent reports find that the level of the Rad51 protein is elevated in some tumor cell lines. Different tumor-derived cell lines, including HeLa and MCF7, have 2–7-fold higher levels of Rad51 protein (compared to some cell lines not derived from tumors) due to transcriptional up-regulation [308]. This work

is supported by earlier studies showing an increased level of Rad51 in invasive ductal breast cancer and in pancreatic adenocarcinoma [309]. However, another study of tissue samples from 179 breast carcinomas found that about 30% had *reduced* levels of Rad51 [310]. Because of these conflicting reports, it is unclear whether Rad51 expression levels play any role in carcinogenesis. In a related study, a cell line expressing the oncogenic tyrosine kinase BCR/ABL had an enhanced level of Rad51, and this increased level was important for the cisplatin and MMC resistance of cells expressing BCR/ABL [311]. The elevated Rad51 resulted from both STAT5-dependent transcription and inhibition of caspase-3-dependent cleavage of Rad51. The BCR/ABL-expressing cells also overexpressed some Rad51 paralogs (Rad51B, Rad51D, and XRCC2) compared to a control cell line, but expressed less Rad51C and XRCC3. This result is interesting in light of the two complexes of Rad51 paralogs seen in human cells, discussed below.

Other studies have evaluated experimentally increased levels of the human Rad51 or Rad52 proteins in cultured cells, but with somewhat conflicting results. Although an earlier study had shown an increased level of HRR following Rad51 overexpression in CHO, a recent report showed a decrease in DSB-induced HRR in both CHO and human cell lines overexpressing human Rad51 and/or Rad52 [312]. In another study, overexpression of the hamster Rad51 in CHO cells unexpectedly increased the spontaneous frequency of nonhomologous recombination, but not the frequency induced by topoisomerase inhibitors [313]. In a third study, overexpression of Rad51 in the HT1080 human fibrosarcoma cell line resulted in decreased plating efficiency and growth rate, and increased apoptosis [314]. The authors suggest that Rad51 overexpression may select for cells resistant to apoptosis, thus, leading to increased tumor progression. Overexpression of Rad52 in human cells can also be deleterious to growth, but results regarding recombination are conflicting [315]. Gene targeting was inhibited by Rad52 overexpression.

5.2. *RAD51 paralogs*

In addition to Rad51, there are five Rad51-related proteins (or paralogs) in human mitotic cells: XRCC2, XRCC3, Rad51B/Rad51Ll, Rad51C/Rad51L2, and Rad51D/Rad51L3. These proteins share 20–30% sequence identity with Rad51 and with each other, and probably arose by gene duplication followed by the development of new functions. Although no inactivating mutations have been isolated in human cells, analyses of mutations in the Rad51 paralogs in hamster and chicken cell lines have shown that these proteins play a major role in HRR (Fig. 1; for review see [5]). Two hamster cell lines that carry *RAD51C* mutations were recently identified, and these cell lines broadly share the phenotype of the *XRCC2* and *XRCC3* mutants [316,317].

The human Rad51 paralogs are probably Rad51 accessory factors, although their precise functions in vertebrate cells have not been determined. *S. cerevisiae* has only two Rad51 paralogs (Rad55 and Rad57). These form a heterodimer that stimulates Rad51-mediated strand-exchange activity by facilitating Rad51's displacement of RPA from single-stranded DNA [318]. Because there are several similarities between the yeast and vertebrate Rad51 paralogs, it is reasonable to expect that some or all of the mammalian Rad51 paralogs might perform an analogous function. Recently the Rad51 paralogs have been shown to form two different complexes (a Rad51C–XRCC3 heterodimer and a Rad51B-C-D–XRCC2 heterotetramer) in the human cell lines tested [319–322]. The Rad51B-C heterodimer purified from insect cells was shown to stimulate Rad51-mediated strand-exchange [319], similar to what was observed with the Rad55/Rad57 heterodimer from yeast.

Although no genetic syndrome associated with a mutation in any of the Rad51 paralogs has been found, there is some conflicting evidence that the paralogs may be involved in carcinogenesis. Convincing evidence suggests that translocations involving the *RAD51B* gene are involved in uterine leiomyomas, which are benign solid tumors [323,324]. A *RAD51B* translocation and loss of the second *RAD51B* allele may play a role in development of uterine leiomyomas with associated ascites and pleural fluid (referred to as pseudo-Meigs' syndrome) [325]. A relatively common polymorphism in XRCC3 (Thr241Met substitution) has been reported to be associated with an increased risk of melanoma, bladder cancer, and breast cancer [326–328]. However, other studies have found no link to cancer [329,330], and, importantly the variant protein has normal function in a recombination

assay for DSB repair [331]. Two groups have found a link between the R188H allele of *XRCC2* and slightly increased breast cancer susceptibility [328,332], but the MMC resistance of cells expressing this allele was essentially normal [332]. Amplification of a chromosomal region containing *RAD51C* correlates with increased breast cancer but the significance of this result is still unclear [333,334].

6. Elevated DSBs and homologous recombination associated with replication arrest in the XP variant

The classical DNA repair disorder xeroderma pigmentosum is caused by two different types of biochemical defects associated with either excision repair or the cell's ability to replicate across *cis*-syn cyclobutane pyrimidine dimers efficiently without making mutations. Only the gene affected in the XP variant (XP-V) is associated with defective lesion bypass. The XP-V polymerase was identified as Pol η, which is encoded by the *PolH/RAD30A* gene [335,336]. XP-V cells have mild UV sensitivity to killing but increased levels of SCEs after UV irradiation [337], suggesting increased HRR in response to UV radiation damage. After UV irradiation, XP-V cells show a fluence-dependent increase in the yield of γ-H2AX foci that closely parallels the production of Mre11 foci [338,339]. Moreover, the nuclear distributions of γ-H2AX and Mre11 spatially co-localize specifically after UV and not x-irradiation. These results show that XP-V cells develop DSBs during the course of UV-induced replication arrest when bypass replication of UV damage becomes inefficient. Thus, inaccurate repair of these DSBs may contribute to skin carcinogenesis in XP-V patients.

7. Concluding remarks

It is apparent that a complex network of highly evolved proteins governs the repair of DNA lesions, particularly DSBs. In addition to the multi-faceted Tp53 [340,341], several large regulatory proteins (ATM, ATR, BRCA1, and BRCA2) participate in numerous, complex functional interactions. Several of the key players (BRCA1, BRCA2, and Tp53) are not represented by homologs in lower eukaryotes, suggesting that their origin is tied to the development of larger genomes that must support great specialization of cell function and that present a greater challenge in keeping their chromosomes intact. Both ATM and BRCA1 have more than 20 interactions, and there is little overlap among these sets of proteins. The proteins that recognize and transduce DSB information to coordinate the cell cycle checkpoint and DNA repair processes determine to a large extent whether a cell can restore the DNA molecule without introducing mutations. In the absence of HRR, DSBs can only be processed through end-joining mechanisms at the expense of mutations in order to preserve chromosome continuity. DSBs arising during DNA replication appear to be a normal consequence of replicating the very long chromosomes of vertebrate cells, and these DSBs are normally only repaired by HRR.

Although this review has emphasized DSBs processed through the ATM kinase, replication-associated DSBs, which are thought to recruit primarily the ATR kinase [40,47,342], are of equal importance. Perhaps no human mutations have been seen in ATR because of its fundamental role in DNA replication. The number of DSBs arising during replication is not known but appears to be 100 or more per cell cycle based on the following considerations. Crossing-over between homologous chromatids was undetectable (<3% of recombination events) in an analysis of HRR events occurring at an I-*Sce*I induced DSB in CHO cells. In other words, >97% of the DSBs are repaired without crossovers. Assuming that spontaneous SCEs, occurring at a frequency of approximately three per cell generation [343], represent HRR-mediated crossovers between sister chromatids, then the lower limit for DSB HRR events would be >90 (3/0.03) per cell cycle.

Certain proteins such as JNK and IKK, which show ATM-dependent activation, operate in oxidative stress signaling pathways to mediate transcriptional responses. A question of considerable interest is the extent to which cytoplasm-initiated responses contribute to the control of DNA damage and genomic stability relative to the contributions of the repair and checkpoint processes [344].

Germline heterozygous mutations in the key proteins BRCA1 and BRCA2 lead to cancer predominantly in the breast and ovary although cancers in other tissues such as prostate and colon also have increased

susceptibility [185]. Elledge has suggested that this tissue specificity is a consequence of the essential functions of these proteins in cell proliferation combined with unique aspects of these tissues that allow newly arising homozygous mutant cells to continue dividing, whereas in other tissues such cells simply die [345]. Continued division would then select for suppressor mutations that further enhance tumor growth.

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