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BRCA1 and BRCA2 in DNA Repair and Genome Stability

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1. INTRODUCTION

Since their discovery, the two major breast-cancer susceptibility genes *BRCA1* and *BRCA2* have challenged clinicians and the basic research community to understand their biological roles, and how their loss contributes to tumorigenesis. Progress has been rapid, accelerating particularly over the last three years, and there is now a foundation of evidence on the functions of the BRCA1 and BRCA2 proteins at the cellular and molecular levels. Each protein has been associated with multiple functions (Table 1). Moreover, BRCA1 and BRCA2 show a striking degree of functional overlap, with roles for both proteins in DNA repair, cell-cycle checkpoints, control of centrosome function, and transcriptional regulation. Much work remains to be done in elucidating the molecular details of how BRCA1 and BRCA2 perform these roles, but it is clear even at this early stage that BRCA1 and BRCA2 are central to the preservation of genomic integrity in mammalian cells. This chapter will review experimental evidence for BRCA1 and BRCA2 function in DNA repair and genomic stability. Not covered here is the voluminous literature on the spectrum of *BRCA1* and *BRCA2* mutations associated with cancer, their population genetics, and their clinical correlates. An overview of this literature can be found in reference *(13).*

2. STRUCTURAL FEATURES AND EXPRESSION OF BRCA1 AND BRCA2

When first described, the predicted protein sequences of human BRCA1 and BRCA2 were conspicuous for two features; their size and their uniqueness. BRCA1, with 1863 amino acids, is a large protein, whereas BRCA2, at 3418 amino acids, is enormous. Early hopes that the sequences of BRCA1 and BRCA2 might shed some light on their functions were thwarted by an almost complete lack of sequence similarity to any other known proteins. Over time, however, some important structural features of each protein have emerged that can now be related to known or proposed functions (Fig. 1).

2.1. The BRCA1 Protein

The human *BRCA1* gene is located on chromosome 17 (17q21). It was originally described as comprising 24 exons *(98).* Exon 4 was later found to be a cloning artifact,

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Table 1
Functional Roles of BRCA1 and BRCA2

Function	BRCA1 (Ref.)	BRCA2 (Ref.)
Transcription-coupled repair	*(2,53)*	(*see* Subheading 5.)
Homologous recombinational repair	*(105)*	(*see* Subheading 6.)
G2/M checkpoint induction	*(46,77,166)*	*(30)*
Centrosome regulation	*(166)*	*(152)*
Transcriptional regulation	*(28,29,37,101,107)*	*(90,99,178)*
Chromatin remodeling	*(64,171)*	*(50,132)*
Ubiquitination/deubiquitination	*(47,66,85)*	

but the original numbering of exons has been retained *(13)*. The *BRCA1* gene gives rise to several distinct mRNAs owing to differential use of transcription start sites and splicing signals, including mRNAs with alternative first exons, a form that omits all of exons 9 and 10, and forms that omit most or all of exon 11, the large central exon that encodes over half of the BRCA1 protein *(86,142,156,160,165)*. The full-length human BRCA1 protein of 1873 amino acids (approx 220 kD) is usually the most abundant form, but smaller isoforms, including ones that correspond to exon 11-deletion splice variant mRNAs have been reported. Nuclear localization signals have been identified within the region encoded by exon 11 *(31,101,142,160)*.

The amino-terminus of BRCA1 contains the cysteine-rich RING motif, a zinc-binding domain that has been identified in a large number of proteins associated with regulatory functions (Fig. 1). RING motifs participate in protein-protein interactions associated with diverse cellular functions *(21)*, and the RING motif in BRCA1 evidently mediates heterodimer formation with BARD1. Another recently proposed general role for RING motifs is in modulating ubiquitination reactions. A number of RING finger proteins, including BRCA1, are able to facilitate the catalytic activity of ubiquitin-conjugating enzymes *(47,85)*, and a deubiquitinating enzyme, BAP1, specifically interacts with the RING motif of BRCA1 *(66)*.

The carboxy-terminus of BRCA1 contains a repeated motif of about 95 amino acids, known as the "BRCT" (BRCA1 carboxy-terminus) domain, versions of which have been identified in a large number of proteins from several eukaryotic species *(23,26)*. The shared attribute of these various proteins is that most have some relation to DNA repair and/or cell-cycle regulation. The BRCT domain appears to function generally in mediation of protein-protein interactions *(92,128,140,176)*, but has also been shown to bind DNA ends *(169)*. Roles ascribed specifically to the BRCT domain in BRCA1 include transcriptional activation or coactivation, chromatin remodeling, and activation of replication, and the mediation of interactions with several other proteins, including p53, RNA helicase A, the transcriptional co-repressor CtIP, and components of the histone deacetylase complex *(4,28,29,64,81,101,107,171,172)*. (Fig. 1).

BRCA1 undergoes changes in phosphorylation during the course of the cell cycle and in response to DNA damage. It becomes hyperphosphorylated late in G1 and in S phase, and undergoes dephosphorylation shortly after M phase *(121)*. G1/S hyperphosphorylation at one site, serine 1497, is evidently mediated by cyclin-dependent kinase 2 *(120)*. In S-phase cells exposed to DNA-damaging treatments, BRCA1 undergoes fur-

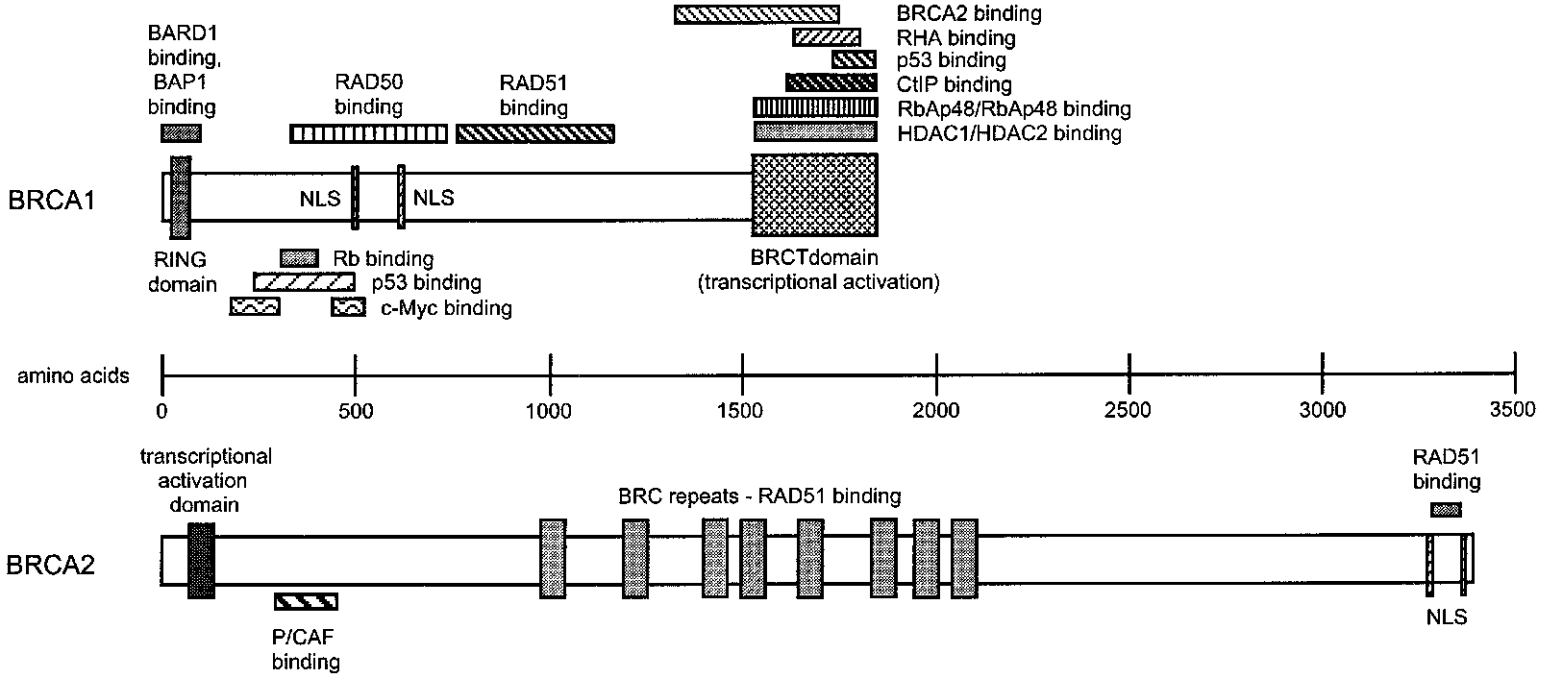

Fig. 1. Functional and protein interaction domains of BRCA1 and BRCA2. The positions of identified functional domains of BRCA1 and BRCA2 are indicated, together with regions that mediate specific protein interactions. This figure is not exhaustive; interactions have been demonstrated, but not yet mapped, for a number of proteins in addition to those shown here (*see* Table 2).

ther phosphorylation, which is accompanied by changes in subnuclear localization *(123).* The damage-induced hyperphosphorylation of BRCA1 is at least partly dependent on the checkpoint protein kinases ATM and CDS1*(41,80).*

The mouse *BRCA1* gene has also been cloned. Its predicted protein is shorter (1812 amino acids), and moderately conserved relative to human, with 58% amino acid identity and 73% similarity overall. The RING domain is perfectly conserved, however, and the BRCT domain is also highly conserved (83% identity), underscoring the probable importance of these domains in the critical functions of BRCA1 *(3,76,127).*

2.2. The BRCA2 Protein

The human *BRCA2* gene resides on chromosome 13 (13q12-q13). Its major transcript comprises 27 exons, but there is a reported splice variant that deletes exon 3 *(178).* Similarly to BRCA1, about half of BRCA2's length is encoded by a large central exon (exon 11). Despite this resemblance in exon structure, BRCA1 and BRCA2 have no significant sequence similarity to each other. BRCA2, like BRCA1, is moderately conserved across mammalian species. The mouse and rat BRCA2 proteins (3328 amino acids) are 59% and 58% identical (73 and 72% similar), respectively, to the human protein *(95,125).*

Exon 3 of human *BRCA2* encodes a region that bears some sequence homology to the transcriptional transactivation domain of c-Jun, and can exert transcriptional activation in yeast or mammalian cells when coupled to DNA-binding domains *(99).* A mutation occurring within this region in some familial breast cancers (tyrosine to cystine at codon 42) sharply reduces transcriptional activation. A splice site mutation that produces an in-frame deletion of exon 3 has been identified in a subset of endrometrial carcinomas that also show molecular evidence of replication and repair errors *(73).* These observations suggest that transcriptional activation may be important in tumor suppression.

Beyond its size, exon 11 of *BRCA2* is remarkable for encoding eight repeats of a loose consensus sequence, variously defined as from 26 to about 80 amino acids long, termed the BRC motif *(19,22).* These are unique to BRCA2, and not to be confused with the BRCT domain of BRCA1. Although exon 11 is poorly conserved overall, the BRC repeats are comparatively well-conserved across mammalian species *(19).* The BRC repeats are now known to mediate interaction between BRCA2 and the RAD51 repair and recombination protein (Subheading 4.2.) and their conservation argues for the importance of this interaction in BRCA2 function. Exon 11 contains other small regions, in addition to the BRC repeats, that are relatively well-conserved, most strikingly a stretch of 25 amino acids between the first and second BRC repeats that is identical in human, green monkey, dog, hamster, and mouse *(19).* No function has been proposed for this motif as yet.

The extreme carboxy terminus of BRCA2, encoded by exon 27, is also notable for two important features (Fig. 1). First, an additional RAD51 interaction domain, distinct from the BRC repeats, has been demonstrated in mouse BRCA2 *(100,126).* This domain is comparatively well-conserved between human and mouse, showing 72% identity. Second, the two functional nuclear localization signals that have been identified in human BRCA2 (amino acids 3263–3269 and 3381–3385) fall within this small region *(135).* Only the first of these is conserved in mouse BRCA2.

2.3. Expression Patterns of BRCA1 and BRCA2

Both BRCA1 and BRCA2 are predominantly nuclear proteins in normal cells *(16,31,101,121,123,142,143,156,160).* Moreover, failure of nuclear localization has been proposed in regard to both proteins as a cause of abnormal function in carcinogenesis. Wild-type BRCA1, although localized to the nucleus in normal cells and in nonbreast tumor cell lines, has been found to be cytoplasmic when expressed in breast-cancer cell lines *(31,36).* The reason for this apparent mislocation specifically in breast-cancer cells is unknown, and its significance has been controversial (reviewed in *38).* For BRCA2, the great majority of the mutations identified in cancers are small sequence changes predicted to result in truncated proteins. Even the most 3′ of known cancer-associated truncating mutations, at codon 3195, results in loss of the nuclear localization signals. It has been proposed that most cancer-associated alleles of BRCA2 are nonfunctional at least in part because the truncated proteins they encode fail to reach the nucleus *(135).* As detailed in later sections, the functions of BRCA1 and BRCA2 relating to DNA repair and genomic stability are expected to operate largely if not entirely in the nucleus.

Expression of both BRCA1 and BRCA2 is regulated during the cell cycle. For both BRCA1 and BRCA2, levels of mRNA and protein rise at the end of G1, prior to the onset of replication, and peak during S and G2 phases *(16,56,118,136,154,155).* For both BRCA1 and BRCA2, mRNA is detectable at low levels during early G1 phase in actively cycling cell populations, but is not detected in quiescent cells *(158).* The cell-cycle expression profiles of BRCA1 and BRCA2, and the absence of expression in non-dividing cells were early indications that both proteins are involved in cell proliferation.

The tissue distributions of BRCA1 and BRCA2 expression give further evidence of involvement in cell proliferation. In mouse, both genes are expressed at high levels during embryonic development, whereas in adult animals, expression of BRCA1 and BRCA2 is highest in rapidly proliferating tissues, including ovary, testis, and thymus, and in breast during puberty and pregnancy *(40,90,117,125).* The tissue localization of the two proteins is generally concordant with proliferating cell nuclear antigen (PCNA) *(20).* Expression is particularly high in the meiotic cells of mouse testis *(20,174),* which first suggested a role in meiosis, and hence in homologous recombination.

3. CELLULAR PHENOTYPES OF BRCA1 AND BRCA2 MUTANTS

Many insights into the functions of BRCA1 and BRCA2 have been gained through the creation of mice bearing targeted mutations of the *BRCA1* or *BRCA2* genes, and from the identification of human tumor cell lines that express only mutant BRCA1 or BRCA2. Analysis of these mutants produced much of the evidence implicating BRCA1 and BRCA2 as proteins involved in DNA repair and essential to genomic stability. Defects in cell proliferation and hypersensitivity to certain forms of DNA damage in *BRCA1* and *BRCA2* mutants were among the first indications of these roles, setting the stage for discovery of specific defects in transcription-coupled repair (TCR) and homologous recombinational repair (HRR), cell-cycle checkpoints, and chromosomal stability.

3.1. Embryonic Lethality and Defects in Cell Proliferation

In mouse, BRCA1 is required for embryonic development. Targeted mutations that truncate, or delete even a small part of the protein, almost always result in the death of

homozygous embryos *(54,60,83,87,129).* The time of developmental arrest is somewhat variable, but in all instances occurs before embryonic day 13, and usually before day 8 or 9. The only exceptions to early death of *BRCA1*$^{-/-}$ embryos are three double-mutant *BRCA1*$^{-/-}$ *p53*$^{-/-}$ mice reported by Cressman et al *(43,44).* The development to term and brief survival of these exceptional animals were proposed to reflect additional, unknown genetic alterations. The more general finding has been that developmental arrest and death of *BRCA1*$^{-/-}$ embryos is only briefly delayed when *BRCA1* mutations are bred into *p53*- or *p21*-null backgrounds *(43,44,59,87,129).*

Cellular proliferation is reduced in *BRCA1*$^{-/-}$ embryos in vivo, and when *BRCA1*$^{-/-}$ blastocysts are explanted their growth is grossly impaired *(60,83,129).* This seems not to be mediated by increased apoptosis *(60).* Growth impairment may result in part from the dramatic overexpression of p21 seen in *BRCA1*$^{-/-}$ embryos *(60).* However, the failure of either *p21*- or *p53*-null mutations to rescue completely embryonic development or blastocyst growth in culture suggests that impaired cell proliferation is owing to something more than the activation of cell-cycle checkpoints *(43,44,59,87,129).* Mouse *BRCA1*$^{-/-}$ cells have generally failed to proliferate in vitro (*see,* for example, *129),* with two notable exceptions: *BRCA1*$^{-/-}$ mouse embryonal (ES) cells and primary fibroblasts from *BRCA1*$^{-/-}$ p53$^{-/-}$ mice derived in the lab of B. H. Koller *(43,53,54).* Early passage *BRCA1*$^{-/-}$ *p53*$^{-/-}$ primary fibroblasts grew more slowly and reached lower saturation densities than control *p53*$^{-/-}$ fibroblasts. While they traversed the cell cycle with kinetics similar to *p53*$^{-/-}$ controls, the *BRCA1*$^{-/-}$ *p53*$^{-/-}$ fibroblasts showed a higher rate of cell death such that the population growth rate was lower.

The influence of BRCA1 on cell proliferation has also been examined in human tumor cell lines, but these experiments are somewhat difficult to reconcile with observations in mouse. In human cells, BRCA1 can inhibit growth. Antisense inhibition of BRCA1 expression accelerates the growth of normal and malignant breast epithelial cells, but not of nonmammary epithelial cells *(145).* Overexpression of wild-type BRCA1 slows the growth of breast and ovarian cancer cell lines, but not cell lines from other tumor types *(62).* In particular, the growth of the *BRCA1*-mutant human breast-cancer line, HCC1937, is inhibited after stable transfection with full-length, wild-type BRCA1 *(2).* HCC1937 carries only the 5283insC frameshift allele of *BRCA1,* encoding a protein that lacks the BRCT domain *(149).* The discrepancy between a requirement for BRCA1 for mouse cell proliferation during development, and the inhibition of growth in human mammary or ovarian cell lines, may be more apparent than real. The growth defect in embryonic mouse cells may reflect a general requirement for BRCA1 in repair processes associated with DNA replication and in chromosome segregation. The inhibition of growth by BRCA1 in human breast and ovarian tumor lines may result from a more specialized growth-regulating function in the hormonal milieu of these cell types.

BRCA2, similarly to *BRCA1,* is essential for embryonic development in the mouse. Mutations that delete the large central exon 11 of *BRCA2,* or terminate translation 5′ to exon 11, result in embryonic death by day 8 or 9 *(87,126,138).* However, mutations that leave exon 11 intact, or that truncate within exon 11, allow embryonic development to term *(39,49,103,109).* These results strongly suggest that the ability of BRCA2 protein to interact productively with RAD51 via the BRC repeats is a critical determinant of embryonic viability (*see* Subheading 4). Mutations that delete all of the BRC repeats

fail to support development, whereas mutations that preserve some or all of the BRC repeats permit embryonic survival, though not without phenotypic consequence.

The ability to create sublethal mutations of *BRCA2* in mouse has made the derivation of *BRCA2*-mutant cells a good deal easier than for *BRCA1*. Primary mouse embryonic fibroblasts (MEF) bearing sublethal truncations of BRCA2 have consistently shown proliferative defects in culture. This is true for two different truncations within exon 11*(39,109),* and also for a milder truncation that removes only the extreme carboxy terminus of BRCA2 encoded by exon 27 *(24,103).* Their growth is markedly slower than wild-type controls, and cellular senescence occurs earlier; typically after only a few passages. Growth arrest is accompanied by high levels of p53 and p21 expression *(39,109).* Immortalized MEF were derived from primary MEF bearing the exon 27 truncation, though only with difficulty. These cells have overcome the block to proliferation seen in primary cells, perhaps through acquisition of other mutations, but still show reduced cloning efficiency relative to immortalized wild-type MEF *(24,103).*

A human *BRCA2*-mutant cell line also exists. The long-established human pancreatic-cancer cell line Capan-1 expresses BRCA2 truncated within the 3′ portion of exon 11 *(1,35,52).* In this way Capan-1 is similar to the targeted mutation in mouse *BRCA2* generated by Ashworth and coworkers *(39,152).* Though immortal, Capan-1 cells grow very slowly in culture and have extremely low cloning efficiency. Because Capan-1 is an aneuploid tumor line and presumably carries numerous mutations, its poor growth characteristics may not be owing (or entirely owing) to loss of BRCA2 function.

3.2. Sensitivity to DNA Damaging Agents

Cells mutated for *BRCA1* show hypersensitivity to ionizing radiation (IR), which induces DNA double-strand breaks (DSBs) and oxidative damage, and to hydrogen peroxide, which produces oxidative damage. Survival of $BRCA1^{-/-}$ mouse ES cells is reduced by about fivefold relative to $BRCA1^{+/+}$ cells after exposure to 8 Gy of γ-radiation, and by about threefold after treatment with 8 m*M* hydrogen peroxide *(53).* Primary fibroblasts from $BRCA1^{-/-}p53^{-/-}$ mice show similar hypersensitivity to IR and to hydrogen peroxide, and a very mild hypersensitivity to ultraviolet (UV) light *(43).* The human *BRCA1*-mutant cell line HCC1937 is also hypersensitive to IR, but much more so than the *BRCA1*-mutant mouse cells, with survival after exposure to 6 Gy of γ-radiation reduced more than 100-fold relative to $BRCA1^{+/+}$ human cells. Although HCC1937 is a tumor line and known to carry other mutations *(149),* its extreme hypersensitivity to IR appears to result mainly from *BRCA1* deficiency, because it can be largely relieved by expression of modified BRCA1 (partial peptides that do not exert the growth-suppressing effect of full-length human BRCA1) *(2).* BRCA1 may also be required for resistance to DNA interstrand crosslinks. Subclones of breast- and ovarian-cancer cell lines with spontaneously acquired resistance to the DNA crosslinking drug cisplatin show increased expression of BRCA1 relative to nonresistant clones *(65).* Antisense inhibition of BRCA1 expression in cisplatin-resistant cells resulted in loss of resistance correlated with reduced repair of crosslinks and increased apoptosis.

BRCA2-mutant cells are similarly hypersensitivity to IR. In early mouse embryos homozygous for a severely truncating (lethal) *BRCA2* mutation, the inner cell mass could be completely ablated by 4 Gy of γ-radiation, an exposure that had only slight effect on wild-type embryos *(126).* Mouse embryonic cells with less severe truncations

of BRCA2 also show exaggerated sensitivity to IR *(103,109),* and in one instance have been shown to repair the DSBs induced by IR less efficiently than wild-type cells *(39).* Capan-1 cells show extreme sensitivity to IR and to chemotherapeutic drugs that induce DSBs, and repairs such breaks less efficiently than human tumor lines not mutated for BRCA2 *(1).*

The observed sensitivities of *BRCA2*-mutant cells to DNA-damaging agents other than IR are somewhat confusing. In mouse cells, one group has observed hypersensitivity to UV light and to the methylating agent methyl methanesulfonate (MMS), but not to the crosslinking agent mitomycin C (MMC) *(109).* A second group found no hypersensitivity to UV, but marked hypersensitivity to MMC *(24,103).* The apparent discrepancies probably derive from differences in experimental methods and from the two very different BRCA2 mutants examined. UV hypersensitivity was seen in primary MEF homozygous for a mutation that truncates the BRCA2 after the third BRC motif *(109).* Absence of UV hypersensitivity was seen in ES cells bearing a milder truncation that deletes only the extreme carboxy terminal domain encoded by exon 27, and leaves all eight BRC repeats intact *(103).* Immortalized MEF bearing the same exon 27 deletion are hypersensitive to MMC when assayed by colony formation *(24).* A colony-formation assay was not possible for the primary MEF tested previously *(109),* and the endpoint used (trypan blue exclusion 48 after exposure) might not have detected mitotic arrest owing to unrepaired DNA crosslinks. Capan-1 cells are also hypersensitive to MMS *(35).* Wild-type monkey cells expressing a fragment of BRCA2 that exerts dominant-negative effects are hypersensitive to cisplatin and IR *(173).*

The hypersensitivity of BRCA1- and BRCA2-mutant cells to IR points to a defect in DSB repair. The association of both proteins with RAD51 (discussed in Subheading 4.) implies that homologous recombination is the affected repair pathway. Increased sensitivity of BRCA1- and BRCA2-deficient cells to cisplatin or MMC is also consistent with this idea, because interstrand crosslinks are thought to be repaired by homologous recombination *(48).* Roles in HRR has now been demonstrated for both BRCA1 and BCRA2 (Subheading 6.).

4. INTERACTIONS OF BRCA1 AND BRCA2 WITH DNA REPAIR PROTEINS, AND SUBNUCLEAR LOCALIZATION IN RESPONSE TO DNA DAMAGE

BRCA1 and BRCA2 participate in specific interactions with a large and growing list of other proteins, as summarized in Table 2. Most of these interactions can be related, directly or indirectly, to some aspect of cellular response to DNA damage.

4.1. BRCA1 and BARD1

BRCA1 interacts with BARD1 (BRCA1-Associated RING Domain), to which it is structurally related *(163).* Human BARD1 is a 777 amino acid protein that, like BRCA1, contains a zinc-binding RING motif near its N-terminus and a bipartite BRCT domain near its carboxy-terminal end. Unlike BRCA1, BARD1 also contains three centrally positioned ankyrin repeats. BARD1 and BRCA1 interact in vitro and in vivo, and it appears that their RING domains are necessary (though not sufficient) for this interaction *(97)* (Fig. 1). The interaction is likely to be important in BRCA1 tumor suppres-

Table 2
Proteins Interacting with BRCA1 and BRCA2

	Protein		References
BRCA1:	BARD1	BRCA1-associated ring domain	*(163)*
	BRCA2		*(33,34)*
	RAD51		*(34,124)*
	RAD50		*(159,177)*
	MRE11		*(159,177)*
	NBS1	Nijmegen breakage syndrome	*(159,177)*
	BLM	Bloom's syndrome – mutated	*(159)*
	RPC	Replication protein C	*(159)*
	ATM	Ataxia Telangiectasia – mutated	*(159)*
	MSH2	MutS homolog 2	*(159)*
	MSH6	MutS homolog 6	*(159)*
	MLH1	MutL homolog 1	*(159)*
	p53		*(28,175)*
	BAP1		*(66)*
	Rb	Retinoblastoma protein	*(7,171)*
	RbAp46/48	Rb binding proteins	*(171)*
	HDAC1/2	Histone deacetylase catalytic subunits	*(171)*
	RHA	RNA helicase A	*(4)*
	γ tubulin		*(63)*
	CtIP	CtBP-interacting protein	*(81,161,172)*
	c-Myc		*(157)*
BRCA2:	BRCA1		*(33,34)*
	RAD51		*(69,100,126,162)*
	p53		*(90)*
	P/CAF	p300/CBP-associated factor	*(50)*

sion. BRCA1 missense mutations within the RING domain that disrupt the BARD1 interaction correlate with breast-cancer susceptibility. Moreover, missense mutations in BARD1 itself, with accompanying loss of heterozygosity, have been found in a small number of breast and uterine cancers *(141).* BARD1, like BRCA1, is a nuclear protein, and its tissue distribution is similar to that of BRCA1 *(9).* Though expression levels of BARD1 are essentially constant over the cell cycle, BARD1 exhibits an S-phase-specific colocalization with BRCA1 within nuclear foci *(67),* and a similar pattern of subnuclear relocalization after DNA damage *(123)* (Subheading 4.2.).

4.2. Interactions of BRCA1 and BRCA2 with RAD51

Early clues that BRCA1 and BRCA2 might function in DNA repair and/or recombination came from findings that both physically associate with the RAD51 (Fig. 1.), that their expression patterns mirror those of RAD51, and that they co-localize with RAD51 in the nuclei of mitotic and meiotic cells. The significance of these findings stems from the central importance of RAD51 itself in homologous recombination and DNA repair. It is likely that the interactions of BRCA1 and BRCA2 with RAD51 lie at the heart of their critical functions in guarding the integrity of the genome and suppressing neoplastic transformation. To appreciate this requires some background on RAD51.

In the yeast *Saccharomyces cerevisiae,* Rad51p is required for the repair of DSBs, and for homologous recombination in both mitotic and meiotic cells. Yeast *rad51* mutants are extremely sensitive to IR and to drugs that induce DSBs or crosslinks, such as MMC *(51,102,115).* Rad51p has the ability to form nucleoprotein filaments with DNA, and to pair or transfer complementary DNA strands *(14),* much like *Escherichia coli* RecA, the prototypical recombinational strand transferase to which it is evolutionarily related.

Vertebrate homologs of yeast *RAD51* were identified on the basis of DNA sequence similarity *(15,17,104,130).* Like yeast Rad51p, mammalian RAD51 forms nucleoprotein filaments with DNA in vitro, has a DNA-dependent ATPase activity *(15),* and has strand-transfer activity in vitro *(10,11).* Expression of the mouse and human *RAD51* genes is cell-cycle dependent, with transcript present from late G1 phase to M phase *(32,168).* From late G1 through the end of G2 phase, RAD51 is localized to the nucleus *(168).* Antibody staining of mouse tissue sections shows that RAD51 is most highly expressed in actively proliferating tissues and in germ-line cells undergoing meiosis *(168).* In meiotic cells, immunofluorescence reveals that mammalian RAD51 associates with synaptonemal complexes *(8,57,114).* RAD51 becomes localized in nuclear foci that appear in premeiotic S phase and persist into metaphase I, suggesting that RAD51 has a role in the initial pairing of homologous chromosomes as well as in subsequent crossing-over. In somatic cells, RAD51 can be detected in discrete nuclear foci or "dots" during S phase and, after exposure to IR or other DNA damaging agents, reaggregates into larger, more irregularly distributed foci *(57,124,139).* These latter nuclear foci coincide with regions of single-stranded DNA, the substrate for formation of RAD51 nucleoprotein filaments, and are interpreted as sites at which recombinational repair complexes have formed *(116).*

The functions of RAD51 are essential in vertebrate cells, not only for repair of exogenous DNA damage but for cell growth and genomic stability. In mouse, knockout of *RAD51* results in early embryonic lethality *(82,151). RAD51*$^{-/-}$ embryos arrest before day six of development, apparently owing to a generalized failure of cell proliferation. The cells of very early *RAD51*$^{-/-}$ embryos are extremely sensitive to IR and, even in the absence of exogenous DNA damage, faltering cell proliferation is accompanied by chromosome loss and apoptotic cell death *(82).* A conditional *RAD51* knockout has been created in DT40 chicken lymphoblastoid cells. These cells grow normally as long as *RAD51* is expressed, but shutdown of *RAD51* expression results in cell-cycle arrest in G2/M phase, with numerous chromosome breaks, followed by cell death *(134).* The functions of RAD51 and the role of HRR in maintaining genomic integrity are more fully reviewed elsewhere *(12,18,131,144; see* also Chapter 9).

Association of BRCA1 with RAD51, in both mitotic and meiotic cells, was initially proposed on the basis of immunofluorescence and co-immunoprecipitation studies *(124).* Fluorescent antibodies to human BRCA1 localized it to discrete nuclear foci during S phase of the mitotic cell cycle. RAD51 similarly occupies discrete nuclear foci during S phase, which significantly (though not completely) overlap BRCA1 foci. Immunoprecipitates from human cell extracts made with antibodies to BRCA1 also contained RAD51, though only a small fraction of total cellular RAD51 was precipitated. Curiously, in the converse experiments, endogenous BRCA1 was not detected in immunoprecipitates made with antibodies to RAD51. However, it was possible to

detect small amounts of exogenously expressed, epitope-tagged BRCA1 in anti-RAD51 immunoprecipitates. In vitro, a peptide corresponding to amino acids 758–1064 of BRCA1 specifically, though weakly, co-immunoprecipitated RAD51. By immunofluorescence, BRCA1 and RAD51 colocalize on synapsing meiotic chromosomes in primary spermatocytes *(124).* Both proteins associate preferentially with the nonsynapsed (axial) portions of developing synaptonemal complexes.

The physiological significance of association between BRCA1 and RAD51 is supported by further studies showing that BRCA1 and RAD51 (and BARD1) disperse from their characteristic S-phase nuclear foci after cells are treated with hydroxyurea (to induce arrest of DNA replication) or DNA-damaging agents (UV, γ radiation, or MMC), and relocalize to regions positive for PCNA *(123).* The latter are presumed to represent sites of DNA replication. The redeployment of BRCA1 to PCNA-positive regions in UV-treated cells is accompanied by a dose-dependent increase in phosphorylation of BRCA1 *(123).* Similar dispersal of BRCA1 nuclear foci is seen in cells subjected to heat shock or herpes virus infection, and in cells infected with adenovirus, BRCA1 is recruited to regions of viral transcription and replication *(94).* It is not yet clear whether the interaction between BRCA1 and RAD51 is direct or indirect. Some aspects of the data suggest that it may be an indirect or perhaps very dynamic interaction, notably the small fraction of total nuclear RAD51 detected in anti-BRCA1 precipitates and the absence of BRCA1 in anti-RAD51 precipitates. It has been proposed that the interaction between BRCA1 and RAD51 might be mediated by a third protein. BRCA2 is a likely candidate, because of its interactions with both BRCA1 and RAD51.

The initial (and somewhat controversial) evidence for interaction between BRCA1 and RAD51 was followed swiftly by several independent demonstrations of physical association between BRCA2 and RAD51. Sharan et al. *(126)* used a yeast two-hybrid screen to detect an interaction between BRCA2 and RAD51 proteins from mouse, and confirmed the interaction in a mammalian two-hybrid system using mouse 3T3 cells. The interaction domains were mapped by deletional analysis to a highly conserved N-terminal region of RAD51 (amino acids 1–43) and a minimal domain comprising 36 amino acids (3196–3232) near the extreme C-terminus of BRCA2 that is also highly conserved (>90% identical to human BRCA2) (Fig. 1). Interaction between RAD51 and the C-terminus of mouse BRCA2 was independently discovered by Mizuta et al. *(100)* in a yeast two-hybrid screen, and confirmed in vitro using an affinity pull-down (GST fusion) assay.

However, it quickly became evident that the BRCA2 interaction with RAD51 involves additional domains. Analysis of human BRCA2 by yeast two-hybrid and affinity pull-down assays identified RAD51-binding domains within the central region encoded by exon 11 *(35,69,162)* and mapped them to the conserved BRC repeats (Fig. 1). Six of the eight BRC repeats, all except repeats 5 and 6, were able to function independently as RAD51-binding domains in the yeast two-hybrid system *(162).* When examined in vitro by affinity pull-down, it was found that all of the BRC repeats, including repeats 5 and 6, were able to independently bind RAD51 *(162).* Yeast two-hybrid analysis was further used to define the minimal region of RAD51 required for interaction with the BRC repeats of human BRCA2. This analysis, in contrast to the previous results for mouse *(126),* revealed that a C-terminal region of human RAD51 (residues 98–339) was required for interaction, and that the N-terminus was not *(69,162).* It appears that

the interaction between the N-terminus of RAD51 and the extreme C-terminal region of mouse BRCA2 may be wholly different from the interactions involving BRC repeats. The extreme C-terminus of human BRCA2 can interact with RAD51 in yeast two-hybrid experiments, but this interaction has not been mapped to a specific part of RAD51 (Z. Shen, personal comunication). BRCA2-RAD51 interaction has been confirmed in vivo by showing that antibodies specific to either protein can readily coimmunoprecipitate the other from cellular lysates *(35,89).*

BRCA1 and BRCA2 associate with each other as well as with RAD51. Antibodies raised against several different epitopes of BRCA1 can co-immunoprecipitate BRCA1 and BRCA2 *(33).* In reciprocal experiments, two different antibodies raised against epitopes in BRCA2 coimmunoprecipitated BRCA2, BRCA1, and RAD51. The part of BRCA1 that mediates interaction with BRCA2 maps to a carboxy-terminal region distal to amino acid 1313 (Fig. 1), but evidently does not include the extreme carboxy terminus, because the mutant BRCA1 in HCC1937 cells, which is truncated after amino acid 1755, can co-immunoprecipitate with BRCA2 *(33).* The part of BRCA2 that participates in association with BRCA1 has not been mapped, but appears to lie within the amino-terminal half of BRCA2, since the mutant protein in Capan-1 cells, truncated after amino acid 1981, co-immunoprecipiates with BRCA1 (and RAD51) *(33).* Like BRCA1 and RAD51, BRCA2 associates with meiotic chromosomes in spermatocytes, localizing preferentially to the nonsynapsed regions of developing synaptonemal complexes *(33).* In mitotic cells, BRCA2 localizes in discrete nuclear foci in S-phase cells, as seen for BRCA1, BARD1, and RAD51, and similarly relocalizes after treatment of cells with hydroxyurea to a distribution that overlaps PCNA foci *(33).* The formation of DNA damage-induced RAD51 nuclear foci may be dependent on BRCA2. Radiation-induced RAD51 foci are sharply reduced in Capan-1 cells, despite the fact that the truncated BRCA2 these cells express is able to bind RAD51 *(173).* Overexpression in wild-type cells of a single BRC repeat from BRCA2 can suppress the induction of RAD51 foci, and increase cellular sensitivity to DNA damage *(30,173).* Formation of RAD51 foci is probably not dependent on BRCA1, because it is unimpaired in HCC1937 cells *(177).*

The physical interactions of BRCA1, BARD1, BRCA2, and RAD51, together with their patterns of subnuclear colocalization and relocalization, suggest strongly that these proteins function together in the response of mitotic cells to DNA damage, and in meiotic recombination. It appears that BRCA1, BARD1, and BRCA2 participate in a protein complex with RAD51, that this complex is assembled specifically during S-phase of the cell cycle, and that it relocates to (or reassembles at) sites of damage in actively replicating DNA.

4.3. Interactions of BRCA1 with the RAD50-MRE11-NBS1 Complex

BRCA1 physically interacts and co-localizes in the nucleus of human cells with the RAD50-MRE11-NBS1 protein complex *(159,177).* This interaction is of great interest because of the central involvement of RAD50-MRE11-NBS1 in DNA damage sensing and repair. *RAD50* and *MRE11,* like *RAD51,* were originally identified in the yeast *S. cerevisiae* as members of the *RAD52* epistasis group, required for wild-type resistance to IR and for genetic recombination in mitotic and meiotic cells. In yeast, Rad50p and Mre11p function in complex with a third protein, Xrs2. Mutations in the *RAD50, MRE11,*

or *XRS2* genes are associated with a sharp reduction in the efficiency of nonhomologous repair of DSBs, with pleiotropic effects (usually reduced frequency) on various classes of homologous recombination events, and with shortening of telomeres. On biochemical and genetic evidence, the RAD50-MRE11-XRS2 complex has been implicated in diverse functions, including: the creation and processing of DSBs required to initiate meiotic recombination; nucleolytic resection of DNA ends in mitotic cells in preparation for HRR; nucleolytic processing and/or spatial juxtaposition of DNA ends for nonhomologous end-joining (NHEJ); and the sensing of DNA damage in connection with induction of the G2/M cell-cycle checkpoint (reviewed in *58).*

In mammals, RAD50 and MRE11 function in complex with NBS1, which is not a sequence homolog of yeast Xrs2p but may be a functional homolog. Functions of MRE11 and RAD50 have been difficult to analyze in mammals; knockout of either gene in mouse results in early embryonic lethality *(88,164).* A conditional knockout of MRE11 has been made in DT40 chicken lymphoblastoid cells *(167).* Shutdown of MRE11 expression results in cell-cycle arrest and chromosome breakage followed by cell death, though not quite so rapidly as shutdown of RAD51 *(134).* The *NBS1* gene is the affected locus in Nijmegen Breakage Syndrome (NBS) *(27,93,153).* Clinically, NBS is associated with retarded growth, immune deficiency, predisposition to cancer, and exaggerated reaction to radiation therapy. Cells from NBS patients exhibit frequent chromosome breakage and rearrangements, hypersensitivity to IR and other DNA damaging agents, and checkpoint abnormalities *(5,6,74,111,113,137,170).* RAD50-MRE11-NBS1 has DNA-unwinding and nucleolytic activities in vitro *(110,150).* In vivo, RAD50-MRE11-NBS1 relocalizes into discrete nuclear foci after treatments that induce DSBs *(91,106).* These foci are reminiscent of, but apparently distinct from, the damage-induced foci formed by RAD51. For a full review of RAD50-MRE11-NBS1, *see* Chapter 7.

BRCA1 is evidently involved in the DNA damage response functions of RAD50-MRE11-NBS1. Antibodies to BRCA1 co-immunoprecipitate RAD50 from human cell extracts and vice versa *(159,177).* As seen for RAD51, only a fraction of total cellular RAD50 coprecipitates with BRCA1. Using synchronized cell populations, the interaction was shown to be cell cycle-specific, with association between RAD50 and BRCA1 appearing during S phase and peaking during G2. In vitro affinity pull-down experiments and a yeast two-hybrid system were used to map the part of BRCA1 required for interaction with RAD50 to a central region (amino acids 341 to 748) that does not overlap the N-terminal RING motif or the C-terminal BRCT domain *(177)* (Fig. 1). The BRCA1-interaction domain of RAD50 is in the N-terminal half of the protein *(177).* IR-induced nuclear foci of BRCA1 substantially overlap induced foci of RAD50-MRE11-NBS1 *(159,177).* Curiously, damage-induced foci of BRCA1 seem to be of two distinct types; those that co-localize with RAD50-MRE11-NBS1, and those that colocalize with RAD51. Individual cells bearing both types of foci have rarely been seen *(106,177).*

Whether induction of nuclear RAD50-MRE11-NBS1 foci is dependent on BRCA1 is controversial. One group *(177)* found that in HCC1937 cells, damage-induced foci of BRCA1 were reduced and RAD50-MRE11-NBS1 foci were nearly undetectable. RAD50, MRE11, and NBS1 are expressed at approximately normal levels in HCC1937 cells, and could be co-immunoprecipitated with antibodies to RAD50, but these

immunoprecipitates did not capture the truncated BRCA1 protein. Transient transfection of HCC1937 cells with a vector expressing full-length BRCA1 restored radiation-induced formation of RAD50-MRE11-NBS1 foci. However, a second group *(159)* reported that damage-induced formation of RAD50-MRE11-NBS1 foci is approximately normal in HCC1937 cells, and is not augmented by expression of full-length BRCA1. BRCA2 probably is not required for the formation of damage-induced RAD50-MRE11-NBS1 foci; they form normally in Capan-1 cells *(173).*

4.4. Interactions of BRCA1 and BRCA2 with Other DNA Repair Proteins

In addition to RAD51 and RAD50-MRE11-NBS1, BRCA1 and BRCA2 associate with several other proteins involved in sensing or repair of DNA damage (Fig. 1 and Table 2). Both BRCA1 and BRCA2 can be co-immunoprecipitated with p53, and both influence transcription in vivo from genes bearing the p53 response element *(28,89,107).* BRCA1 stimulates transcriptional activation by p53 *(28,107),* while BRCA2 inhibits it *(89).* RAD51 enhances the inhibition of p53 transactivation by BRCA2. Interaction of BRCA1 with RAD50-MRE11-NBS1 has recently been proposed to occur within a supercomplex of protein complexes that function in DNA repair and replication, termed BASC (BRCA1-Associated genome Surveillance Complex) *(159).* BASC was defined by immunoprecipitation studies in which the majority of cellular BRCA1 was recovered in a large protein complex with mass exceeding 2 MD. The anti-BRCA1 immunoprecipitates may contain as many as 40 specifically associated proteins. Among the identified components are RAD50-MRE11-NBS1; ATM; the RecQ-type helicase BLM; three subunits of replication factor C (RPC); and the mismatch repair proteins MSH2, MSH6, and MLH1 (RAD51 and BRCA2 are apparently not present). In reciprocal immunopreciptation experiments, many of these proteins also interact with each other. Mutations in the *BLM* gene cause Bloom's Syndrome, which is characterized by chromosomal instability and predisposition to cancer. Co-immunoprecipitation of BLM with BRCA1 is especially robust, and BLM was seen by immunofluorescence to form nuclear foci, co-localizing with BRCA1, RAD50, and MRE11 nuclear foci, in cells treated with hydroxyurea to inhibit replication. The BLM-BRCA1-RAD50-MRE11 foci also colocalize with foci of PCNA, reinforcing the idea that BASC is a damage surveillance and repair supercomplex operating in conjunction with replication. Whether BASC exists in vivo as a stable, integrated surveillance and repair "machine," or rather as a dynamic system of transient interactions remains to be established.

5. BRCA1 AND BRCA2 IN TRANSCRIPTION-COUPLED REPAIR

BRCA1 copurifies with RNA polymerase II and other components of the basal transcription complex *(122),* an association that may be mediated by direct interaction between BRCA1 and RNA helicase A *(4).* These findings suggest a general function in transcriptional regulation, possibly related to transcriptional transactivation by the BRCT domain of BRCA1 *(29,101).* The evidence relating BRCA1 to functions in transcriptional regulation has been recently reviewed *(37).* However, although there is no evidence for a general transcription defect in BRCA1 mutants, recent evidence indicates that BRCA1 has a role in transcription-coupled repair (TCR) of DNA damage, the process whereby damage is preferentially repaired on the transcribed strand of actively expressed genes. TCR has been reviewed by Leadon *(78).*

Gowen and coworkers compared TCR of damage occurring within the dihydrofolate reductase (DHFR) gene in mouse ES cells bearing either wild-type BRCA1 or a targeted mutation that deletes amino acids 223-763 of BRCA1 *(53,54).* In $BRCA1^{+/+}$ or $BRCA1^{+/-}$ ES cells, TCR of oxidative damage inflicted by exposure to IR or hydrogen peroxide is observed much as it has been previously in other cell types. However, TCR of oxidative damage is absent in ES cells homozygous for the $BRCA1^{\Delta 223-763}$ allele. The role of BRCA1 in repair of oxidative damage is specific to TCR, because the overall efficiency of oxidative damage repair for the genome as a whole is not reduced in $BRCA1^{-/-}$ cells. Curiously, BRCA1 is not required for TCR of UV-induced damage. The TCR defect in $BRCA1^{\Delta 223-763}$ ES cells correlates with sensitivity to DNA-damaging agents, as measured in colony-formation assays. $BRCA1^{\Delta 223-763}$ ES cells are significantly more sensitive to IR and to hydrogen peroxide than wild-type or heterozygotes, but have no increased sensitivity to UV.

TCR has recently been examined in the BRCA1-mutant human cell line HCC1937. Abbott et al. *(2)* found that HCC1937 is extremely sensitive to IR, and that this hypersensitivity can be substantially relieved by transient or stable expression of a modified form of BRCA1 in which amino acids 702–834 have been deleted. Use of this modified BRCA1 was necessary because expression of full-length BRCA1 inhibits the growth of human mammary cells in culture *(62,133,145).* Like $BRCA1^{\Delta 223-763}$ mouse cells, HCC1937 cells lack TCR of radiation-induced oxidative damage. TCR was restored, in parallel with radiation resistance, by expression of the modified BRCA1 (Δ702–834) protein. In the same study, HCC1937 cells were examined for overall efficiency of DSB repair, by pulsed-field electrophoresis of whole cellular DNA after γ-irradiation. DSB repair was not obviously defective in HCC1937 cells, and showed only a very slight improvement in HCC1937 cells transfected with $BRCA1^{\Delta 702-834}$. The results imply that the IR sensitivity of HCC1937 is owing more to defective TCR than inability to repair DSBs.

It is not yet clear whether the requirement for BRCA1 in TCR of oxidative damage is direct or indirect. It may reflect a direct participation in damage sensing or repair, or alternatively, an indirect function as a transcriptional regulator of proteins required for these processes. Leadon *(78)* argued that a requirement for BRCA1 as a transcriptional regulator is less likely, because the genes activated would have to be specific for repair of oxidative damage, because BRCA1 is not required for TCR of UV-induced damage. Another possibility is that BRCA1 is required to recognize oxidative DNA damage on the transcribed strand of actively expressed genes and recruit repair proteins to the site. Long before the discovery of BRCA1, it was proposed that RNA polymerase stalled at a site of damage may be the signal that directs repair preferentially to the transcribed strand *(96).* The observed associations of BRCA1 with RNA pol II and RNA helicase A *(4,122)* are consistent with such a role.

BRCA2 also has a role in TCR, though perhaps a more limited one than BRCA1. In ES and MEF cells bearing an exon 27 truncation of BRCA2, TCR of damage induced by IR is reduced but not abolished, though TCR of thymine gylcols produced by hydrogen peroxide, as well as UV damage, appears unaffected (S. A. Leadon, personal communication).

6. BRCA1 AND BRCA2 IN HOMOLOGOUS RECOMBINATIONAL REPAIR

Reports that BRCA1 and BRCA2 physically associate and co-localize with RAD51 suggested that both proteins are involved in homologous recombination. Moreover,

BRCA1- and BRCA2-mutant cells exhibit phenotypic traits similar to two other mammalian cell lines known to be deficient for HRR; those mutated for XRCC2 and XRCC3. These traits include reduced cellular viability, a specific spectrum of sensitivities to DNA damaging agents, and gross chromosomal instability (*see* Subheadings 3. and 8.). The similarity of phenotypes between *BRCA1* and *BRCA2* mutations and mutations in the *RAD51*-related genes *XRCC2* and *XRCC3* strongly support the idea that BRCA1 and BRCA2 have critical involvements in HRR.

For BRCA1, there is now direct evidence for such a role. Moynahan et al. *(105)* used the same *BRCA1*$^{-223-763}$ ES cell line previously used to demonstrate a defect in TCR *(53)* to examine the effects of BRCA1 deficiency on two classes of homologous recombinational events: gene targeting and HRR of a chromosomal DSB. Relative frequencies of gene targeting at two different loci in *BRCA1*$^{-223-763}$ cells were reduced by 20 to 50-fold as compared to heterozygous controls, reflecting both decreased targeting and, in one case, increased nonhomologous vector integration. To assess HRR of a chromosomal DSB, a *neo* direct-repeat recombination substrate was installed in *BRCA1*$^{-223-763}$ cells and heterozygous controls by gene targeting. A chromosomal DSB was induced in vivo by expression of the highly site-specific endonuclease I-*Sce*I. DSB-induced HRR was reduced by five- to six-fold in the *BRCA1*$^{-223-763}$ cells as compared to heterozygous controls. The molecular analysis used in this study also allowed for a measurement of DSB repair by imprecise NHEJ, which was unaffected or even slightly increased by *BRCA1* mutation.

The defect in HRR in *BRCA1*-mutant ES cells can be placed in perspective by comparison to the defects caused by mutations in the *XRCC2* and *XRCC3* genes. *XRCC2* and *XRCC3* are members of the *RAD51* gene family *(84).* Hamster cell lines mutated for each gene have been examined using chromosomally integrated recombination substrates similar to those employed by Moynahan et al. In XRCC2-mutant cells, HRR of I-*Sce*I-induced breaks occurred at a low basal level, but increased some 100-fold upon complementation by expression of the human *XRCC2* gene *(68).* Similarly in XRCC3-mutant cells, the frequency of DSB-induced HRR increased upon complementation with the human *XRCC3* gene by 25 to 30-fold in one study *(112),* and by 34-fold to 200-fold in a second study *(25).* The apparently greater severity of defects in the *XRCC2*- and *XRCC3*-mutant hamster cells as compared to *BRCA1*$^{-/-}$ mouse cells should be interpreted with caution, as it could be due partly to the species difference, or to differences in genetic background (e.g. p53 status) and experimental design. Even so, it appears that the deficiency in HRR inflicted by loss of *BRCA1* function in mouse cells is comparatively moderate.

For BRCA2 as well, there is now direct evidence for a function in HRR. Two groups have independently reported that expression of BRCA2 fragments containing a single BRC repeat (the RAD51-interacting domains of BRCA2) results in hypersensitivity to DNA damage by IR, MMS or cisplatin, reduces formation of subnuclear RAD51 foci after IR exposure, and suppresses induction of the G2/M cell cycle checkpoint *(30,173).* These effects are presumed to come about by a dominant-negative mechanism, i.e. through competitive interference with the normal protein-protein interaction between BRCA2 and RAD51. The dominant-negative effect of BRC repeats has been exploited to assess the dependence of HRR upon the BRCA2-RAD51 interaction. A recombination substrate comprising inverted repeats of the *pac* (puromycin resistance) gene was

installed in HT1080 human fibrosarcoma cells, such that high frequencies of HRR occur following induction of a chromosomal DSB within one *pac* repeat using *I-SceI* endonuclease. When expression of I-*Sce*I in these cells is accompanied by expression of a BRC repeat peptide, HRR of the induced DSB is reduced by at least fifteen-fold compared to I-*Sce*I-only controls (Brenneman, Chen, and Nickoloff, unpublished results). Other types of homologous interactions are also affected by BRCA2 deficiency. In Capan-1 cells, the frequency of homologous recombination between extrachromosomal plasmid substrates increases by about eight-fold upon complementation with wild-type BRCA2, and gene targeting frequency is increased by more than ten-fold (S. N. Powell, personal communication).

7. BRCA1 AND BRCA2 IN CELL-CYCLE CHECKPOINTS

Normal cells exposed to acute DNA-damaging treatments such as IR respond by activating cell-cycle checkpoints. Activation of the G1/S checkpoint causes arrest at the G1/S transition, such that cells bearing unrepaired damage are prevented from beginning DNA synthesis until repair can be effected, while the G2/M checkpoint arrests cells that have sustained chromosomal damage during or after replication, preventing them from progressing to mitosis. It has been proposed that both BRCA1 and BRCA2 function in DNA damage-induced cell-cycle arrest, and that failure to restrain cell-cycle progression in the face of DNA damage may underlie the heightened sensitivity of BRCA1- and BRCA2-mutant cells to DNA damage, as well as their tendencies to genomic instability and neoplastic transformation. This in no way contradicts the idea that BRCA1 and BRCA2 function directly in DNA repair. There is growing evidence that both kinds of defects, repair and checkpoint, contribute to the cellular phenotypes and carcinogenic potential of BRCA1 and BRCA2 mutations. This is particularly so for BRCA1, which is better studied in this regard.

BRCA1 is evidently required for the G2/M damage-induced checkpoint *(46,77,166).* MEF bearing a targeted deletion of exon 11 in BRCA1 showed little reduction (or even an increase) in mitotic index after γ-irradiation, under conditions that reduced the mitotic index of wild-type cells by as much as 70% *(166).* The same study demonstrated that the checkpoint function of BRCA1 is specific to certain kinds of damage. The G2/M checkpoint response of BRCA1-mutant cells after exposure to UV light or MMS was not affected. This correlates in an interesting way with BRCA1 involvement in DNA repair, because BRCA1-mutant cells are defective for TCR of oxidative damage (a major product of γ-radiation) but not UV-induced photodamage *(53),* and impaired for HRR of DSBs (also a product of γ-radiation) *(105).* It appears that BRCA1 triggers G2 arrest only in response to types of DNA damage that it participates in repairing.

BRCA2 apparently is also required for the G2/M checkpoint. In a human tumor cell line that expresses normal BRCA2, overexpression of a BRCA2 fragment containing the fourth BRC repeat motif exerts a dominant-negative effect, as evidenced by increased sensitivity to γ-radiation and by a reduction in formation of radiation-induced RAD51 nuclear foci *(30).* Cells expressing the dominant-negative peptide exhibit little or no reduction in mitotic index after γ exposure, indicating abrogation of the G2/M checkpoint. Loss of G2/M checkpoint function was not seen in primary mouse cells with sublethal targeted truncations of BRCA2 *(103,109).* However, a different method of assessing G2/M checkpoint function was used in these studies (flow-cytometric scor-

ing of cells having 4N DNA content), and it is important to note that these are mutations intended to preserve some BRCA2 function.

The G1/S checkpoint is evidently not affected by BRCA1 or BRCA2 deficiency. Mouse cells homozygous for a targeted mutation of BRCA1 show a similar G1/S checkpoint to wild-type controls *(166),* and human cells expressing a dominant-negative BRCA2 fragment also show a wild-type G1/S checkpoint *(30).* That the G1/S checkpoint is intact in BRCA1- and BRCA2-mutant cells reinforces the idea that mutation of these genes must be accompanied by mutations in other checkpoint genes such as p53 in order for tumorigenesis to proceed *(39,79,129).* Although p53 mutations are generally common in human tumors, they are significantly more common in $BRCA1^{-/-}$ and $BRCA2^{-/-}$ tumors *(55,119).*

The loss of G2/M checkpoint function in BRCA1- and BRCA2-deficient cells suggests that, even in the absence of exogenous DNA damage, chromosomes bearing unrepaired spontaneous damage incurred in the course of replication will enter mitosis with abnormally high frequency. The resulting errors of segregation may underlie the gross chromosomal instability seen in mouse cells with mutations in *BRCA1* or *BRCA2 (24,129,152,166).*

8. BRCA1 AND BRCA2 IN MAINTENANCE OF CHROMOSOMAL STABILITY

Gross chromosomal instability has emerged as another feature characteristic of cells mutated for BRCA1 or BRCA2, and indicative of defects in cellular response to DNA damage. Cells of mouse embryos with a targeted deletion of *BRCA1* exon 11 ($BRCA1^{11-/-}$) have abnormal numbers of chromosomes, either greater or less than the normal number of 40, in up to 30% of metaphases examined *(129).* Wild-type embryos had numerical changes in only about 3% of metaphases. When the $BRCA1^{11-/-}$ mutation was bred into a checkpoint-defective ($p53^{-/-}$) background, the chromosome number abnormalities increased to over 70% of metaphases. Structurally abnormal chromosomes including translocations and dicentrics were also seen in mutant embryos. In culture, $BRCA1^{11-/-}$ primary MEF rapidly accumulate chromosome abnormalities, both structural and numerical *(166).* These cells are also defective for the G2/M cell cycle checkpoint (*see* Subheading 7.). Most remarkably, they frequently contain multiple centrosomes that give rise to multipolar mitotic spindles and aberrant chromosome segregation at cell division *(166).* This indicates that BRCA1, in addition to DNA repair and checkpoint functions, has some role in regulating centrosome replication. This role might be accomplished indirectly through the transcriptional regulatory functions that have been attributed to BRCA1. However, in monkey and human cells BRCA1 is localized to the centrosomes during mitosis, and physically interacts with γ-tubulin *(63),* which suggests that its effects are exerted directly, via protein-protein interactions.

BRCA2 mutation is also associated with gross chromosomal instability in mouse cells. Primary MEF with a targeted truncation of BRCA2 within exon 11 (retaining only three of the eight BRC motifs) had structural chromosome abnormalities, most notably chromatid breaks and chromatid exchanges, in about 40% of metaphases examined at the first passage after dissociation from embryos, increasing to almost 70% by the third passage *(109).* Curiously, the structural chromosome abnormalities in these

cells (as well as their growth defect in culture) can be largely suppressed by expressing dominant-negative mutant forms of p53 or the spindle-assembly checkpoint kinase Bub1 *(79).* Primary MEF bearing a milder truncation (retaining all the BRC repeats, but deleting the extreme carboxy-terminal RAD51-binding domain encoded by exon 27) similarly displayed elevated frequencies of structural chromosome abnormalities that increased with time in culture, such that 50–60% of metaphases were abnormal by the third passage *(24).* Spontaneously immortalized MEF with the same exon 27 deletion also showed evidence of ongoing chromosomal instability *(24).* The modal chromosome number in these cells was 60, intermediate to the normal diploid number of 40 and the approximately tetraploid chromosome numbers usually seen in newly immortalized mouse cells *(148).* Such sub-tetraploid chromosome numbers may reflect tetraploidization followed by chromosome loss. These cells were also conspicuous for the presence of one to several abnormally shortened chromosomes in every metaphase examined. By fluorescence *in situ* hybridization analysis, the majority of the abnormally small chromosomes either lacked telomeres or lacked a centromere, indicating that they are chromosome fragments. Chromosome fragments lacking a centromere are subject to loss by segregation errors, whereas fragments that lack telomeres can be expected to undergo progressive shortening, and further cycles of fusion and breakage. Dicentric chromosomes were also frequently seen, and may reflect the latter process.

Most recently, mutation of BRCA2 in mouse cells has been associated with centrosome amplification and segregation errors, much as for BRCA1 *(152).* Primary MEF with an exon 11 truncation of BRCA2 were found to show spontaneous micronucleus formation (indicating the presence of broken and/or incorrectly segregated chromosomes) in about 30% of cells at the second passage after dissociation from embryos, increasing to more than 50% by passage 3. Aneuploid chromosome numbers were present in more than 80% of metaphases examined, even at passage 2. Immunofluorescent staining with antibodies to γ- and β-tubulins revealed extra centrosomes and abnormal spindles in 44% of cells at passage 2, rising to 65% at passage 3.

In human cells, it is not yet clear whether the effects of BRCA1 or BRCA2 loss on chromosome stability are as severe as in mouse cells. The human BRCA1-mutant tumor cell line HCC1937 and the human BRCA2-mutant tumor cell line Capan- 1 both have highly aneuploid and variable karyotypes *(75,149),* but it cannot be known whether this was a cause or a result of neoplastic transformation (or both). More generally, breast cancers from BRCA1 and BRCA2 germ-line mutation carriers show markedly higher frequencies of chromosome rearrangements than sporadic breast cancers *(146,147).*

9. CONCLUDING REMARKS

The roles of BRCA1 and BRCA2 have begun to emerge in outline, though it is by no means certain that the activities identified so far are all that will ultimately be found. It is now clear that both proteins are involved in at least three types of function essential to the preservation of genomic integrity: DNA repair, cell-cycle checkpoint activation, and control of centrosome replication. The actions of BRCA1 and BRCA2 in chromatin remodeling and transcriptional regulation might subserve any or all of the aforementioned functions. Far less clear is how BRCA1 and BRCA2 participate in these functions at the molecular level. It seems likely that much of what BRCA1

and BRCA2 do, they do together. The similarity of mutant phenotypes in mouse support this view, as do the similar patterns of subnuclear localization and re-localization in response to DNA damage. The physical interaction and co-localization of both proteins with RAD51 also suggest that they participate in a single pathway, at least in regard to HRR. The involvement of both BRCA1 and BRCA2 in TCR suggests that they may function together in this repair pathway as well. That mutation of either BRCA1 or BRCA2 compromises activation of the G2/M checkpoint, but not the G1/S checkpoint, implies that they act together in signaling the presence of DNA damage in replicating chromosomes. The redundant centrosomes and abnormal chromosome segregation seen with mutation of either gene suggest that BRCA1 and BRCA2 also work together in control of centrosome replication. However, numerous interactions of BRCA1 and BRCA2 with other proteins have been detected (Fig. 1 and Table 2), as discussed in previous sections, and additional interactions probably await discovery. Full understanding of BRCA1 and BRCA2 will require a focus on their roles as members of multiprotein complexes.

The involvement of BRCA1 in both TCR and HRR suggests that it may perform analogous functions in the two repair pathways; possibly the sensing of DNA damage. The association of BRCA1 with the RNA polymerase II holoenzyme *(122)* may reflect such a function in TCR, serving to target repair to the template strand. If stalling of RNA polymerase at a lesion in the template strand is the signal that triggers TCR *(96),* then it can be imagined that BRCA1 acts to sense and transduce this event, and that it might sense and transduce the analogous event (stalling of DNA polymerase at a lesion) in the context of HRR or lesion bypass associated with replication. Though no direct association of BRCA1 with DNA polymerase has been uncovered, the co-localization of BRCA1 with the essential replication protein PCNA after DNA damage and its physical association with replication factor C are consistent with such a role *(123,159),* as is the recently reported ability of BRCA1 to bind DNA ends and nicks *(169).* But if the function of BRCA1 is to sense DNA damage, then how is the presence of damage transduced? Is the next event recruitment of DNA repair proteins? Direct activation of a checkpoint signaling cascade? Indirect activation of repair or checkpoint functions via transcriptional regulation? BRCA1 may function in a combination of these processes.

The robust physical association of BRCA2 with RAD51 and the iterated RAD51-binding domains in BRCA2 imply an intimate functional interaction. Targeted truncations of BRCA2 in mouse suggest that all of the RAD51-binding domains of BRCA2 are required for a fully functional interaction. One potential explanation for this is that the relevant ligand of BRCA2 is not monomeric RAD51, but rather multiple units of RAD51 assembled with single-stranded DNA into a nucleoprotein filament. In this scenario, BRCA2 might function to facilitate the loading or compacting of RAD51 monomers onto single-stranded tails produced by processing of DSBs. Alternately or additionally, BRCA2 might signal the presence of RAD51-nucleoprotein filaments (and hence that HRR is active) through interaction with BRCA1, via the transcriptional activation domain in its amino-terminus, or via other functional domains yet to be identified in this enormous protein.

Many of the identified involvements of BRCA1 and BRCA2 relate directly to cell proliferation, and this is consistent with the growth defects associated with *BRCA1* and *BRCA2* mutations in mouse cells and embryos. Defects in homologous recombi-

nation occurring in conjunction with replication may be the proximate cause of growth impairment. *In E. coli,* recombinational processes are required for repair and bypass of template defects during replication and for the restart of collapsed replication forks (*42,71,72; see* also Chapter 2). A growing body of evidence supports the conservation of this requirement in eukaryotes *(108,144).* Frequent errors of chromosome segregation may also contribute to growth impairment, by rendering the products of many cell divisions inviable. However, the association of growth defects with BRCA1 and BRCA2 mutations poses a paradox in relation to carcinogenesis: how can mutations that severely impair cell proliferation promote development of a tumor? It has been proposed that loss of BRCA1 or BRCA2 can promote carcinogenesis only if it is accompanied by mutations that disable other checkpoint and apoptotic functions *(55, 61, 79).* This raises the question of whether loss of BRCA1 or BRCA2 acts as an initiating event in carcinogenesis, by causing genomic instability that drives the loss of other growth-controlling genes, or whether loss of BRCA1 or BRCA2 is more usually a secondary event, contributing to carcinogenesis in cells that have already lost the ability to arrest or self-destruct in the face of accumulating genomic damage.

Regardless of whether it is typically the first or a later event, it is probable that mutation of BRCA1 or BRCA2 promotes tumorigenesis by causing genomic instability at the chromosomal level, i.e., deletion and rearrangement of chromosome parts, and loss or gain of entire chromosomes. In mouse cells, the chromosome instability associated with *BRCA1-* and *BRCA2* mutation now appears to be driven by three defects operating in conjunction: failure to repair DNA damage that arises during or after replication; failure to arrest cell-cycle progression at G2 in the presence of unrepaired damage and; and failure to correctly segregate chromosomes during mitosis owing to unrepaired chromosome breaks and centrosome/spindle malfunction. To the extent this genome-destabilization scenario holds in human cells, it may go far in explaining the carcinogenic effect of *BRCA1* and *BRCA2* mutations. In this regard, both BRCA1 and BRCA2 fit well into the category of "caretaker" genes, as conceptualized by Kinzler and Vogelstein *(70).*

An important question still outstanding is why loss of BRCA1 or BRCA2 in humans is associated predominantly with breast and ovarian cancers. Their roles in DNA repair, checkpoint induction, and regulation of centrosome function would seem equally important in any rapidly proliferating cell population. Why then do tumors arise with highest frequency in estrogen-responsive tissues? One possibility is suggested by the observation that BRCA1 can restrain the growth of normal and malignant breast epithelial cells, but not tumor cells derived from other tissues *(145).* It may be that BRCA1 (perhaps in conjunction with BRCA2) has been recruited to perform a growth-regulating function specific to the developmental program of breast and ovary, i.e., modulation of the mitogenic stimulus of estrogen. BRCA1 can inhibit signaling by the ligand-activated estrogen receptor (ER-α) and block its transcriptional activation function *(45).* Another possibility, raised by Livingston and coworkers *(33,34),* is that certain metabolic products of estrogen, by forming DNA adducts, add to the load of endogenous DNA damage in cells that concentrate estrogens. Either or both of these possibilities, added to the general effects of BRCA1 or BRCA2 deficiency on DNA damage response and genomic integrity, might account for the heightened risk of cancer in breast and ovary.

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11

DNA Repair and the Generation of Immune Diversity

The Agony and the Ecstasy

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1. INTRODUCTION

Lymphocytes endow the immune system with the capacity to specifically recognize and destroy a vast array of pathogenic microorganisms. To accomplish this task, lymphocytes express clonally unique antigen receptors that transduce signals that activate humoral (B cell) or cellular (T cell) effector functions upon recognition of a suitable antigenic ligand. Lymphocytes require the faithful execution of DNA repair processes first to generate, and then to refine the recognition specificity of their antigen receptors. Antigen receptor diversity is generated somatically during lymphocyte development through a DNA breakage and rejoining process called V(D)J recombination. Site-specific recombination of variable (V), diversity (D), and joining (J) gene segments assembles the variable region exon of an antigen receptor chain in each newly generated lymphocyte. In mice and humans, substantial germline diversity exists in tandem genomic arrays of dozens to hundreds of each gene segment. The random recombination of particular V, D, and J segments in immature lymphocytes combinatorially expands this germline repertoire. Importantly, imprecision in the joining process further diversifies the available germline repertoire by several orders of magnitude. V(D)J recombination occurs early in lymphocyte development, but more mature B cells undergo V gene hypermutation and/or additional rounds of V(D)J recombination to further refine their antigen specifities. Finally, the immune effector function of the immunoglobulin (Ig) heavy chain can be modified by class-switch recombination, a transaction involving targeted, but not site-specific, breakage and rejoining of different constant region genes. Thus, lymphocytes are clearly unique in their dependence on multiple DNA repair processes for their maturation and function. Defects in these pathways can result in profound immune deficiencies. Furthermore, genetic rearrangement and hypermutation occur in lymphocytes that have great proliferative potential, so infidelity in these processes poses a risk of genomic instability and growth dysregulating

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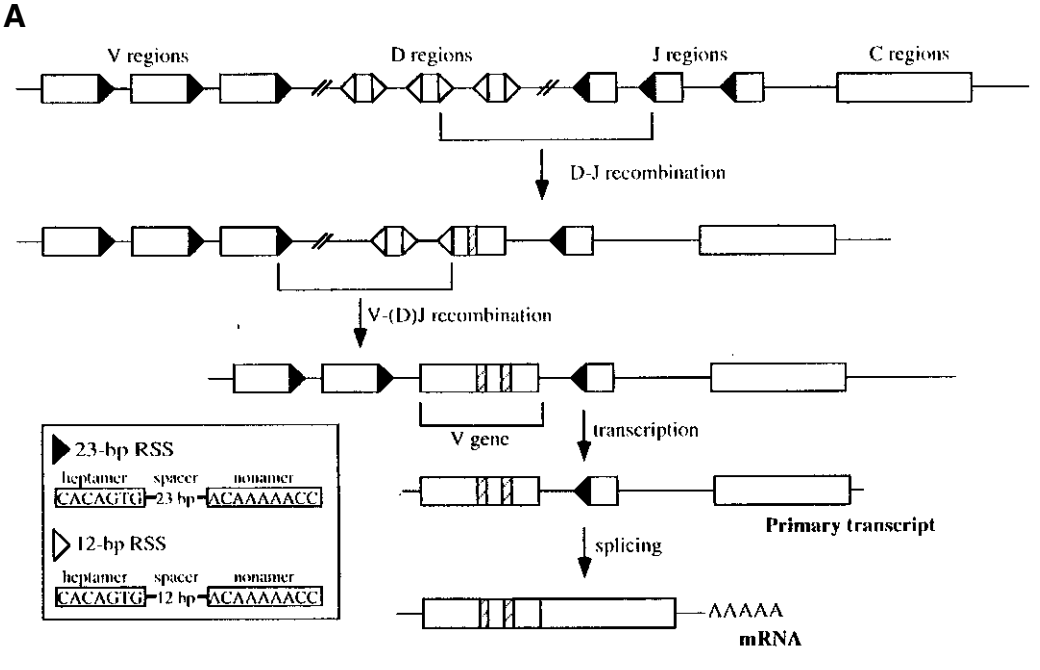
mutations. DNA damage checkpoints help to ensure that DNA breakage and rejoining at antigen-receptor genes are carefully controlled, and to eliminate lymphocytes containing potentially dangerous mutations. This chapter will explore the molecular regulation of V(D)J recombination and other physiological DNA repair mechanisms operative in lymphocytes, emphasizing recent, and sometimes conflicting, data in a rapidly expanding field of DNA repair. The pathological consequence of defects in these processes will also be addressed in the context of rodent models and human disease. We hope that the reader will come to appreciate the complex molecular choreography required to create, modify, and deploy antigen-reactive lymphocytes, processes that are not yet fully understood.

2. LYMPHOCYTE DEVELOPMENT

2.1. Structure of Antigen-Receptor Proteins and Genes

Most T cells express on their surface a T cell antigen receptor (TCR) consisting of disulfide-linked α and β chains, but a minor T-cell sublineage expresses an alternative TCR, composed of a γδ heterodimer. TCR-αβ and some TCR-γδ recognize cell-bound antigen fragments complexed with major histocompatibility complex (MHC) proteins. In contrast, the B-cell antigen receptor (BCR) is a membrane-bound Ig that is able to capture soluble antigens. Monomeric Ig is a dimer of dimers, consisting of two Ig heavy (IgH) chains and two Ig light (IgL) chains. Each IgL chain is disulfide linked to one IgH chain, and the two IgH chains are disulfide-linked to each other. There are two IgL isotypes (κ, λ) generated from distinct genetic loci, but each B cell only expresses one IgL isotype. Following ligand binding by the IgH/IgL and TCR-αβ or TCR-γδ heterodimers, a noncovalently associated invariant polypeptide complex (Ig-αβ and CD3, respectively) transduces intracellular signals to activate immune effector functions *(168,198).* The clonally unique N-terminal variable region of both antigen-receptor chains determines antigen specificity in T and B cells. The constant regions mediate effector functions and/or structural roles. Most antigen receptor loci encode only one or two functionally identical constant (C) region genes. However, there are many IgH C region genes (μ, δ, γ1–4, α, ε), and class-switch recombination between them generates multiple IgH subclasses that mediate diverse effector functions (Subheading 7.3).

Fig. 1. Schematic representation of V(D)J recombination. **(A)** V(D)J recombination involves the site-specific recognition and cleavage of chromosomal DNA by lymphoid-specific proteins (RAG1/2), to bring variable (V), diversity (D), and joining (J) gene segments of antigen receptors together to create functional transcripts. The site-specific cleavage occurs at recombination signal sequences (RSSs) represented here by shaded triangles. The inset panel shows the DNA sequence of the RSSs. **(B)** Two types of V(D)J recombination can occur: deletional and inversional. Deletional rearrangement occurs between gene segments in the same transcriptional orientation. Inversional recombination occurs between gene segments in opposite transcriptional orientation. Transcriptional orientation is indicated by the arrows within gene segments. **(C)** Extrachromosomal recombination substrates (ECRSs) are used to assay V(D)J recombination in cell culture. Prior to recombination, the two RSSs flank a transcriptional terminator (STOP). Transcription is initiated at a bacterial promoter (p), but is interrupted by the terminator. Successful RSS-mediated rearrangement deletes the terminator and transcription of a marker gene, most often encoding resistance to chloramphenicol (CAM), occurs allowing selection of the rearranged product.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D-J recombination
V-(D)J recombination
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Primary transcript
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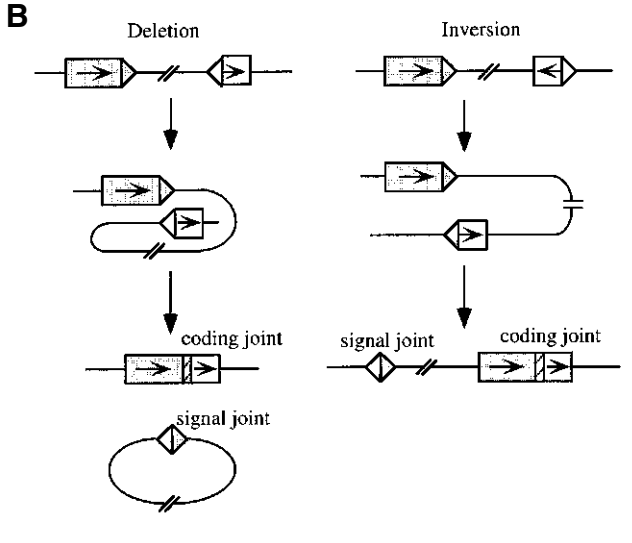
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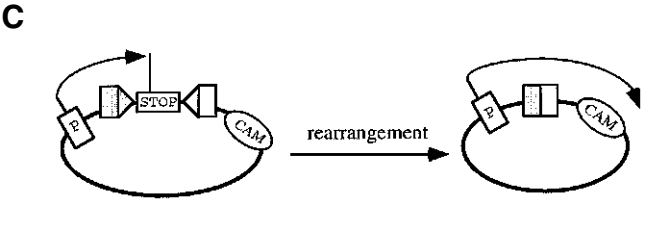
C
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CAM

The organization of the antigen receptor genes reveals how a limited set of genes codes for receptors capable of binding an enormous variety of antigens (Fig. 1A). Variable region genes are encoded by clusters of discontinuous gene segments, spread over vast regions of a chromosome (reviewed in *149).* Somatic rearrangement brings these gene segments together in the process of V(D)J recombination. TCR-β, TCR-δ, and IgH V regions consist of V, D, and J gene segments, while only V and J gene segments comprise TCR-α, TCR-γ, and IgL V regions. Thus, each antigen receptor contains a V(D)J chain bound to a VJ chain. The developmental sequence in which the gene segments are rearranged is highly ordered. D and J segments (of TCR-β and IgH loci) are generally joined first, and then a V segment is joined to the DJ segments, yielding a template for RNA transcription. As the gene segments are connected, nucleotides may be removed or added in a non-templated manner at the joint. The processes of somatic DNA rearrangement and imprecise DNA joining create a diverse repertoire of antigen receptors, enabling the detection and clearance of a myriad of infectious agents.

It should be pointed out that gene rearrangement is not the major mechanism for diversifying the germline repertoire in all animals (reviewed in *167,293*). Chickens have a single functional IgH and IgL V element, resulting in a rather limited primary B-cell repertoire. Interestingly, this repertoire becomes highly diversified following antigenic stimulation of mature B cells by a gene-conversion process. The donor sequences are provided by a large family of highly homologous and clustered pseudo-V_H and pseudo-V_L genes located upstream of the functionally rearranged V_H or V_L gene. Rabbits have approx 200 V_H gene segments, but only about half are functional, and the initial rearrangement in most B cells is subsequently diversified using functional and nonfunctional upstream V_H elements. Superficially, V region diversification by gene conversion seems similar to the mating-type switch in *Saccharomyces cerevisiae.* Though it seems likely that targeted gene conversion of V elements involves the generation of double-strand DNA breaks (DSBs), a site-specific endonuclease, analogous to the HO endonuclease that initiates the mating-type switch in yeast, has not been identified.

2.2. *Regulation of Lymphocyte Development by V(D)J Recombination*

The tremendous somatic diversification of the germline repertoire that is afforded by V(D)J recombination comes at a high price. Because of the variable nucleotide loss or addition at V(D)J junctions, each joining event has only a one-third chance of being in-frame. Because generating both chains of an antigen receptor requires at least 5 joining events, most developing lymphocytes will fail to express a functional antigen receptor, even if they attempt rearrangement on both chromosomes. The evolutionary solution was to connect intimately lymphocyte development to the timing and success of V(D)J recombination. Accordingly, antigen receptor-mediated developmental checkpoints regulate the development of T and B lymphocytes in similar ways (reviewed in *119,287).* Recombination is first activated at the IgH and TCR-β loci in pro-B or pro-T cells, respectively (Fig. 2). Successful (in-frame) rearrangement allows expression of pre-BCR or pre-TCR in which the IgH/TCR-β protein is paired with a "surrogate" IgL or pre-TCR-α chain encoded by nonrearranging genes. Expression of these surrogate chains is limited to immature lymphocytes, and contributes to the assembly of pre-BCR or pre-TCR complexes, which signal progenitors to cease rearrangement, proliferate extensively, and to develop to the pre-B or pre-T stage where rearrangements of IgL and

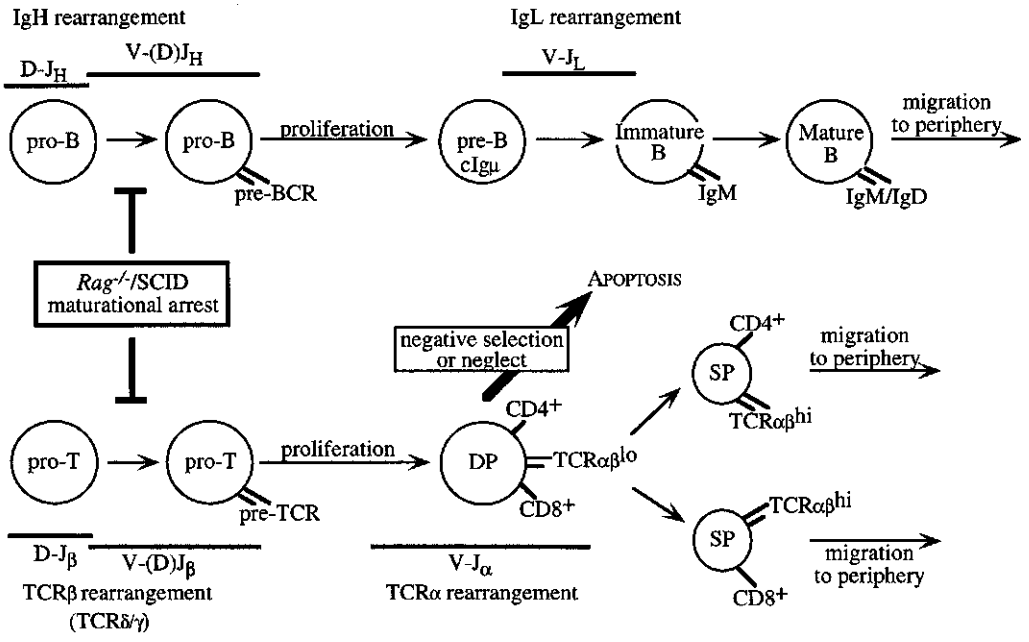

Fig. 2. Schematic representation of B- and T-lymphocyte development. Productive rearrangement of IgH or TCR-β genes and signaling through the pre-BCR and pre-TCR, respectively, stimulates precursor proliferation, allelic exclusion of unrearranged IgH or TCR-β, initiation of IgL or TCR-α rearrangement, and subsequent maturation. RAG-deficiency or the SCID mutation arrests development at the pro-B or pro-T cell stages, though the mechanistic reason for arrest is different. *See* Subheading 2.2. for details.

TCR-α loci begin. Thus, this first developmental checkpoint serves to clonally expand the rare progenitors that successfully rearrange IgH or TCR-β, and to induce their developmental progression to the next phase of rearrangement. Because of the random nature of V(D)J recombination, most pre-T cells will express TCR-αβ that do not recognize foreign antigens complexed with self-MHC proteins, and are useless to the immune system. A second developmental checkpoint, known as positive selection, ensures that only pre-T cells expressing self-MHC-specific TCR-αβ mature into helper and cytotoxic T cells *(87).* B cells expressing IgH/IgL with high affinity for foreign antigens are also positively selected, but only after they enter the peripheral circulation (Subheading 7).

The vast majority of developing lymphocytes (90–99%) is eliminated by apoptosis before completing their development (Fig. 2). Although it is clear that some of this loss is accounted for by precursors failing to make functional TCR or Ig rearrangements, a second developmental consequence of V(D)J recombination is that it has the capacity to generate antigen receptors specific for self, rather than foreign, antigens. To minimize the potential for autoimmunity, a receptor-mediated negative-selection process induces apoptosis in immature (or sometimes mature) T and B cells with high-affinity autoreactive BCR or TCR *(97,138).* Thus, diversification of the repertoire by V(D)J recombination has necessitated that developing lymphocytes evolve strategies for coping with the inefficiency of generating functional receptors, and the danger of generating autoreactive ones.

Dramatic evidence for the pivotal role of V(D)J recombination in lymphocyte development was provided by studies of mice homozygous for the spontaneous severe combined immune deficiency (SCID) mutation and mice bearing targeted deletions of either of the recombination activating genes, *Rag1* or *Rag2* (Subheading 3.). B and T lymphocyte development in SCID, RAG1-and RAG2-deficient mice is arrested owing to failure to generate pre-BCR or pre-TCR *(47).* Because developmental arrest occurs prior to the proliferative expansion elicited by pre-BCR and pre-TCR signals, RAG-deficient and SCID mice contain drastically reduced numbers of immature and mature lymphocytes, rendering the animals susceptible to opportunistic infections. Although the cellular basis of immune deficiency is largely indistinguishable in RAG-deficient and SCID mutant mice, the mechanistic failure in V(D)J recombination is distinct in these two models, and provides insight into the molecular steps in this DNA breakage and repair process, as discussed below.

3. BREAKING IT UP: THE V(D)J CLEAVAGE REACTION

V(D)J recombination is a site-specific, but nonconservative, DNA cleavage and repair process that apposes antigen-receptor gene segments (Fig. 1A). Lymphoid-specific endonucleases, RAG1 and RAG2 initiate this process (Fig. 3). The RAG proteins recognize recombination signal sequences (RSSs) flanking the V, D, and J gene segments, and a synaptic complex is formed between two segments. DSBs are introduced at the boundary between the RSS and the V, D, or J coding segment, generating two types of intermediates: hairpin-terminated coding ends (CEs) and blunt recombination signal ends (SEs). As discussed in Subheading 4, a complex of DNA repair proteins mediates CE ligation to form coding joints and SE ligation to form signal joints. V(D)J recombination can occur either by deletion or inversion, allowing recombination of V gene segments in either transcriptional orientation relative to the (D)J germline elements (Fig. 1B). Deletional rearrangement occurs between two gene segments in the same transcriptional orientation flanked by RSSs in opposite (head to head) orientations. This process generates a coding joint between germline elements, and excision of the intervening region, which is then religated to form an extrachromosomal circle (reviewed in *149).* Inversional recombination occurs between two gene segments in opposite transcriptional orientations that are flanked by RSSs in the same (head to tail) orientations. In this case, both coding and signal-joint formation occur on chromosomal DNA, and both are required to re-establish a patent chromosome.

V(D)J recombination can result in two types of "nonstandard" V(D)J products, called hybrid and "open-and-shut" joints. Open-and-shut joints result from the cleavage and reunion of adjacent CE and SE, whereas hybrid joints result from the cleavage and joining of a CE from one gene segment with the SE from a different segment (reviewed in *76,149).* Although rarely observed, these nonstandard events can apparently rejoin DSBs in a RAG-dependent fashion, without a full complement of repair proteins *(183).*

3.1. RSSs Mark the Spot: Recombination Signal Sequences

RSSs are evolutionarily highly conserved, and are absolutely required for V(D)J recombination. The RSS consists of highly conserved palindromic heptamers and conserved A/T-rich nonamers separated by a spacer region of either 12 or 23 base pairs (bp) in length (Fig. 1A, inset panel; *149).* Both in vitro and in vivo studies have demon-

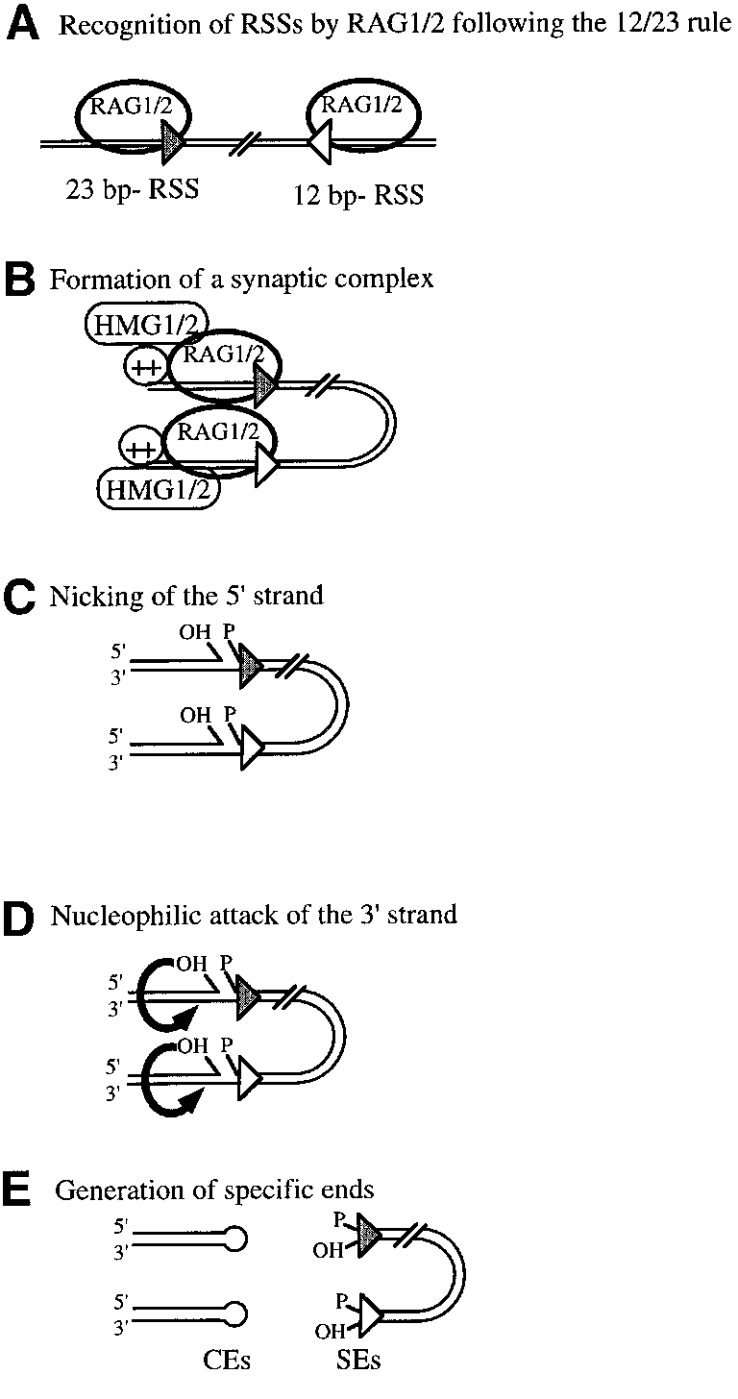

Fig. 3. The V(D)J recombination cleavage reaction. **(A)** RAG proteins (represented by ovals) recognize and bind to RSSs. The stoichiometry of RAG1/2 complex binding to the RSSs is not known. One tetramer may recognize both RSSs or different RAG1/2 tetramers may recognize different RSSs. **(B)** A synaptic complex forms, containing the RSS, RAG1/2 proteins, HMG proteins, and divalent cations. **(C)** The 5′ strand of the RSS is nicked by the RAG1/2 proteins. For simplicity, the synaptic proteins are not shown. **(D)** Nucleophilic attack by the hydroxyl group breaks the phosphodiester bond of the 3′ strand, creating a hairpin loop. **(E)** Covalently closed hairpin CEs and blunt 5′-phosphorylated SEs, the intermediates of V(D)J recombination, are the result.

strated that although the sequence of the spacer is not important, the length is crucial *(4,45,124,219,222,283,292).* The phasing of the spacer on the DNA helix may correctly position the nonamer and heptamer for binding by the RAG proteins. Recombination only occurs between a coding segment flanked by a 12-bp spacer RSS and a second one flanked by a 23-bp spacer RSS, a selection know as the "12/23 rule." Site-recognition, DSB formation, and rejoining of the gene segments are influenced by both RSS and the adjacent coding-segment sequences *(4,45,52,102,106,156,193,222,232,261,264,284).* Cellular assays and cell-free reactions using naked DNA templates containing RSSs with point mutations have highlighted critical RSS residues for each of these steps *(76,222,264).* Superimposed upon the influence of RSS and coding-sequence flanks, the timing and locus specificity of V(D)J recombination in developing lymphocytes is controlled by the chromatin conformation surrounding the antigen-receptor gene clusters.

3.2. Lymphoid Specificity and Accessibility

Extrachromosomal recombination substrate (ECRS) assays have been extremely important for dissecting many aspects of the V(D)J recombination process *(102,103,152,153,163).* These specialized shuttle vectors, containing both eukaryotic and prokaryotic origins of replication, are used to examine the frequency and fidelity of V(D)J recombination under readily manipulable conditions (Fig. 1C). The eukaryotic cells of interest are transfected with ECRS plasmids and, if necessary, with *Rag1/Rag2* expression constructs. Extrachromosomal DNA is harvested 48 hours later and transformed into *Escherichia coli.* The ECRS substrates contain 12 and 23 RSSs flanking a transcription terminator lying upstream of a prokaryotic promoter driving a chloramphenicol resistance (CAM) gene. Recombination at the RSSs deletes the terminator allowing expression the CAM gene, and survival of bacteria harboring recombined ECRS following CAM selection. Depending on RSS orientation, deletional and/or inversional recombination events can be assessed, and manipulation of the RSS and flanking coding sequences can reveal details of their influence on the process. These assays have been instrumental in revealing aspects of lineage and developmental specificity in the regulation of V(D)J recombination.

V(D)J recombination at each antigen-receptor locus displays lymphoid cell-type specificity, developmental-stage specificity, and a highly regimented order of rearrangement steps for each antigen-receptor locus *(149,246,262).* Lymphoid specificity of V(D)J recombination is achieved in part by restricted expression of RAG1 and RAG2 proteins in lymphoid precursors *(200,245).* Fibroblasts transfected with *Rag1* and *Rag2* expression constructs can recombine ECRS but fail to recombine their endogenous antigen-receptor loci, indicating additional levels of control on this process *(200,245).* Using intact nuclei from T- or B-lymphoid precursors of defined maturational stage, chromatin accessibility at each locus was shown to control the imposition of DSBs at RSSs *(262).* The cell-type and developmental stage-specificity of particular loci accessible to cleavage observed in the purified nuclei were identical to those observed in these cells in vivo *(262).* It should be noted that some cross-lineage V(D)J recombination can occur, because IgH rearrangements can be found in T cells, and TCR-β rearrangements are occasionally seen in B cells. However, these rearrangements are always limited to D-J joining and are usually out of frame. Thus, the lineage specificity of IgH vs TCR-β rearrangement is imposed at the V to DJ joining step in T- and B-cell precursors.

Changes in chromatin accessibility presumably reflect higher-order changes in chromatin structure that occur during lymphocyte development to regulate which RSSs are targeted by the recombinase proteins at each developmental stage *(248,256,262).* Locus accessibility to the recombinase is likely modulated by the binding of trans-acting factors to *cis*-acting regulatory sequences, such as transcriptional promoters *(253,299)* and enhancers *(99,180).* These elements may alter chromatin accessibility and influence recruitment of the RAG proteins *(180a,248,299)* and other repair components *(99).* Transcriptional activity at the locus may regulate accessibility, given observations that germline transcription often correlates with V(D)J recombination at a given locus *(98,256).* However, germline transcription is not absolutely required for V(D)J rearrangement of some V genes in the Igμ locus *(5).* Recently, the positioning of the RSS on the nucleosome and association of the high mobility group protein 1 (HMG1) have been shown to influence locus accessibility *(135).* Other DNA binding proteins, *cis*-acting elements, and additional molecular mechanisms may be involved in regulating accessibility *(98,248,256),* continuing to make this an intriguing area of study.

3.3 Structure of RAG proteins

Much is known about the function of the RAG proteins, owing to recent success at reconstituting the V(D)J recombination reaction in vitro using purified proteins. A major technical hurdle in establishing "V(D)J in vitro" was producing large amounts of purified RAG1 and RAG2 proteins, both of which are stubbornly insoluble when expressed as full-length bacterial fusion proteins. Deletion analysis identified core regions of RAG1 and RAG2 that retained RSS recognition and cleavage activity in vitro and in ECRS assays, and had greater solubility than the full-length proteins *(46,238,254).* The RAG1 core protein includes residues 384–1008 (N-terminal deletion of approx 30% of the protein) and the RAG2 core protein includes residues 1–387 (C-terminal deletion of approx 25% of the protein; *237,238).* However, the recombination efficiency of the full-length RAG proteins exceeds that of RAG core domains in ECRS assays *(177,265)* and on intact chromosomal substrates in vivo *(233).* Additional evidence that the RAG core regions are functionally essential has come from identification of mutations affecting these regions that cause severe combined immune deficiencies in humans *(249,286).*

Numerous motifs in the RAG proteins are thought to contribute to protein-protein or protein-DNA interactions. The most extensively studied is a helix-turn-helix DNA binding motif in the RAG1 core domain, which binds to the RSS nonamer in vitro *(52,261).* Other regions of RAG1 also influence DNA binding *(72,177,239).* RAG1 contains basic amino acid motifs that mediate binding to proteins involved in nuclear import *(42,44,147,260),* as well as other domains that may be required for interactions with other proteins *(229,233).* RAG2 also has potential protein-binding motifs *(237),* and several well-characterized phosphorylation sites, some of which affect cell-cycle regulation of RAG2 *(81,164,165,260).* Interestingly, the C-terminal region of RAG2 that is missing from the core protein has been implicated in the V_H to DJ_H joining step in vivo *(126).* It has been suggested that efficient disassembly of the post-cleavage complex and locus targeting require functions located outside of the RAG core domains *(195,265).* Further analysis of the full-length RAG proteins is needed to understand the protein partners and DNA interactions operating in V(D)J recombination.

3.4. RAG-Mediated DNA Cleavage

3.4.1. Recognition and Binding of the RSS

The first step of V(D)J recombination is the recognition of the RSS by the RAG proteins and formation of the synaptic complex (Fig. 3). In vitro studies have defined the initial recognition and binding of RAG1 within the minor groove of the RSS nonamer *(52,193,261,267)* and interaction with additional nucleotides in the spacer *(193).* These binding interactions occur through the RAG1 helix-turn-helix DNA binding motif *(52,261).* Interactions between RAG1 and the heptamer *(52,106,222,261,267)* and the coding flank appear to stabilize RSS-RAG interactions *(106).* Although RAG2 is absolutely required for cleavage at the RSS/coding border, direct binding of RAG2 to DNA has not been demonstrated *(3,52,193,261).* However, there is good evidence that RAG2 binding stabilizes RAG1/DNA interactions *(3,156,267).* Both RAG proteins can be isolated in a high molecular-weight complex, and the presence of RAG2 in this complex is dependent on the presence of RAG1 *(147).* The regions of RAG1 required to interact with RAG2 have been defined *(179),* and it has been demonstrated that they exist as a mixed tetramer with two of each molecule in solution and bound to DNA *(8a).* Although much remains to be discovered about these interactions, it is clear that both proteins are required for RSS binding and for formation of the synaptic complex.

3.4.2. Formation of the Synaptic Complex

It has long been postulated that preceding the recombination of two coding elements, their RSSs are physically apposed in a synaptic complex. This notion has been validated by recent studies in cell-free systems (Fig. 3B). This DNA-protein complex includes 12-bp and 23-bp spacer RSSs, the RAG1 and RAG2 proteins, a divalent cation, and likely HMG1 and HMG2, which may structurally distort or bend the DNA at the 23-bp spacer *(57,76,82,105,106,124,156,176,180,244,281,283,295).* The structural distortion introduced by HMG1/2 may potentiate RAG binding and recognition of the 23-bp spacer, permit the RAG proteins to distinguish 12-bp from 23-bp spacers, promote interactions with other proteins *(244),* and/or partially unwind the DNA revealing single-stranded DNA regions *(124).* Participation of numerous additional proteins in the process is likely *(244).* Assembly of this synaptic complex occurs before *(124,156,267)* and persists throughout cleavage, perhaps to tether the DNA ends *(2,57,82,105).*

3.4.3. DNA Cleavage Reaction

After assembly of the synaptic complex, RAG1 and RAG2 cleave at the RSS heptamer/coding sequence border in two successive steps. Cleavage yields four DNA ends: two hairpin-terminated CEs and two blunt SEs (Fig. 3C–E; *176).* Extensive in vitro analyses have defined essential roles for both RAG1 and RAG2 during cleavage *(3,57,106,156,176,200,282).* First, the RAG proteins act as an endonuclease to nick one strand of DNA at the boundary between the coding sequence and the 5′ end of the RSS heptamer, yielding a 3′-hydroxyl group. In the second step, this 3′-hydroxyl group attacks the phosphodiester bond of the complementary DNA strand through trans-esterification. Under suitable in vitro conditions, cleavage at the 12-bp and 23-bp RSSs occurs almost simultaneously in a coupled reaction *(57,284),* generating a covalently closed hairpin CE and a blunt 5′-phosphorylated SE *(176,220,283).* Though recent data

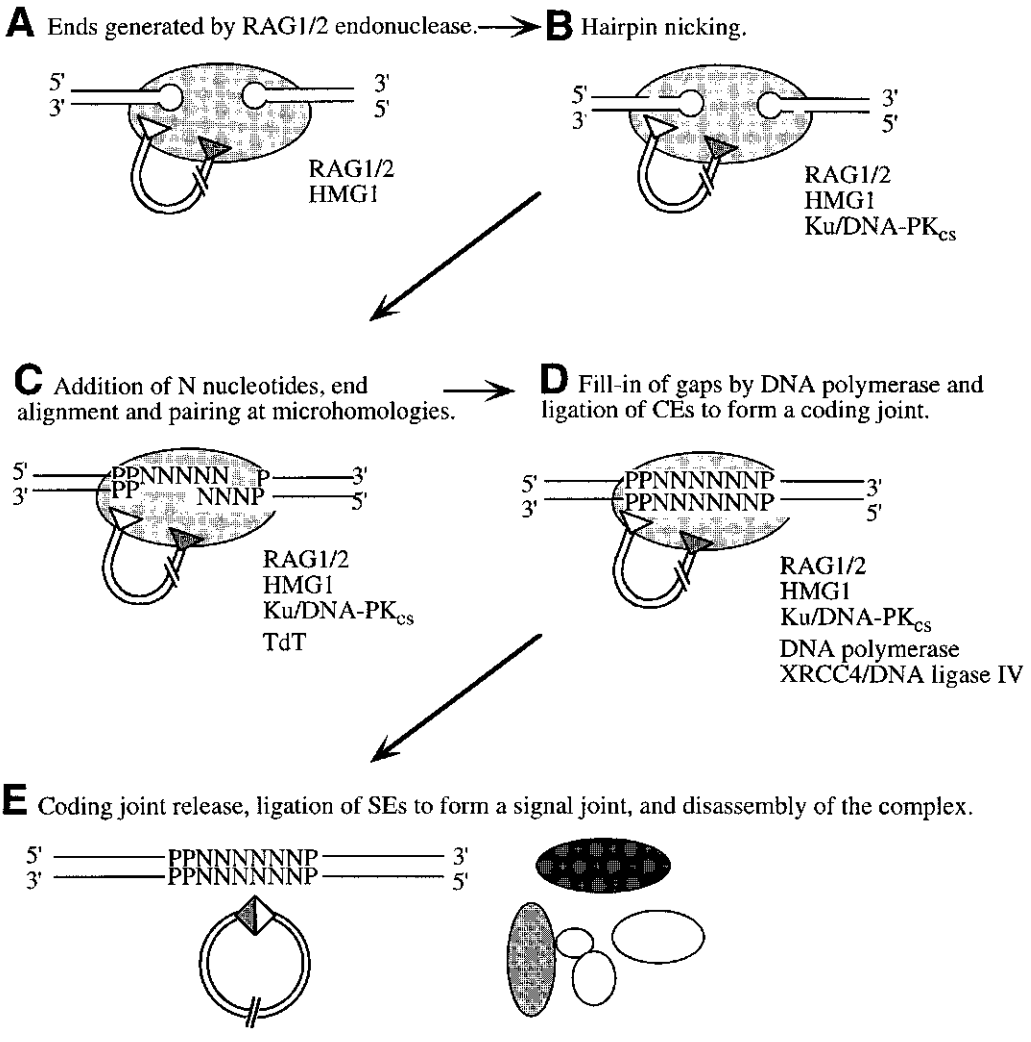

Fig. 4. A model of end-joining in V(D)J recombination. Ligation of V(D)J ends into coding and signal joints is a carefully regulated multistep process involving many proteins. The gray oval represents a multiprotein complex involved in V(D)J joining. **(A)** Ends generated by the RAG1/2 endonuclease are held together in a postcleavage complex. **(B)** The recruitment of "joining factors" facilitates hairpin nicking and opening. This process may be carried out by RAG itself. **(C)** Once opened, CEs are processed with the addition of N nucleotides by TdT, followed by end-alignment, and pairing at microhomologies. **(D)** Prior to ligation by DNA ligase IV, gaps must by filled in by a DNA polymerase. **(E)** Release of the coding joint may precede signal-joint formation, which is quickly followed by the disassembly of the complex. Spatial relationships are neither known nor implied by this figure. *See* Subheading 6. for further details.

implicate the formation of a synaptic complex in vivo, under physiological conditions low levels of single RSS cleavage can be seen at nonconsensus RSSs, suggesting that the 12/23 rule can sometimes be violated in vivo *(264).* This observation, coupled with the suggestion that the genome may be littered with nonconsensus, or "cryptic," RSSs *(151),* implies that proper regulation of the cleavage reaction may be crucial for limiting oncogenic translocations in lymphoid precursors.

3.4.4. Additional Roles for RAG Proteins

In vitro reactions using RAG-supplemented cell extracts demonstrate persistent association of RAG proteins with CEs and SEs following coupled cleavage. This complex contains additional proteins believed to be involved in the joining reaction *(2,106).* Many details of this complex remain to be defined, for example whether a single complex contains CEs and SEs, and how the(se) complex(es) is (are) remodeled over time. The importance of a stable post-cleavage complex is emphasized by observation that coding joint formation is facilitated when the cleavage and joining reactions are temporally linked *(146).* One role for RAG proteins in the post-cleavage complex may be the opening of hairpin-coding ends *(13).* Other potential roles of RAG proteins include the retention of DNA ends in close proximity to facilitate ligation, the protection of DNA termini from nucleases, and/or the regulation of subsequent steps of V(D)J recombination *(82,105,223).* For example, the post-cleavage complex may recruit DNA repair or signaling proteins *(45).* There is evidence for direct involvement of RAG proteins in facilitating the joining of coding and signal ends by other DNA repair proteins *(41,265,294).*

Recent studies support the idea that *Rag* genes may have evolved from an ancient transposase. Multiple mechanistic similarities between V(D)J recombination and transposition have been identified. In both reactions, a recombinase recognizes sequences that flank the mobile element, introduces a DSB which dissociates the signal sequences from the transposed element, and then cellular DNA repair machinery is co-opted to rejoin the DSB *(234).* Furthermore, the two *Rag* genes encoding the recombinase are closely linked, and found only in vertebrates in which antigen-receptor gene rearrangements generate antigen-specific immunity *(154).* This genomic arrangement and coincident evolutionary introduction of *Rag* genes and RSS elements suggest that both may have been introduced together by an ancestral transposon *(1,107,154,234,274,283).* Recent in vitro evidence shows that RAG proteins can act as a transposase, mediating cleavage and insertion of SEs into a target site *(1,107).* A molecular signature of retroviral integrases is left behind: short duplications of the target DNA immediately flanking the mobilized fragment *(1,107).* Currently there is no evidence that the V(D)J reaction causes integration of one piece of DNA into another in cells *(1,107).* The excitement generated by the in vitro evidence of transposase-like behavior ensures intensive future efforts to discover whether RAG proteins can catalyze other DNA transactions in lymphocytes. RAG-mediated transposition is a possible mechanism by which an antigen receptor locus could be misdirected to an unrelated genomic region, a frequent characteristic of chromosomal translocations isolated from lymphoid malignancies. Thus, the same mechanism that may have established the capacity for antigen-specific immunity in early vertebrates may have nefarious potential to compromise genomic stability.

4. GETTING IT TOGETHER: THE V(D)J JOINING REACTION

Following RAG1/2-mediated cleavage at RSS, two types of ends must be rejoined, blunt SEs and hairpin CEs. SEs are ligated directly without the addition or deletion of any nucleotides. CEs are processed, both to open the hairpin and to add or delete nucleotides, before ligation. This nonconservative end-joining process is similar to the process of nonhomologous end joining (NHEJ). NHEJ is a major DSB repair pathway in mammalian cells and functions to a lesser extent in yeast *(114).* V(D)J recombination

is a unique form of NHEJ because nontemplated nucleotides can be added at the coding joints, creating junctional diversity that vastly increases the antigen-receptor repertoire.

4.1 Coding End Processing and Joining

The first step in CE processing is the opening of the hairpin ends produced by RAG1/2-mediated cleavage. The endonuclease activity responsible for hairpin opening has not been identified. However, recent work has shown that RAG1/2 can nick hairpins in vitro as part of a post-cleavage complex *(13).* Hairpin opening is dependent on the DNA-dependent protein kinase (DNA-PK; Subheading 4.1.2.) because hairpin ends accumulate in lymphoid cells of both SCID *(73,235)* and *Ku80*$^{-/-}$ mice *(309).* However, SCID cells can nick and ligate transfected hairpin substrates, suggesting that the putative role of DNA-PK in hairpin opening differs for extrachromosomal and chromosomal substrates *(150).*

Addition of nontemplated nucleotides (N-regions) at coding joints is catalyzed by the lymphoid-specific terminal deoxynucleotidyl transferase (TdT) prior to coding joint ligation *(78,132).* Although TdT contributes to the generation of diversity, it is dispensable for V(D)J joining itself *(76,82).* Recent work has shown that nucleotide addition and loss at the CE may be influenced by sequence motifs within the coding flanks *(192).* Removal of nucleotides is not completely random *(247)* and may result from the nicking on both sides of the hairpin *(192).* The addition of self-complimentary or palindromic (P) nucleotides results from the asymmetric opening of hairpin CEs. Before joining, CEs may be aligned by short regions of microhomology *(149).* One could imagine a dynamic equilibrium at the CE, with TdT activity lengthening the DNA ends and an exonuclease shortening the ends. Because microhomology-mediated recombination may involve as little as a single bp match *(205),* TdT activity could increase the probability of creating microhomology. Thus, N nucleotide addition may function both to increase diversity of variable regions and to facilitate V(D)J joining.

Identification of the proteins involved in SE and CE joining during V(D)J recombination was propelled by studies of X-ray cross complementation mutants *XRCC4 – XRCC7,* cell lines bearing DNA repair-deficiencies that were subsequently shown to have defects in V(D)J recombination (Table 1). Several ubiquitously expressed genes responsible for the radiosensitivity and V(D)J defects have been identified. They are the DNA-PK (XRCC7), the autoantigens Ku70 and Ku80 (XRCC5), and XRCC4, which interacts with DNA ligase IV the enzyme that joins coding and signal ends *(43,85).*

4.1.1. Biochemical Characterization of DNA-PK$_{cs}$

DNA-PK$_{cs}$ was recognized as a kinase activated by free double-strand DNA (dsDNA) ends in human cell extracts *(29,144).* Initial biochemical purification revealed that this kinase activity co-purified with three components: a heterodimer of Ku70/Ku80 and the catalytic subunit called DNA-PK$_{cs}$ *(34).* DNA-PK$_{cs}$ activity was identified in a wide range of organisms, including mouse, hamster, *Xenopus,* and *Drosophila (64),* and homologs have now been cloned from humans *(19,39,94,214),* mice *(6,68),* and horses *(252).* Partial cDNAs have also been isolated from hamster *(19)* and *Xenopus (136).* DNA-PK$_{cs}$ belongs to a family of serine-threonine kinases involved in DNA damage surveillance and repair that are related to phosphatidyl inositol-3-kinase (PI3-kinase; *94).*

Table 1
V(D)J Recombination on ECRS Assays in DSB Repair Mutants

		Signal joints		Coding joints		
Species	Mutant/parent cell line	Relative frequency[a]	Fidelity %[b]	Relative frequency[a]	Mutation	References
Mouse	SX9/SR-1	0.10	12	0.03	DNA-PK Leu_{3191}→Pro	*(69)*
Mouse	SCID	0.1~1.0	50~80	<0.01	DNA-PK Tyr_{4046}→Stop	*(6,17,19,48,162)*
Mouse	*DNA-PK*$^{N/N}$ ES line	0.29~0.86	100	0.004~0.02	DNA-PK knockout	*(73)*
Hamster	V3/AA8.4	0.22~0.54	70	0.03	DNA-PK, not identified	*(19,215,269)*
Hamster	*irs*-20/CHO-10B2	0.12	80	0.03	DNA-PK, Glu_{4120}→Lys	*(215)*
Hamster	XR-C1/CHO9	0.01~0.03	n.d.	0.01~0.44	DNA-PK, not identified	*(60)*
Horse	SCID	n.d.	n.d.	n.d.	DNA-PK, 5-bp deletion frame shift	*(252,300)*
Human	MO59J/MO59K[c]	1.0	95	<0.001	DNA-PK, not identified	*(133,145)*
Hamster	*xrs-6*/CHO-K1	0.05	0	0.02	*Ku80,* 13-bp insertion frame shift	*(188,255,270)*
Hamster	XR-V15B/V79	<0.01	n.d.	<0.02	KU80, deletion codons 317–417	*(61)*
Mouse	*Ku70*$^{-/-}$ KO cell line[d]	0.11	0	<0.004	*Ku70* knockout	*(86)*

[a] As compared to parental or wild-type cell lines.
[b] Percentage of signal joints formed that could be cut with ApaL 1.
[c] MO59J/MO59K are independent cell lines derived from a human glioblastoma. MO59J has no detectable DNA-PK activity, whereas MO59K does.
[d] Mouse-embryo fibroblasts with *Ku70* targeted disruption.

DNA-PK$_{cs}$ is activated by DNA in the absence of Ku, but Ku increases the selectivity of DNA-PK$_{cs}$ for dsDNA ends and stabilizes DNA binding by DNA-PK$_{cs}$ *(90,296,305).* This stabilization increases DNA-PK activity *(90).* These data led to a model in which Ku binds dsDNA ends and recruits DNA-PK$_{cs}$ to these sites. Ku then translocates along the DNA allowing DNA-PK$_{cs}$ to contact the dsDNA end, thereby activating the kinase. Blocking Ku translocation causes Ku and DNA-PK$_{cs}$ to remain in an enzymatically nonproductive complex *(90,296).* This model accounts for the inability of DNA nicks, hairpins, and cisplatin-damaged DNA to activate DNA-PK: even though Ku can bind to these discontinuities, it fails to expose dsDNA ends to recruit and/or activate DNA-PK$_{cs}$ *(90).*

4.1.2. On the SCIDs: DNA-PK$_{cs}$ Mutants

The expression of *DNA-PK$_{cs}$* rescues the radiation sensitivity and V(D)J recombination defects of SCID and hamster *XRCC7* mutant cell lines proving the genetic identity of these defects *(127,134,294,305).* The SCID mutation was identified as a nonsense mutation at codon 4046, resulting in the deletion of the C-terminal 83 amino acids *(6,19,48),* a region highly conserved among proteins involved in DNA damage surveillance *(48).* Although the murine SCID phenotype proved to be as radiosensitive as mutants defective in either Ku subunit or XRCC4, the V(D)J phenotype affected primarily coding joint formation (Table 1). In contrast, the equine SCID and targeted *Ku$^{-/-}$* mutations severely affected both coding- and signal-joint formation *(300).* Both murine and equine SCID cell lines were shown to lack detectable DNA-PK activity, while retaining normal levels of Ku DNA end-binding activity by direct measure *(300)* or complementation *(48,127,271).* The phenotypic discrepancy between murine and equine SCID and between murine SCID and Ku-deficient cells led several groups to hypothesize that the murine SCID DNA-PK retained some residual activity allowing the generation of near-normal signal joints.

This hypothesis was supported by the characterization of three additional mutant cell lines (Table 1). The murine cell line SX9 and the hamster cell line *irs-20* were found to contain *DNA-PK$_{cs}$* point mutations. These cell lines, along with another hamster cell line defective in DNA-PK$_{cs}$, XR-C1, had profound defects in both signal- and coding-joint formation. Given this evidence, it was suggested that the SCID DNA-PK$_{cs}$ mutation allowed the production of V(D)J signal joints and rare coding joints, the latter of which explains the "leaky" development of low numbers of mature T and B cells in aged SCID mice *(22,49).* However, human DNA-PK$_{cs}$-deficient cell lines could not mediate coding-joint formation on ECRS, despite apparently normal signal-joint formation *(133).* One interpretation of these seemingly paradoxical data was that the defective signal-joint formation in SX9 and *irs-20* cells resulted from a dominant negative effect of mutant DNA-PK$_{cs}$. If this is true, this dominant negative effect must be reversible by wild-type DNA-PK$_{cs}$ because both radiosensitivity and V(D)J recombination phenotypes were complemented by somatic-cell fusions with wild-type cells or by transfection of wild-type *DNA-PK$_{cs}$ (69,127,215,269,294).*

This debate can now be resolved, at least for mice. Gao et al. *(73)* generated a targeted knockout of the murine *DNA-PK$_{cs}$* gene by insertion of a *neo^{r}* cassette in exon 6, antisense to DNA-PK$_{cs}$. These mice, *DNA-PK$^{N/N}$*, have V(D)J recombination and lymphocyte development defects very much like that of SCID mice. In contrast, a mouse with a deletion of the DNA-PK$_{cs}$ kinase domain *(DNA-PK$^{-/-}$)* has a significant population of CD4+ CD8+ T cells *(268).* In *DNA-PK$^{N/N}$* embryonic stem (ES) cells, normal,

precise signal joints are detected on ECRS under conditions where coding-joint formation is almost completely abrogated. Similarly, normal TCR-δ signal joints were detected in the *DNA-PK*$^{-/-}$ mouse, although some D-J_H coding joints were detected *(268).* These differences may be owing to the different knockout constructs used. Analysis of the products of RAG1/2-mediated cleavage in SCID, *DNA-PK* $^{N/N}$, and *DNA-PK*$^{-/-}$ thymocytes showed that although SEs are normal *(73,268),* CEs accumulate as hairpin structures *(73,235).* Taken together, these data are consistent with a role for DNA-PK in the formation of coding, but not signal, joints.

Although both *DNA-PK*$^{N/N}$ and *DNA-PK* $^{-/-}$ mouse embryo fibroblasts are radiosensitive *(73,268),* the *DNA-PK*$^{N/N}$ ES cells were not *(73),* suggesting the presence of a DNA-PK-independent DSB-repair pathway that is not present in more differentiated cells. Similarly, cell fusions made between two SCID-like cell lines, $SCGR_{II}$xV3 or V3xV3, still manifest defective coding-joint formation, but are partially complemented for radiosensitivity *(269),* again invoking a DNA-PK-independent DSB-repair pathway. Perhaps this pathway involves homologous recombination such that in tetraploid fused cells the extra homologous templates facilitate this usually inefficient mode of DSB repair. It has also been shown that homologous recombination is stimulated by DSBs and may be more active in ES cells than in more differentiated cells *(55,158,228,259).*

4.1.3. Biochemical Characterization of Ku

Ku was initially described as a heterodimer of 70 kDa and 80 kDa protein subunits with DNA end-binding activity. Ku is able to translocate to internal sites on the DNA in a sequence-and ATP-independent manner *(50,307).* DNA-PK activity may regulate this translocation during end joining *(25).* DNA-dependent ATPase and nucleotide-dependent helicase activities for Ku have also been described *(27,279).* As with DNA-PK_{cs}, understanding of the role played by Ku in DSB repair came from studies of mutant cell lines *(224).* Mutations in *Ku80* were shown to be responsible for both the radiosensitivity and V(D)J recombination deficiency of the *XRCC5* complementation group *(59,61,188,255).* Although Ku alone does not have any kinase activity *(90),* it is a DNA-PK substrate in vitro *(33).* Phosphorylation of Ku and autophosphorylation of DNA-PK_{cs} in vitro causes the dissociation of the DNA-PK complex from dsDNA ends and cessation of DNA-PK kinase activity, although the in vivo significance of these phosphorylation events is unclear *(33).* Neither the ATPase nor the helicase functions of Ku are essential for DSB repair because site-directed mutagenesis of key residues in these motifs does not affect the ability of Ku80 to rescue *xrs-6* radiosensitivity. Furthermore, mutagenesis of some of the serine residues phosphorylated by DNA-PK also does not affect Ku80 rescue of *xrs-6* radiosensitivity *(255).* Several studies have demonstrated that DNA-PK_{cs} and Ku can simultaneously bind and bridge two DNA molecules *(30,203,221,305).* In addition, Ku transferred from one DNA molecule to another when the DNA fragments had complementary ends as short as 4 bp in length *(18),* and could activate DNA ligase activity under some conditions *(221).* These data are consistent with a model where Ku, DNA-PK_{cs} and/or DNA-PK function as scaffolding components during DSB repair.

Further evidence for DNA-PK_{cs} and Ku function in V(D)J recombination comes from studies of in vitro V(D)J recombination reactions. Cortes and her colleagues recapitulated signal- and coding-joint formation in separate reactions *(41,294).* In vitro sig-

nal-joint formation required the presence of core RAG1/2 proteins and cell nuclear extracts. These signal joints were precise and the dependence on nuclear extracts could be relieved by addition of T4 DNA ligase *(41,223).* Immunodepletion of the nuclear extracts with anti-Ku antibodies abolished signal joint formation, confirming the importance of Ku in this process. Coding joint formation required RAG1/2 and a fraction of HeLa cell nuclear extract that contained Ku70, Ku80, DNA-PK_{cs}, and a ligase *(294).* Furthermore, immunodepletion of extract with anti-DNA-PK_{cs} antibodies abolished coding-joint formation, while immunodepletion using anti-Ku antibodies only decreased coding-joint formation by 50%. These data are consistent with a primary function of Ku in chromatin remodeling at CEs, service as a SE bridge, or in stabilizing DNA-PK activity at the post-cleavage complex. If Ku-independent DNA-PK_{cs} activity is sufficient to mediate coding-joint formation, in vitro coding-joint formation would not be completely abrogated by immunodepletion of Ku. Therefore, although Ku, DNA-PK_{cs} and ligase activities are all essential for V(D)J recombination, Ku probably has functions in V(D)J recombination independent of its role in DNA-PK activation. It seems likely that future in vivo studies will reveal additional players recessed from the ends of DNA, perhaps involved in chromatin accessibility and remodeling.

4.1.4. You Don't Know What You've Got 'Til It's Gone: Ku Mutants

Cell lines with mutations in *Ku80* show defective coding- and signal-joint formation (Table 1), unlike the murine SCID defect, which primarily affects coding-joint formation. Studies using endonuclease-generated DSB in *xrs-6* cells showed that Ku was needed for the NHEJ of chromosomal DNA ends but not homologous recombination-mediated repair *(159).* Ku function is specific to the joining phase of V(D)J recombination, and is not required to protect SEs from nuclease degradation *(91).*

The generation of Ku80- and Ku70-deficient mice has allowed for a careful comparison of these phenotypes with SCID mice. In dramatic contrast to DNA-PK_{cs} mutations, *Ku80*$^{-/-}$ and *Ku70*$^{-/-}$ mice are proportional dwarfs as well as being lymphocyte-deficient *(86,197,202,309).* Expression of both Ku proteins is compromised in either single mutant, consistent with previous findings that co-expression is essential for the stability of each protein *(37,59,61,86,202,255).* In both types of Ku-deficient mice, B-cell development is arrested at the pro-B cell stage, probably because the rearrangement of Ig genes is severely compromised *(86,197,202,309).* SEs and CEs are full length in immature Ku$^{-/-}$ lymphocytes, and when present, V(D)J joints are reminiscent of those seen in SCID mice *(86,309).*

Although the stunted growth, radiosensitivity, and B-cell development phenotypes are very similar between *Ku70* and *Ku80* knockout mice, T-cell development shows some surprising differences, possibly indicative of subunit-specific Ku functions. In *Ku80*$^{-/-}$ mice, >90% of thymocytes are arrested at the CD4- CD8- DN stage and successful TCR rearrangement is rare *(197,309). Ku70*$^{-/-}$ mice, however, have a significant population of CD4+ CD8– and CD4– CD8+ TCR-β+ T cells in the thymus and secondary lymphoid organs *(86,202).* Further, *Ku70*$^{-/-}$ mice die of either intestinal neuronal disorders or of T-cell lymphomas, often of a CD4+ CD8+ TCR-β+ phenotype *(155).* Primary mouse *Ku70*$^{-/-}$ fibroblasts show multiple hallmarks of genomic instability: increased rate of sister chromatid exchange, radiosensitivity, increased rate of in vitro transformation, and 100% tumor growth in adoptive transfers to nude mice *(155).*

The authors suggested that Ku70 is a tumor suppressor in T cells, and proposed that the induction of a secondary DSB-repair pathway rescues thymocyte development in *Ku70*$^{-/-}$ mice. In the context of an inherent DSB-repair defect, developing *Ku70*$^{-/-}$ thymocytes are susceptible to oncogenic transformation. At present it is difficult to harmonize the phenotypic outcomes of mutations in DNA-PK, Ku80, and Ku70 with a simple model of their functions in V(D)J recombination. Rather, it will likely emerge that these proteins subserve both cooperative and independent functions in DSB repair, DNA replication, and transcription.

4.1.5. Biochemical Characterization of XRCC4 and DNA Ligase IV

XRCC4 was identified by complementation of the radiosensitive and V(D)J recombination-defective phenotypes of XR-1 cells by a human cDNA. However, sequence analysis of XRCC4 revealed no similarity to known proteins *(157).* The XRCC4 mutation in XR-1 cells reduces coding- and signal-joint formation in ECRS assays by at least 100-fold, with the rare coding joints formed resembling microhomology-directed events seen in SCID cells *(92,157,271).* At a biochemical level, XRCC4 is a disulfide-linked homodimer or multimer that can complex with DNA, Ku, and DNA-PK in vitro *(141).* DNA-PK phosphorylation of XRCC4 in vitro can abrogate XRCC4 DNA binding *(189).* Although XRCC4 is an effective substrate for DNA-PK and other kinases *(43,141),* the phosphorylation sites are dispensable for DNA repair, and may not be physiologically relevant in this context *(141).*

DNA ligase IV was identified as an XRCC4-interacting protein by virtue of both co-purification and co-immunoprecipitation *(43,83).* XRCC4 was shown to be a potent activator of DNA ligase IV activity both in transfected cell lines and in vitro *(83,189)* and stabilizes DNA ligase IV protein levels in XR-1 cell lines *(24).* Further, only those XRCC4 deletion mutants that interact with and activate DNA ligase IV rescue V(D)J recombination in *XRCC4* mutant cells *(85).* In contrast, V(D)J recombination is normal in cells deficient in DNA ligase I and DNA ligase II/III *(109,211,276).* These data led to the hypothesis that DNA ligase IV, in association with its activator, XRCC4, is the ligase used in V(D)J recombination. This hypothesis has been confirmed in both knockout cell lines and mice (Subheading 4.1.6.)

4.1.6. Unexpected Links between Immune and Nervous Systems: XRCC4 and DNA Ligase IV Mutants

V(D)J recombination and DSB repair are abolished in a human pre-B cell line disrupted at both alleles of DNA ligase IV *(84)* and in cells from DNA ligase IV knockout mice (*LigIV*$^{-/-}$; *65).* Rescue of V(D)J recombination in these mutant cells can be effected by DNA ligase IV, but not by DNA ligases I or II/III *(84).* Interactions between DNA ligase IV and XRCC4 are crucial, as mutations that abrogate their contact blocked this rescue *(84).* Examination of fetal lymphoid tissues in *LigIV*$^{-/-}$ or *XRCC4*$^{-/-}$ mice revealed an arrest of T- and B-lymphocyte development at the pro-T and pro-B cell stages (Fig. 2; *65,74).* Overall lymphoid-precursor cell number is decreased, perhaps owing to death of progenitors that initiate, but cannot complete, V(D)J recombination. The rare coding joints detected in *XRCC4*$^{-/-}$ mice have large deletions reminiscent of those seen in SCID mice *(74).* Taken together, these data demonstrate that DNA ligase IV, in association with XRCC4, is essential for both V(D)J recombination and DSB repair, but do not rule out the possibility that other ligases might also be involved *(65).*

Interestingly, *LigIV*$^{-/-}$ and *XRCC4*$^{-/-}$ knockouts cause early embryonic lethality. Histological examination of the embryos reveals a severe defect in neurological development resulting from increased cell death *(74).* These observations led to the examination of neuronal development in other NHEJ mutant mice. Gu et al. demonstrated increased neuronal apoptosis in *Ku70*$^{-/-}$ and *Ku80*$^{-/-}$ embryos, but not in *DNA-PK*$^{-/-}$ embryos *(86a).* In all cases examined, the onset and peak of neuronal apoptosis correlates with neuronal development. Furthermore, the degree of neuronal apoptosis is inversely related to the degree of leakiness in signal joint formation in ECRS assays in mutant MEFs. While the roles of these proteins in neurological development are unknown, it is intriguing that these phenotypes provide another link between neurological defects and DSB repair. Additionally, 25% of *Ku70*$^{-/-}$ knockout mice die as a result of intestinal neuron degeneration *(155).* Patients with mutations in the DNA damage surveillance gene, ataxia telangiectasia mutated (*atm;* Section 8.4.), or with mutations in p95, a component of the Mre11/Rad50 complex (*See* Section 5.), also show neurological defects. These observations are suggestive of an as yet undefined, but essential, role for DNA repair in neurological development.

Two alternate, though not exclusive, hypotheses have been proposed for the role of DNA repair in neurological development *(37a,73a,86a,143a).* The first invokes targeted DNA rearrangements as an essential part of neuronal development, perhaps responsible for the generation of neuronal diversity. The second suggests that developing neurons are exquisitely sensitive to DNA damage and undergo apoptosis as a consequence to perturbations in the mechanisms of DNA repair. The source of DNA damage could arise from site-specific cleavage during development or be the result of normal cellular processes, such as replication or oxidative metabolism. While searches for a potential target of neuronal DNA rearrangement have not yet yielded fruit (*86a,* unpublished data), recent genetic evidence suggests that neuronal lethality is the result of the cellular response to unrepaired DNA as opposed to defective NHEJ per se *(73a).* The lethality of *XRCC4*$^{-/-}$ and *LigIV*$^{-/-}$ can be rescued by mutations in the DNA damage checkpoint involving ATM and p53 (Sections 8.3. and 8.4.). Neither *ATM*$^{-/-}$ *LigIV*$^{-/-}$ *(143a)* nor *p53*$^{-/-}$ *XRCC4*$^{-/-}$ *(73a)* embryos show increased neuronal apoptosis, and mice of both genotypes survive to birth with morphologically normal CNS. However, *ATM*$^{-/-}$ *LigIV*$^{-/-}$ mice die two days postnatally, whereas *p53*$^{-/-}$ *XRCC4*$^{-/-}$ mice succumb to pro-B cell lymphoma at 6 weeks of age. Neither genotype rescues V(D)J recombination defects, but the *p53*$^{-/-}$ *XRCC4*$^{-/-}$ pro-B cell lymphoma have translocations between the IgH locus and the c-*myc* oncogene *(73a,143a).* The early postnatal death of ATM-deficient *LigIV*$^{-/-}$ mice suggests that other organ systems may be affected or that the CNS may not be entirely functional, despite the abrogation of postmitotic neuronal apoptosis. These data support the hypothesis that an alternative DNA repair process functions in cells with unreapired DNA damage that are not eliminated by the p53-dependent apoptotic pathway.

5. LESSONS FROM YEAST NHEJ

The studies previously discussed have revealed many similarities between V(D)J recombination and NHEJ, both with respect to the genes involved (Table 1) and the DNA joints produced *(114,150).* It has been well-established that the yeast *S. cerevisiae* repairs DSBs primarily by homologous recombination, involving members of the

RAD52 epistasis group (reviewed in *114,118*) and is discussed further in Chapter 16 in Vol. I. However, the identification of the yeast Ku homologs, *HDF1* (Ku70;)*(62)* and *HDF2* (Ku80; *186)* revealed a second repair pathway with striking parallels to mammalian NHEJ (reviewed in *277*). The information garnered from studies with yeast provide us with a road map for the exploration of the proteins that catalyze NHEJ and by analogy, the joining of V, D, and J ends during antigen receptor gene rearrangement.

In addition to NHEJ, Ku has multiple functions in genome maintenance in yeast. These include essential roles in telomere silencing, length maintenance, structure and subnuclear organization *(23,80,137,196).* Other genes involved in chromatin silencing and telomere-length maintenance, (*MRE11, RAD50,* and *XRS2*), also function in Ku-dependent NHEJ, possibly as an exonuclease *(23,196,278),* although a recent report suggests nuclease-independent functions in NHEJ *(190).* Human MRE11 and RAD50 homologues have been identified *(53,212).* p95 is encoded by the gene mutated in Nijmegen breakage syndrome (NBS), a human disorder characterized by mental retardation, cellular radiosensitivity, chromosomal instability, early-onset lymphoma, agammaglobulinaemia, and mild to moderate lymphopenia *(116,280).* Detection of p95 as the NBS protein relied in part on the complex formed with human RAD50 and MRE11 in vivo *(28,175,285).* This complex can unwind dsDNA and cleave hairpin DNA *(206).* The genetic tools available for yeast have been powerful in elucidating the roles of shared players in NHEJ in yeast and mammals. It is important to note that although sequence and function are conserved among some proteins (e.g., Ku70/80) involved in NHEJ across species, function is not always conserved (e.g., *MRE11,* by noncomplementation), and sequence is not conserved among others (e.g., *XRS2,* p95/NBS1). Moreover, no member of the *RAD53/MEC1*/DNA-PK_{cs} PI3-kinase family has been implicated in yeast NHEJ beyond the DNA damage detection stage. This pattern of shared and distinct proteins is consistent with unique participants in NHEJ in response to pathological vs physiological DNA damage, and for species-specific components.

6. TOWARDS A MODEL OF V(D)J JOINING

Although far from complete, the data support an emerging model for DNA-PK, Ku70/80, XRCC4, and DNA ligase IV function in V(D)J recombination. An early step may be Ku recruitment of DNA-PK to SEs at the post-cleavage complex (Fig. 4B). SEs could be the major activators of DNA-PK because hairpin-ended DNA does not appear to activate DNA-PK activity in vitro *(258).* Translocation of Ku along the DNA may then activate DNA-PK. The accumulation of hairpin ends in SCID and DNA-$PK^{N/N}$ mice *(73,235)* suggests that DNA-PK function is required for hairpin nicking in this chromosomal context. DNA-PK may enable RAG1/2 to nick the hairpins, remodel the post-cleavage complex, allow access of TdT and other joining factors, and/or facilitate CE release from the post-cleavage complex. Although the model in Fig. 4 shows coding-joint formation occurring within the post-cleavage complex, there is no direct evidence that this occurs. By analogy to Ku function at yeast telomeres, Ku could remodel chromatin to expose the CE for processing, and/or to align the ends for joining (Fig. 4C). Subsequent to hairpin opening, CE processing occurs. A dynamic equilibrium between the action of TdT and exonuclease activity may exist to produce short regions of homology at CEs facilitating pairing prior to coding-joint formation. A DNA polymerase would fill in any gaps, perhaps by an error-prone mechanism to increase diver-

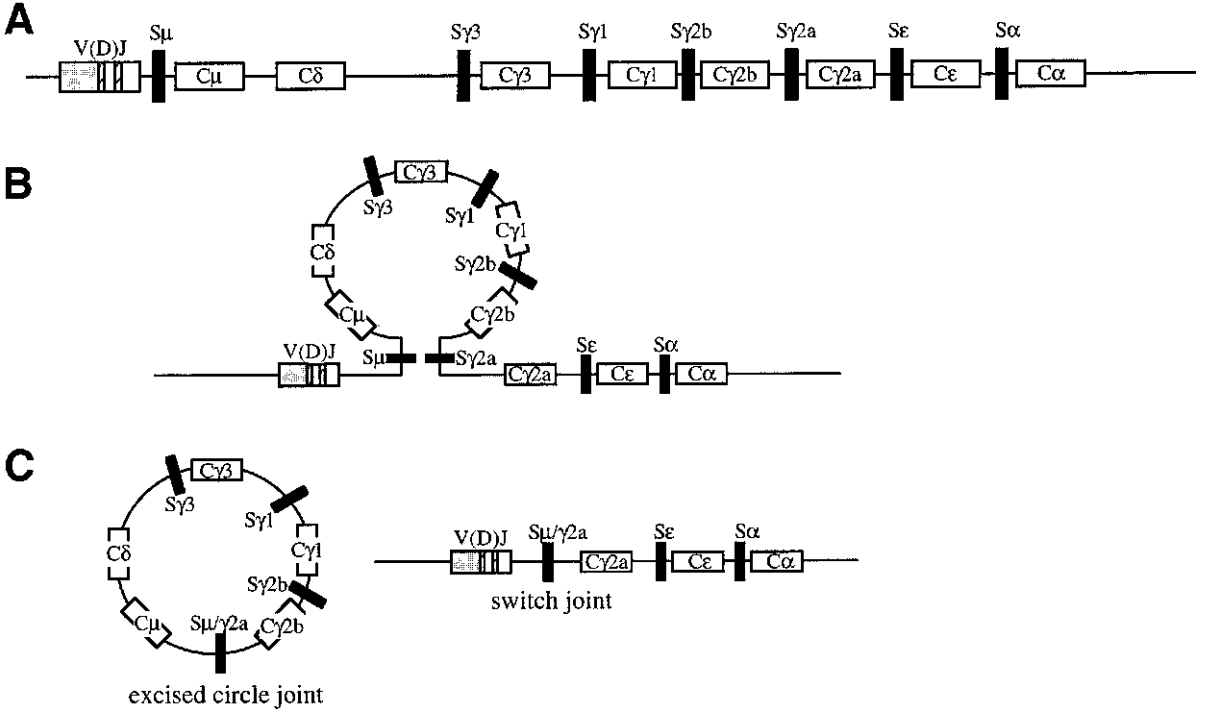

Fig. 5. CSR at the mouse heavy-chain locus. **(A)** S regions are present 5′ of each C_H gene, except Cδ. **(B)** Switch recombination is a multistep process. It likely involves the synapsis of the participating S regions followed by the introduction of DSBs in each S region. **(C)** Subsequent DNA repair juxtaposes the heavy-chain constant region of the switched gene and the V(D)J gene region at the switch joint. The intervening DNA is deleted from the chromosome as a circle. Drawing is not to scale and not all V, D, and J segments, or constant region exons, are shown.

sity *(111).* The large size of DNA-PK, the majority of which has no known function, makes it an attractive scaffolding protein. XRCC4 has been shown to interact with DNA-PK and Ku on DNA *(141),* so XRCC4 might recruit and activate DNA ligase IV to ligate CEs (Fig. 4D).

Although it is clear that DNA-PK function is not essential for signal-joint formation, studies of both DNA-PK mutant cells lines and mice has led to the hypothesis that DNA-PK function improves the frequency and fidelity of signal joints *(20).* DNA-PK function, in addition to being required for the opening of hairpin CEs, may facilitate signal-joint formation by recruitment of XRCC4/DNA ligase IV to the SE. DNA-PK autophosphorylation after end-joining may trigger disassembly of DNA-PK/Ku/DNA complexes *(33)* and the entire joining complex (Fig. 4E). Viewed from this perspective, V(D)J recombination is a carefully regulated process requiring coordinated activity of several proteins converged at dsDNA ends. Regulation of these steps probably involves control of both protein function (e.g., cleavage vs hairpin nicking by RAG1/2) and access to the V(D)J ends. Although several key players have been identified, their precise roles in V(D)J recombination remain enigmatic. Over the next several years, in vivo and in vitro experiments should provide a comprehensive picture of the molecular mechanisms of V(D)J recombination.

7. MAKING A GOOD THING BETTER: *DIVERSIFICATION OF ANTIGEN SPECIFICITY AND EFFECTOR FUNCTION IN MATURE B LYMPHOCYTES*

The antigen-binding specificity of B cells is not fixed once functional IgH and IgL rearrangements are completed in B-cell progenitors. IgV_H and IgV_L regions, as well as IgC_H regions can be altered at later times in a B cell's life-span. These alterations occur by a variety of mechanisms that involve targeted (but not site-specific) introduction of DSBs, though not all are rearrangement processes. Interestingly, these diversification events take place in a specialized microenvironment, called the germinal center (GC), which develops in secondary lymphoid organs, such as spleen, lymph node, and Peyer's patches *(216).* Prior to antigenic exposure, B cells reside in the primary follicles of lymphoid organs, whereas T cells reside in a distinct area. Following immunization, activated T and B cells migrate to the perimeter of the primary follicles where they interact to induce focal B-cell proliferation to generate GCs *(75).* GCs are the primary sites for three important modifications of Ig genes: hypermutation of V genes, receptor editing by V gene replacement, and class-switch recombination.

7.1. Somatic Hypermutation and Affinity Maturation

Somatic hypermutation is a process that refines V-region sequences in a template-independent fashion *(167,195,293).* The V-gene hypermutation rate of 10^{-3}–10^{-4} bp/cell division is 5–7 orders of magnitude higher than the spontaneous mutation rate. Unlike spontaneous alterations, hypermutation is selective for the 1–2 kbp surrounding a rearranged (but not germline) V_H or V_L gene. V-gene hypermutation exhibits a bias for base transitions *(15,195),* although insertions and deletions also occur *(301).* Some data suggest that purines in the coding strand are preferentially altered, but recent data suggest both strands are hypermutable *(56,187).* Finally, these mutations occur in a stepwise manner, such that a single B cell can accumulate multiple mutations during the many cell

divisions it undergoes in the GC. Most evidence suggests that TCR V genes do not undergo hypermutation, despite one study that claimed to find hypermutated TCR Vα regions in GC-derived cells *(308).* Though clearly an intriguing observation, this study did not involve purified T cells. Given that TCR genes are sometimes rearranged in B cells, it remains possible that GC B cells were the source of the mutated TCR Vα genes *(8).* In most mammalian species, hypermutation is an antigen-driven process operative in GC B cells. However, in sheep, hypermutation is an antigen-independent component of a developmental program used to diversify the primary Ig repertoire *(293).*

Comparisons of germline (unmutated) V_H/V_L gene sequences with the highly mutated ones derived from antigen-stimulated B cells reveal a significant clustering of mutations in the complementarity-determining regions (CDRs) that are crucial to antigen contact. Although mutational hotspots in the CDR appear to be intrinsic to the hypermutation mechanism *(15,195,218,306),* CDR clustering largely reflects profound selection for high-affinity antigen binding during the GC reaction. Once B cells cease proliferating in the GC, they express their modified BCR on the surface and migrate to the GC interior. This region also contains specialized antigen-presenting cells (APCs) whose surface is decorated with antigen-antibody complexes. B cells that can recognize antigen with high-affinity survive and re-enter the circulation as antibody-secreting plasma cells or long-lived memory B cells, and the remainder die by apoptosis *(172).* Thus, somatic hypermutation generates very high-affinity ($K_d = 10^{-8}$–10^{-9}) Ig from the low to moderate affinity ($K_d = 10^{-7}$) Ig produced in the primary repertoire. Strikingly, this affinity enhancement can be effected by as few as 3 amino acid substitutions. Although V gene hypermutation can be elicited under appropriate conditions in vitro *(117),* studies of mutant mice with defects in GC formation demonstrate that the specialized GC microenvironment is critical for affinity maturation in vivo *(40,71,122,173,174).* Interestingly, a subset of patients with common variable immune deficiency was reported to have defects in somatic hypermutation, identifying this process as critical for normal immune function *(148).*

The molecular mechanism of V-gene hypermutation remains mysterious, despite a recent torrent of studies in this area. An important clue was provided by studies implicating the transcriptional machinery in this process *(167,266).* Though hypermutation does not depend on an Ig promoter *(14),* sequences subjected to hypermutation are delimited by the promoter at the 5′ end of Ig loci. Strikingly, deletion of two IgL enhancers abolishes hypermutation without impacting expression of an IgL transgene, arguing that distinct *cis*-acting elements control Ig transcription and hypermutation *(14).* An IgH enhancer regulates hypermutation of V_H in a manner distinct from its regulation of transcription *(7).* Finally, hypermutation can be targeted to the IgC region by insertion of an IgL promoter immediately upstream *(210).* These data are consistent with the involvement of a transcription-coupled DNA repair process, such as excision repair, in V-gene hypermutation. Disappointingly, however, mice and humans with mutations in the excision repair genes *XPD* or *XPB* have normal V-gene hypermutation *(113,125,289).* Inactivating mutations of the *CSA, CSB,* or *XPA* excision repair genes also failed to debilitate hypermutation *(113,125,302).* Homologous recombination and base-excision repair are also not required for V-gene hypermutation, because this process is normal in mice with mutations in RAD54 or in 3-methyladenine-DNA glycosylase *(113).*

There has been substantial interest in a potential role for mismatch repair in somatic hypermutation. The mismatch repair pathway could facilitate hypermutation by processing transient DNA secondary structures in the V region. Alternatively, it could limit the extent of hypermutation by correcting mismatch mutations. Several groups reported that hypermutation following a primary immune response is normal or only slightly diminished in mice carrying a targeted mutation of the *Msh2* or *Pms2* mismatch repair genes *(12,66,113,213,302).* However, V genes from PMS2-deficient mice had significantly more adjacent base-pair substitutions *(302),* and *Msh2* mutant mice displayed more intense focusing of the mutations to the intrinsic mutational hot spots *(218),* than wild-type mice. Thus, rather than orchestrating hypermutation, the mismatch repair pathway may qualitatively alter the spectrum of mutations after they are introduced. However, hypermutation frequencies were diminished three to fivefold when Peyer's patch B cells from unimmunized mutant mice were examined *(66,218).* Even more surprising, hypermutation frequency was reduced 10-fold in PMS2-deficient mice *(31).* However, these mice also had very limited, quasi-monoclonal IgH and IgL V genes, causing tremendous selective pressure. In addition, the microsatellite instability associated with defective mismatch repair may be exacerbated during the extensive proliferation of antigen-activated B cells in the GC, and may lead to their premature death. A reduced life span of the mutant B cells could account for the paucity of hypermutated V genes accumulating large numbers of mutations, as well as their abnormal maturational status *(66,218,288).* Based on these observations, it was suggested that in contrast to most other cells, B cells may "fix" mismatches in the genome by using the newly synthesized strand of the V gene, rather than the parental strand, as the template for mismatch repair *(31).* Validation of this idea awaits further characterization of B-cell proliferative and maturational defects in mismatch-repair mutant mice, as these may indirectly affect hypermutation frequencies.

In summary, the molecular mechanism of V-gene hypermutation remains elusive, but recent studies are proving informative. Sale and Neuberger obtained evidence that DNA strand breaks are scattered within the mutation domain *(240).* Such breaks have been postulated but have been difficult to observe because, in contrast to RAG-mediated breaks, they would not likely be site-specific. To reveal the transient presence of DNA breaks during V-gene hypermutation, this group ectopically expressed TdT, reasoning that it would add nontemplated nucleotides to accessible 3′ ssDNA or dsDNA ends. The nature (ss vs ds) of the breaks and the mechanism by which they are induced remain obscure, but the authors propose a role for a DNA polymerase. Similarly, Bertocci et al. *(12)* suggest that hypermutation involves the recruitment of an error-prone DNA polymerase that mediates short-patch DNA synthesis occurring outside of global DNA replication. Whatever the mechanism, a potential role for dysregulated V-gene hypermutation in B-cell transformation and immune deficiencies provides continued incentive to define it *(79,148,185,250).*

7.2. Receptor Editing by V-Gene Replacement

Until recently, RAG expression was thought to be permanently extinguished once immature lymphocytes expressed functional antigen receptors. It is now clear RAG expression and V(D)J recombination can be re-elicited in Ig^+ B cells (reviewed in *199*). Self-antigen binding to immature B cells with high-affinity autoreactive BCRs induces

RAG-mediated V-gene replacement, generally with nonautoreactive V genes *(36,182,207).* This process presumably prevents the apoptotic elimination of self-reactive cells by editing their receptor specificity. Similarly, secondary rearrangements of TCR-α genes occur in immature T cells that fail to be positively selected by self-MHC, presumably to give them another chance to express a useful TCR *(290).*

More recently, RAG1 and RAG2 expression has been observed in antigen-activated B cells recovered from spleens and Peyer's patch GCs, and new V(D)J rearrangements were observed in these cells *(93,104,204).* The biological function of this renewed V(D)J activity is still unclear, given that it could generate autoreactive as well as useful B-cell clones. However, in contrast to receptor editing in immature B cells, editing in peripheral B lymphocytes is inhibited by antigen receptor cross-linking *(100,101,181),* suggesting this process is triggered only in GC B lymphocytes with diminished antigen-binding affinity. Not surprisingly, only B lymphocytes expressing low-affinity BCRs are able to induce a new round of V(D)J recombination *(100),* resulting in a drive towards improved antigen-receptor affinity. The locus organization of BCR genes suggests that these improvements occur primarily at the IgL locus as simple joining of two alternate V and J regions during each round of V(D)J recombination. New V(D)J recombination at the IgH locus is impossible as the unused D segments are deleted during the V-to-DJ joining. However, V segment replacement, using an RSS-like sequence within the IgH coding region, has been documented *(35,130,226).* Mature T cells may also undergo receptor editing, but the reasons and regulation are not yet clear *(178).*

7.3. Class-Switch Recombination

Like V-gene hypermutation and some forms of receptor editing, class-switch recombination (CSR) is an antigen-dependent process that takes place in the specialized GC microenvironment. However, in this case, mature B cells change antibody-effector functions without altering antigen specificity (reviewed in *38,263*). The different antibody classes differ in their ability to fix complement, activate APCs and natural killer (NK) cells, and induce cytokine production. These functions are essential for efficient immune responses to foreign antigens and destruction of virally infected and transformed cells. Like V(D)J recombination, CSR involves the generation and repair of DSBs. Although the mechanism of CSR has not been defined, it involves intrachromosomal recombination between the switch (S) regions 5′ of the participating heavy chains (C_H; Fig. 5). S regions contain tandemly repeated segments of DNA, 1–10 kbp in length, located 5′ of each C_H gene, except Cδ. While V(D)J recombination occurs only at RSSs, class-switch events occur within the S regions of the switched partners, but without apparent sequence specificity *(263).* Switches involving Igμ can even occur outside of the Sμ *(38,142).* Another difference between V(D)J recombination and CSR is that although productive V(D)J rearrangement at IgH results in allelic exclusion, CSR occurs on both the productive and nonproductive C_H alleles *(263).*

The proteins involved in CSR have not been fully elucidated. CSR occurs ex vivo in pre-B cells from RAG2-deficient mice under appropriate stimuli, demonstrating that the RAG recombinase is not essential for CSR *(231).* Recently, a putative B-cell-specific switch recombinase activity was isolated from spleen-cell nuclear extract and identified as a multiprotein complex *(21).* One component of the complex, SWAP-70, was suggested to be a specific recruiting element, assembling the switch recombinase from universal compo-

nents, very similar to the process seen in V(D)J recombination. This study used extrachromosomal switch-recombination templates to assay switching, however the switched products were not sequenced. Confirmation of this complex as the elusive switch recombinase awaits confirmation of S-region specificity and genetic analyses of knockout mice.

Although switch recombinase activity is unique to B cells, evidence is mounting that, as with V(D)J recombination, the joining of switch regions depends on the general DNA repair components DNA-PK, Ku70, and Ku80 *(32,171,231).* CSR cannot be induced in ex vivo SCID pre-B cells, indicating an essential role for DNA-PK in CSR *(231).* Casellas et al. *(32)* used *Ku80*$^{-/-}$ mice with targeted rearranged VDJμ heavy and VJ_κ light chain transgenes to show that Ku80-deficient B cells could respond to switch signals by upregulating germline C_H transcripts and introducing DSBs into switch regions. However, these DSBs could not be resolved and neither mature Igγ1 transcripts nor Sμ-Sγ1 DNA recombination was detected. Using a similar Ig transgenic approach, Manis et al. *(171)* demonstrated that B cells from *Ku70*$^{-/-}$ mice respond to class-switch signals, but neither the DNA products of class switching nor serum IgG or IgE were detected. Experiments using targeted, rearranged VDJ heavy and VJ light-chain genes in a SCID background could determine whether DNA-PK function is required for processes other than V(D)J recombination and CSR. These could also determine if, as with V(D)J recombination, impaired SCID DNA-PK function affects the two ligation products of CSR asymmetrically, the switch joint vs the excised circle joint (Fig. 5C).

Several pathogenic consequences of defective CSR have been identified. The X-linked hyperimmunoglobulin M (X-HIM) syndrome is characterized by severely restricted antibody repertoire owing to a defect in the CD40 ligand gene that results in impaired T-cell function *(70).* This inherited immune deficiency causes recurrent infections and an increased susceptibility to B-cell lymphoproliferative disease. The latter may result from the absence of CD40 receptor engagement for the induction of Fas-mediated apoptosis of proliferating B-cells *(70).* Studies of multiple myeloma tumors and cell lines have indicated that chromosomal translocations involving the IgH switch regions are an early step in disease development *(11).* Burkitt's lymphoma in humans and murine plasmacytomas result from the translocation of c-*myc* to the IgH locus, likely during abnormal CSR *(217,251,272).* It is clear that careful regulation of CSR is necessary both for the development of an effective immune system and for the prevention of detrimental chromosomal translocations.

8. V(D)J GONE WRONG: *ONCOGENIC POTENTIAL OF V(D)J RECOMBINATION*

The DSBs generated by V(D)J recombination, as well as those resulting from genotoxic stress, are repaired by NHEJ *(257).* V(D)J misjoining events can transcriptionally activate oncogenes or inactivate tumor-suppressor genes, resulting in dysregulated cell growth and accumulation of mutations *(217).* Because development of lymphoid-cell precursors requires physiological DSBs coupled with bursts of proliferation, they may be especially vulnerable to the oncogenic consequences of such events. To minimize the occurrence of aberrant recombination events, RAG-generated DSBs must be placed precisely adjacent to target gene segments and the synaptic complex must promptly join the resulting gap. Poor fidelity in either process has serious consequences for the genomic stability of an organism.

8.1. Aberrant Rearrangements

Misdirected RAG activity during V(D)J rearrangement has been implicated in defects associated with unusual chromosomal rearrangements. Trans- or interlocus V(D)J rearrangements can occur in cells from normal mice and humans and may increase the immune repertoire *(129).* However, the presence of these aberrant receptor-gene rearrangements may herald genetic instability with potentially dangerous consequences. Indeed, translocations between proto-oncogenes and antigen receptor genes are a common feature of human lymphoid malignancies *(129,217),* where over 50% of the cases examined have a translocation between an antigen receptor and a growth-promoting gene (reviewed in *128*). The mechanism of these rearrangements is uncertain but it has been suggested that during V(D)J recombination, RAG proteins may catalyze a DSB at one legitimate RSS and at one "cryptic-signal sequence" at an unrelated chromosomal site *(107).* Cryptic-signal sequences resembling authentic RSSs are numerous in the genome and may be targets of RAG activity *(151).* Alternatively, chromosomal translocations may arise from defective alignment of the DNA strands before or after DNA cleavage at two authentic RSSs. The frequency of such aberrant chromosomal rearrangements is likely restricted by chromatin accessibility, limiting RAG proteins from binding sites not intended for V(D)J recombination. Ultimately, the potentially devastating effects of an aberrant recombination event are limited by cellular selection. Lymphocytes harboring "illegitimate" rearrangements would fail to be selected unless they also express the products of normal V(D)J rearrangements *(151).* However, cells in which a growth- or survival-promoting gene (e.g., a proto-oncogene) is activated by juxtaposition to the potent enhancers within the antigen-receptor locus may proliferate despite the absence of normal selection signals. Thus, uncoupling V(D)J recombination from normal cellular selection for functional antigen receptors puts lymphocytes at risk of neoplastic transformation into lymphoma or leukocytic leukemia *(217).* The consequences of putative mistargeting of RAG activity are restricted to lymphocytes by virtue of tissue-specific expression of these enzymes. In contrast, the components involved in V(D)J joining are ubiquitously expressed and have other DNA repair and regulatory functions in the cell.

8.2. Cell Cycle and V(D)J Recombination

The signaling pathways responsible for the mobilization of DNA repair systems are still being defined, both in terms of identity and temporal relationships of the component molecules. The response to DNA damage involves surveillance molecules (detectors) conveying signals that elicit appropriate cellular responses (effectors). These responses include cell-cycle arrest and activation of DNA repair, or programmed cell death (apoptosis). Cell-cycle arrest facilitates DNA repair preventing the replication of mutated DNA strands (G1-S transition) and the transmission of damaged chromosomes to daughter cells (G2-M transition). As was discussed previously, the initiation of V(D)J recombination is coordinated with the cell cycle, in part by restricting the accumulation RAG2 protein to G0/G1 *(51).* It is not surprising that the activity of certain DNA repair components is also the highest at this time *(77,110,143,157).* Similar to yeast and higher eukaryotic cells exposed to genotoxic agents, lymphoid progenitors undergoing V(D)J recombination are arrested in G0/G1 *(108,209),* presumably ensuring that V(D)J-specific DSBs are joined prior to the onset of S phase *(246).*

8.3. p53 and V(D)J Recombination

The *p53* tumor-suppressor gene encodes a tetrameric transcription factor that plays a central role in the response to signals from damaged DNA by activating genes involved in cell-cycle arrest and apoptosis *(131,298).* Basal levels of p53 are low in most cells *(63,201,225,230).* In response to DNA damage insults as discrete as a single DSB *(88,89,110,120,166,170,194),* p53 accumulates owing to post-transcriptional stabilization, localizes to the nucleus *(67),* and binds to specific DNA sequences to activate target genes *(275).* Illustrating the efficiency of DSB management during normal V(D)J recombination, p53 protein is undetectable in wild-type thymocytes actively engaged in TCR rearrangement *(88).* In addition, lymphocyte-compartments of p53-deficient mice are normal, indicating that p53 is not essential for the for V(D)J recombination *(54),* including the associated G1 arrest *(88).* However, p53 is vital for management of DNA damage as indicated by the development of aneuploidy in p53-deficient cells treated with DNA-damaging agents *(131).* This loss of genomic stability underlies the cancer-prone phenotypes of the inherited Li-Fraumeni cancer syndrome *(169)* and $p53^{-/-}$ mice *(54,112,139).* Seventy to ninety percent of the spontaneous tumors observed in p53-deficient mice are lymphomas, with a mean onset at 4–5 mo of age *(54,95,96,112).* This lymphoma susceptibility remains in the absence of V(D)J recombination, as $Rag^{-/-}p53^{-/-}$ double mutant mice also display a high incidence of thymic lymphoma *(161).* However, evidence for the protective role of p53 in the context of V(D)J recombination is evident in SCID mice where checkpoint and repair defects collide. SCID lymphoid precursors accumulate CEs *(235,310)* and these DSBs are sufficient to cause p53 upregulation in SCID thymocytes *(88).* Disruption of the p53 DNA-damage checkpoint in $p53^{-/-}$ SCID double mutant universally results in pro-lymphocyte aneuploidy and lymphoblastic leukemia with a mean onset at 2 mo of age *(88,191).* In addition to the p53 response to physiologic V(D)J breaks in SCID cells, p53 expression is also induced in response to γ-irradiation, indicating that stabilization and function of p53 is not dependent on wild-type DNA-PK *(88,191).* An attractive candidate gene for a role in p53 stabilization/activation was identified by studying patients with ataxia telangiectasia (AT), and subsequently mice with targeted *Atm* mutations, whose cellular response to DNA damage, including p53 induction, is defective *(121,123).*

8.4. ATM and V(D)J Recombination

AT is a rare, autosomal recessive disease characterized by progressive cerebellar ataxia and occulocutaneous telangiectases, immune deficiencies, increased predisposition to lymphoid malignancies, and extreme sensitivity to ionizing radiation (reviewed in *140,236*). In $Atm^{-/-}$ mice, the development of thymoma is dependent on the initiation of V(D)J recombination *(160).* Cellular abnormalities include cell-cycle defects, sensitivity to γ-irradiation, and an increased incidence of chromosomal translocations, all of which result in increased genetic instability. AT was mapped to mutation of the *ATM* gene. *ATM* encodes a 370 kDa protein, that along with DNA-PK, is part of the large superfamily of PI3-kinase related proteins *(242,243).* Despite identification of many spontaneous *ATM* mutations in AT patients, the exact role of this protein remains unclear. ATM may have a direct role in DNA repair *(115),* however, the kinetics of DNA repair are normal in ATM-deficient cells *(236).* Importantly, ATM may be a regulator of signal transduction in response to DNA damage, activating cell-cycle check-

points, DNA repair mechanisms, or apoptosis *(236).* ATM may also affect chromatin conformation, thereby regulating susceptibility to DNA damage as well as access of recombination/repair machinery to DNA lesions *(129).* It is now generally accepted that *ATM* mutations result in DNA damage checkpoint defects that account for the genomic instability in ATM-deficient cells *(115,236,273).*

ATM and p53 are likely co-participants in at least one pathway *(297)* in which the ATM-dependent dephosphorylation of p53 leads to its association with a 14-3-3 protein resulting in enhanced p53 stabilization *(291).* Additionally, recent evidence suggests that ATM directly phosphorylates p53 in response to ionizing radiation *(9,26,236).* Characteristic of the genetic instability associated with *ATM* mutations, AT patients' lymphocytes show increased chromosomal aberrations, many involving chromosomes 7 and 14 in the vicinity of TCR and Ig genes *(128,140).* Although a DNA recombination defect was first suspected, one study using ECRSs suggests that V(D)J recombination is normal in cells of AT patients *(109).* If so, the mechanism of immune deficiency in AT patients and $Atm^{-/-}$ mice is completely unclear. Developmental studies in ATM-deficient mice revealed phenotypic differences between their T- and B-lymphoid precursors and those of $Atm^{+/-}$ or wild-type controls *(10,58,184,303,304).* Most striking is the depletion of the more mature T-cell populations. However, neither a profound arrest in lymphocyte development nor a striking V(D)J recombination defect characteristic of SCID mice was observed. Given that p53-deficient mice do not show a deficit in either precursor or mature lymphocytes, the effect of ATM on lymphocyte development is likely p53-independent, perhaps affecting cellular proliferation or pre-senescent apoptosis. Thus, mechanistic connections between DNA-PK, p53, and ATM in lymphocyte development, V(D)J recombination, and the cellular response to DNA damage are likely, but not yet well-understood.

Ongoing inquiry into the regulation of V(D)J recombination will no doubt involve exploration of molecular pathways already identified to serve in the context of generalized DNA repair. The involvement of 14-3-3 proteins in the stabilization of p53 is reminiscent of a pathway involved in activation of cyclin-dependent kinase Cdc2, primarily examined in yeast models. In response to DNA damage, Cdc2 is inhibited by specific tyrosine phosphorylation resulting in a G2 arrest. The phosphorylation status of Cdc2 is maintained by kinases (wee1p and mik1p in *S. pombe*) and Cdc25 phosphatase (*S. pombe* and human; *227).* Cdc25 itself is phosphorylated by the Chk1 protein kinase *(241)* and subsequently sequestered in a complex with 14-3-3 *(208).* An interesting connection between Chk1 and p53 is suggested by the finding that cdc2 is involved in p53 phosphorylation *(16).* Indeed, Chk1 may be activated via phosphorylation by DNA damage-responsive protein kinases such as *S. pombe* rad3p, or human ATM or ATM-related protein, ATR. Mutations in DNA-PK_{cs} or Ku result in inefficient DSB repair and lead to impaired lymphocyte development (immunodeficiency) and genomic instability resulting in neoplastic transformation. The ability to detect DNA damage and engage appropriate signaling pathways is an important prerequisite for the mobilization of DNA repair proteins and cell-cycle arrest. Indeed, mutations in known DNA-damage detector and effector components, such as ATM and p53, result in profound tumor susceptibility, particularly in the lymphoid lineages. As yet, these molecules have not been implicated in V(D)J recombination, CSR, or somatic hypermutation.

9. CONCLUDING REMARKS

V(D)J recombination is a highly regulated process that couples successful DNA rearrangement with lymphocyte proliferation and maturation. The introduction of V(D)J-specific DSBs is regulated at two levels: through the lymphoid-specific expression of the RAG1/2 endonuclease and by control of chromatin accessibility to RAG1/2. Once cut, the DNA is rejoined using the general cellular DNA-repair machinery, involving DNA-PK_{cs}, Ku, XRCC4, and DNA ligase IV, although RAG1/2 may have specialized roles for V(D)J end processing. Further understanding of the process of NHEJ, in yeast and mammals, should facilitate understanding of V(D)J recombination.

The oncogenic potential of aberrant V(D)J recombination and CSR is evidenced by the high occurrence of translocations between antigen receptor and growth-promoting genes in human lymphoid malignancies. Additionally, the increased frequency of lymphocytic tumors in mice and humans with mutations in DNA repair and checkpoint genes, such as *Ku, DNA-PK, ATM, NB51,* and *p53,* demonstrate the importance of careful coordination of V(D)J recombination and cell-cycle regulation. Potentially oncogenic translocations may result from the (mis)targeting of the RAG1/2 endonuclease or perturbation of the DNA damage and cell-cycle checkpoints. V(D)J recombination provides a physiological context in which investigators can examine the complex mechanisms that maintain genomic stability.

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12

Interaction of Cell-Cycle Checkpoints with Muscle Differentiation

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1. INTRODUCTION

Damage to genomic DNA occurs spontaneously in all living cells, and represents a significant and constant problem. In addition, chemical or physical mutagens can cause a variety of DNA lesions, including base modifications, intra- and interstrand crosslinks and single- or double-strand breaks *(31)*. If left unrepaired, these DNA lesions can lead to mutations or loss of viability. Thus, cell-cycle checkpoints and DNA-repair mechanisms have evolved to ensure cellular survival in the face of DNA damage. Multicellular organisms have additional issues to deal with, including differentiation programs, as well as the existence of a limited number of stem cells required for renewal and repair of differentiated tissues. Because cell-cycle checkpoints and differentiation utilize the same key cell-cycle regulatory factors to mediate cell-cycle arrest, multicellular organism must integrate these two processes simultaneously. The subject of this chapter is the intersection of genetic alterations in the DNA damage-response pathway that interfere with overt differentiation.

2. CELL-CYCLE CONTROL BY ATM AND ATR

ATR is a mammalian gene with homology to *Schizosaccharomyces pombe rad3 (3a), Saccharomyces cerevisiae ESR1/MEC1 (77a), Drosophila mei-41 (39a),* and human ataxia telangiectasia mutated *(ATM) (46a). ATR* also shows homology to *FRAP/mTOR* and has been reported as *FRP1 (12a).* This recently identified family of proteins share common structural features, being large proteins (>2000 amino acids) with highly conserved carboxy-terminal kinase domains homologous to PI3-kinases. Mutations in the *ATM* gene lead to pleiotropic defects in cell-cycle regulation following DNA damage, including loss of the G1/S and G2/M checkpoints (reviewed in refs. *41a,42a*). Like ATM, ATR is a nuclear protein with protein kinase activity *(46a).* In the mouse, both ATM and ATR can directly associate with broken chromatin, as both proteins show complementary staining foci on meiosis I recombining chromosomes *(46a).* Recently, disruption of the *ATR* gene in mice has indicated that ATR functions early in develop-

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ment, as *ATR*$^{-/-}$ embryos die by day 7.5 of development *(8,18).* In culture, *ATR*$^{-/-}$ blastocyst cells display mitotic catastrophes and die of caspase-dependent apoptosis. Evidence that ATR has a DNA damage induced cell-cycle-checkpoint function come from analysis of kinase-inactive mutants of ATR (ATR-ki). Overexpression of ATR-ki has been shown to cause sensitivity to DNA-damaging agents and defects in cell-cycle checkpoints in response to ionizing radiation (IR) as well as ultraviolet (UV) light *(13,97).* Furthermore, overexpression of ATR-ki interferes with the late-phase Ser-15 phosphorylation of p53 in response to IR and to block UV-induced Ser-15 phosphorylation of p53 *(92).* One attractive interpretation of these results is that ATR-ki functions in a dominant-negative manner to interfere with the normal cell-cycle checkpoint functions of ATR. However, in the absence of information about ATR null cells exposed to DNA damage, it is difficult to determine whether ATR-ki is truly functioning as a dominant-negative, especially given that overexpression of wild-type ATR interferes with at least some p53 functions *(85).*

The *S. cerevisiae* Mec1p and the *S. pombe* rad3p proteins are involved in all of the known DNA-structure checkpoints in yeast (reviewed in ref. *11a*). In *S. cerevisiae,* there are two members of this large lipid kinase motif family, Mec1p and Tel1p. Mec1p has the highest degree of homology to ATR, and is an essential protein required for all the known DNA-structure checkpoints. The *TEL1* gene, which shows the highest degree of homology to ATM, is not an essential gene and does not show checkpoint defects when mutated. However, the double mutant *(mec1 tel1)* has increased radiation sensitivity. Furthermore, overexpression of *TEL1* can suppress the radiation sensitivity of *mec1* mutants. These results suggest a partial functional overlap between Mec1p and Tel1p in the control of DNA-damage-induced checkpoints. Taken together results suggest that, like Mec1p and Tel1p, ATM and ATR control cell-cycle checkpoints in mammalian cells.

3. CELL-CYCLE CHECKPOINTS AND p53

Mammalian cells with DNA-damage arrest in G1, S, and G2 phases of the cell cycle. In contrast, differentiating cells terminally arrest their cell cycles from the G1 phase of the cell cycle in a state known as G0. Primary among mammalian G1 checkpoint genes is p53. p53 is required for the G1 checkpoint, in which cells arrest in G1 in response to DNA-damaging agents such as IR *(46).* Utilizing cells from knockout mice, it has become clear that the p53 gene is critical for G1 arrest following γ-irradiation *(22).* In addition, p53 has been shown to be required for maintenance of the G2 arrest following IR *(9).* Following DNA damage, p53 protein levels are significantly increased in vertebrate cells grown in vitro *(16,46,51,53),* as well as in vivo *(38,57).* Although p53 mRNA levels do not change in response to DNA damage in many cell systems, the levels of p53 protein increases rapidly. The half-life of p53 protein increases substantially after DNA damage *(52,53,69),* and increased translation of p53 mRNA also contributes to p53 induction *(32,46,60).* The relative contributions of increased half-life and enhanced translation remain largely undefined. In addition to an increase in the levels of p53 protein, DNA damage is thought to result in activation of p53's ability to bind sequence-specific DNA and consequently transactivate gene expression *(83).* Furthermore, ATM has been implicated in regulation of p53, because cells from AT patients do not activate p53 normally in response to DNA damage (reviewed in ref. *24).* Activation

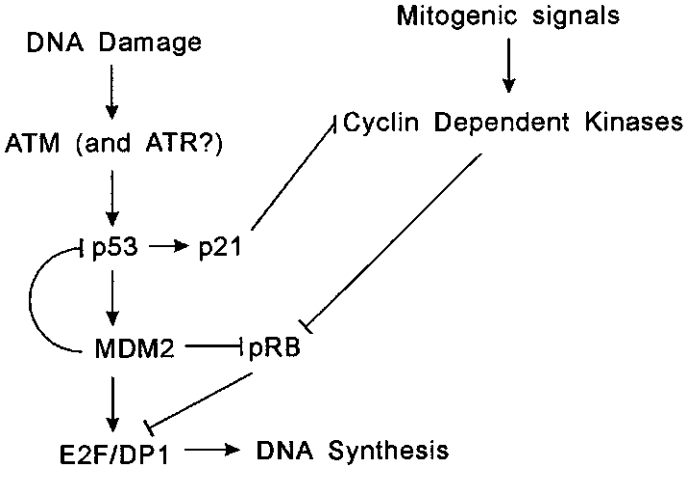

Fig. 1. Model for the G1 checkpoint.

of p53 DNA binding activity is thought to involve phosphorylation of serine 15 of p53 by ATM *(1,10)* and by ATR *(10).* In addition, dephosphorylation of p53 by an unknown phosphatase and subsequent association with 14-3-3 proteins leads to an increase in the affinity of p53 for sequence-specific DNA *(93).*

In response to IR, G1 arrest is mediated at least in part, through induction of the cyclin-dependent kinase (CDK) inhibitor p21/WAF1/Cip1 by p53 *(23).* p21/WAF1/Cip1 forms a complex with cyclin/CDKs, and represents one of the most studied p53 response genes *(25,40,47,78).* In normal cells, p21 is found associated with a variety of cyclin/CDK complexes, including the G1 cyclins CDK4/Cyclin D *(101,102).* In addition, p21 can inhibit the kinase activity of all of the cyclin/CDK complexes *(100).* A current model for the G1 checkpoint is illustrated in Fig. 1.

4. MUSCLE DIFFERENTIATION

Differentiating muscle cells fuse to form multinucleated myotubes, thereby withdrawing permanently from the cell cycle. In mice, specification as well as differentiation of skeletal muscle cells is dependent on four muscle-specific basic helix-loop-helix transcription factors, the *MyoD* family of muscle-determination genes. Targeted disruption of each family member *(MyoD, Myf-5, myogenin,* and *MRF4)* in mice has demonstrated the importance of these factors for vertebrate myogenesis. Inactivation of *Myf-5* results in defects in early myotome formation with presumptive muscle precursors adopting nonmuscle cell fates *(7,88,89).* However, this early myotomal defect is compensated by *MyoD,* leading to apparently normal muscle at birth. Targeted disruption of *MyoD* alone also does not dramatically affect muscle specification or differentiation, as these mice have grossly normal muscle *(76).* The functional overlap between *Myf-5* and *MyoD* was demonstrated by generating mice with disruption of both genes, resulting in a complete absence of proliferating myoblasts as well as differentiated muscle fibers *(77).* In contrast, mice with disruption of the *myogenin* gene contain normal numbers of proliferating myoblasts, but these cells fail to differentiate, indicating that myogenin has a unique role in the transition from determined myoblasts to a fully differentiated

myotubes *(41,61)*. Lastly, inactivation of the *MRF4* gene results in a very mild muscle phenotype, consistent with the notion that *MRF4* may have functions that are redundant with the other *MyoD* family members *(6,68,104)*. These experiments have led to a simple model for vertebrate-skeletal myogenesis that involves Myf-5 and MyoD functioning early to establish or determine myoblast-cell fate, and myogenin mediating the terminal differentiation of myoblasts.

Vertebrate-skeletal muscle is derived from cells in the prechordal and somitic mesoderm that give rise to committed myogenic cells of the somite, which become the skeletal muscle of the head, trunk, and limbs. The different myofiber and myogenic constituents are thought to be formed from different lineages of myogenic cells *(58)*. In the mouse, primary myofibers develop first at 8.5 d of gestation, followed by secondary myofibers at day 14. Recent experiments have suggested that MyoD and Myf-5 do have distinct roles for determining these myogenic lineages during development *(45)*. Although *MyoD*$^{-/-}$ embryos display normal development of paraspinal and intercostal muscles in the body proper, muscle development in limb buds and brachial arches is delayed by about 2.5 d. By contrast, *Myf-5*$^{-/-}$ embryos display normal muscle development in limb buds and brachial arches, and markedly delayed development of paraspinal and intercostal muscles. Taken together, these observations strongly support the hypothesis that Myf-5 and MyoD play unique roles in the development of epaxial and hypaxial muscle, respectively. In addition, satellite cells, the muscle stem cells of adult muscle, arise around day 17 as a unique myogenic lineage (for review, *see* ref. *5)*. Satellite cells are mitotically quiescent but are induced to enter the cell cycle in response to stress induced by exercise or injury. The activated satellite cells undergo multiple rounds of division before fusing with existing myofibers resulting in repair and/or hypertrophy. Recently, MyoD has been shown to play a novel role in satellite-cell function *(56)*. *MyoD*$^{-/-}$ mice interbred with the *mdx* mouse (a model for Duchenne and Becker muscular dystrophy) exhibit increased penetrance of the *mdx* phenotype characterized by reduced muscle hypertrophy and increased myopathy. This condition is thought to arise owing to a defect in muscle regeneration. Consistent with this hypothesis, the single mutant *MyoD*$^{-/-}$ knockout mice show defects in skeletal-muscle regeneration following injury *(56)*.

5. CONTROL OF MYOD ACTIVITY BY pRB

Tissue-culture experiments have been useful in elucidating the sequence of events that result in terminal cell-cycle withdrawal and differentiation of muscle cells. This process is controlled by regulatory interactions involving MyoD family members and various cell-cycle-related proteins (reviewed in refs. *48,66)*. Myf-5 and MyoD are present in proliferating myoblasts and initiate a cascade of events, including activation of myogenin expression when myoblasts are deprived of mitogens, resulting in terminal differentiation. Furthermore, MyoD transactivation of muscle-specific genes to high levels requires pRB *(34,63)* or high levels of the pRB related protein p107 *(79)*. Loss of pRB function in muscle cells has two prominent phenotypes: (1) the level of activation of terminal differentiation markers is drastically reduced, and (2) new DNA synthesis can occur in the nuclei of the rare differentiated myotubes. During muscle differentiation, pRB expression increases *(15,26,54)* and assumes a hypophosphorylated and activated state *(34,91)*. These studies show that pRB plays a critical role in myogenesis.

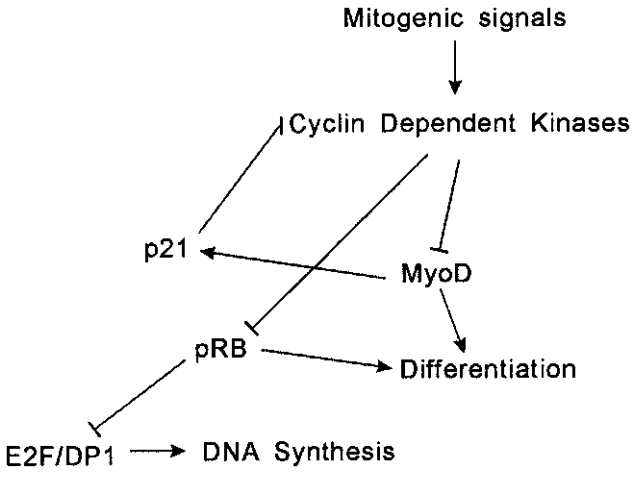

Fig. 2. Model for muscle-cell differentiation.

However, the precise mechanism by which pRB participates in myogenesis remains unclear. Direct protein-protein interaction between pRB and MyoD has been proposed to account for the pRB requirement *(34).* However, it remains possible that the affects of pRB on muscle differentiation is an indirect affect via one or more cell-cycle changes mediated by pRB. In addition, MyoD is thought to induce cell-cycle arrest during differentiation, at least in part, by inducing expression of the cyclin-kinase inhibitor p21, which would in turn inhibit the G1-phase cyclins and allow pRB to become activated *(35,37).* A current model for muscle-cell differentiation is shown in Fig. 2.

6. MYOD AND ONCOGENESIS

In addition to promoting tumor formation, anchorage-independent growth, and cellular immortalization, expression of transforming oncogenes inhibits cellular differentiation in several different cell lineages *(4,14,21,29,30,43,49,70).* In muscle cells, for example, expression of oncogenic tyrosine kinases (v-src and v-fps), growth-factor receptors (v-erbB), nuclear oncogenes (v-myc, c-myc, v-erbA, and E1A), and the activated form of signal-transducing G proteins (H-ras and N-ras) can inhibit terminal differentiation to varying extents *(27,29,33,43,67,80,95).* Furthermore, several hypotheses have been proposed to explain how the MyoD family of proteins are kept in check during proliferation: (1) inhibition of the MyoD family members by interaction with the Id family of negative HLH factors *(3);* (2) inhibitory phosphorylation of the MyoD family members by protein kinase C *(39,50);* and (3) inhibition by cyclin-D-dependent kinases *(73,84).* However, none of these interactions have been shown to be causative in the generation of the abnormal proliferation or lack of differentiation of skeletal-muscle tumors.

7. MYOD AND SKELETAL-MUSCLE TUMORS

Rhabdomyosarcomas are skeletal-muscle tumors and are one of the more common solid tumors of childhood, representing 4–8% of all malignancies in humans under 15 years of age. Tumors arise de novo from skeletal muscle. Sarcomas have traditionally been classified as rhabdomyosarcomas based on morphology and expression of muscle-

specific structural genes, such as myosin heavy chain or desmin. Expression of *MyoD* has been shown to be the most sensitive marker for classifying sarcomas as rhabdomyosarcomas *(20,81).* This is paradoxical, because MyoD has been shown to induce muscle differentiation in a wide variety of primary cells and transformed cell lines *(96),* yet rhabdomyosarcomas have a low propensity to differentiate into myotubes. Thus, even though MyoD is expressed in rhabdomyosarcomas cells, it would appear to be nonfunctional in inducing differentiation in these cells.

Rhabdomyosarcomas are grouped by histologic and cytogenetic criteria as either embryonal or alveolar rhabdomyosarcomas: a balanced translocation between chromosomes 2 and 13, t(2:13)(q35;q14), is associated with alveolar rhabdomyosarcomas *(2).* The *PAX3* gene has been shown to be fused to a forkhead gene-family member *(FKHR)* in the t(2:13) translocation *(2,82).* More recently, the *PAX7* gene, located at 1p36, has been shown to be translocated to the *FKHR* gene as a 1:13 translocation (p36;q14) in some cases of alveolar rhabdomyosarcoma *(17).* Loss of heterozygosity on the short arm of chromosome 11 encompassing 11p15 is associated not only with embryonal rhabdomyosarcomas *(81)* but also with several other solid tumors *(62),* suggesting the location of one or more tumor-suppressor genes for multiple tumor types in this region. In addition, gene amplification has been observed in both embryonal and alveolar rhabdomyosarcomas. Comparative genomic hybridization showed that in primary alveolar rhabdomyosarcomas the most frequent amplicons are localized to 2p24 and 12q13-14, with both amplifications occurring in 4 out of 10 tumors *(94).* The 2p24 amplicon had previously been shown to involve the *MYCN* gene *(19),* whereas the genes involved in the 12q13-14 amplicon have not yet been fully defined. Two distinct chromosome 12q13-14 amplicons have been described in other types of sarcomas *(87)* as well as in gliomas *(74).* Mapping of these two amplicons implicates *MDM2* or *CDK4* and *SAS* as likely targets of the amplification events *(74).* The frequency of these two different amplicons in primary rhabdomyosarcomas is currently unknown.

8. INHIBITORS OF MUSCLE DIFFERENTIATION

8.1. Amplification of MDM2

One obvious phenotype of tumor cells is a lack of terminal differentiation. We have conducted a series of somatic-cell genetic experiments designed to identify genetic loci present in rhabdomyosarcoma cells lines that are capable of inhibiting muscle differentiation *(28,85,90).* Initially, we showed that rhabdomyosarcomas could be classified as either dominant or recessive with respect to MyoD activity and terminal differentiation *(90).* Subsequent analysis of two different dominant types of tumors indicated that the loss of differentiation could be mapped to individual loci. First, microcell-mediated chromosome transfer of a derivative chromosome 14 from the rhabdomyosarcoma cell line Rh18 into the differentiation competent myoblast cell line C2C12 inhibits muscle differentiation and the ability of MyoD to transactivate reporter constructs. The derivative chromosome 14 contains a region of amplified DNA originating from chromosome 12q13-14, and contains several genes often amplified in sarcomas *(28).* Testing the amplified genes for the ability to inhibit muscle-specific gene expression indicates that forced expression of *MDM2* inhibits MyoD function, and consequently inhibits muscle differentiation. Thus, amplification and overexpression of *MDM2* in rhabdomyosarcomas inhibits MyoD function, resulting in dominant inhibition of muscle differentiation *(28).*

The oncogenic properties of MDM2 have been postulated to result from direct interaction with several cell-cycle regulatory proteins. MDM2 interacts directly with p53 *(64),* and blocks p53-mediated transactivation by inhibiting the activation domain of p53 *(12,36,59,65,98,103).* In addition, MDM2 interacts with the activation domain of E2F1, resulting in stimulation of E2F1/DP1 transcriptional activity *(55).* Furthermore, MDM2 has been shown to interact directly with pRB, resulting in stimulation of E2F/DP1 transcriptional activity and inhibition of pRB growth-regulatory function *(99).* Taken together, these results suggest that MDM2 not only relieves the proliferative block mediated by either p53 or pRB, but also promotes proliferation by stimulating the S phase-inducing transcriptional activity of E2F/DP1. Thus, the most obvious role for amplification of *MDM2* in tumorigenesis would be to inactivate p53 and/or pRB. In addition, we have shown that amplification of *MDM2* inhibits MyoD activity and consequently inhibits normal muscle differentiation *(28).* These studies have identified a previously unknown activity for the MDM2 protein, and broadens the role of MDM2 in cell-growth control to include inhibition of differentiation.

Because MDM2 is expressed in proliferating myoblasts, and MDM2 can inhibit muscle differentiation *(28),* one possibility is that MDM2 inhibits differentiation of myoblasts during cellular proliferation. Furthermore, activation of muscle-specific genes to high levels requires members of the pRB family *(34,63,79).* During muscle differentiation, pRB expression increases *(15,26,54)* and assumes a hypophosphorylated, activated state *(34,91).* Because MDM2 interacts with pRB and inhibits the growth-regulatory functions of pRB *(99),* it is possible that pRB in turn regulates the growth-stimulatory effects of MDM2. Consistent with this possibility, we find that forced expression of pRB can restore MyoD activity in cells with amplified *MDM2* (unpublished observations). Because pRB and MDM2 interact, these results suggest that an additional role for pRB during muscle-cell differentiation is to bind and inactivate MDM2.

8.2. Duplication of ATR

In a second screen for inhibitors of differentiation, we found that microcell-mediated chromosome transfer of an isochromosome 3q [i(3q)] from a different rhabdomyosarcoma cell line into the differentiation-competent myoblast cell line C2C12 also inhibits differentiation. In addition to inhibition of differentiation, the i(3q) causes abnormal centrosome amplification, resulting in aneuploidy, and abolishes G1 arrest following DNA damage *(85).* We have extended these observations by showing that forced expression of *ATR* (located at 3q24) results in a phenocopy of the i(3q) containing hybrids. These findings may have implications for nonmuscle tumors as well. Comparative genomic hybridization was used to demonstrate that 3q is a hotspot for increased DNA copy number in several different types of cancers. In head and neck squamous-cell carcinoma, the most frequently observed increase in DNA copy number was from chromosome 3q, occurring in 10 of 13 primary tumors *(86).* Karyotypic analysis of these cells showed the presence of an i(3q) in 30–40% of tumors *(11,44,72).* Similarly, the most frequent increase in DNA copy number in primary small-cell lung carcinomas was also on 3q, occurring in 10 out of 13 cases *(75).* Furthermore, the formation of an i(3q) has been observed in small-cell lung carcinomas *(71).* Prior to our study, the most compelling argument for an involvement of 3q in tumorigenesis came from analysis of

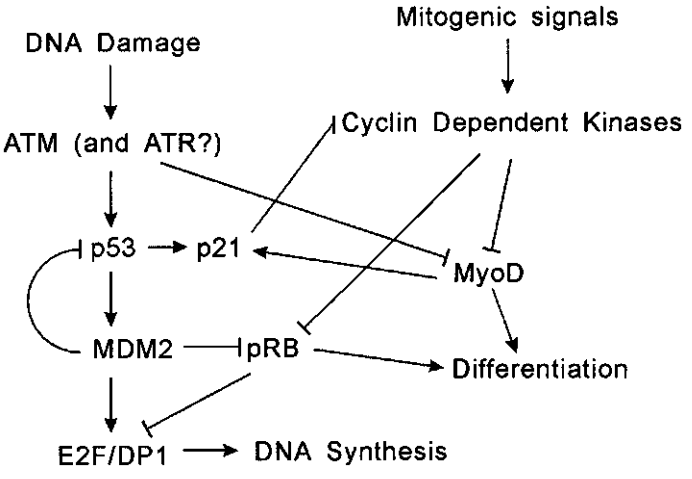

Fig. 3. Model integrating G1 checkpoint and muscle-cell differentiation.

cervical carcinomas *(42).* Gain of 3q is the most consistent chromosomal aberration in cervical carcinomas and was present in 9 out of 10 tumors. Furthermore, this alteration occurs during the progression from severe dysplasia to invasive carcinoma *(42).* Interestingly, the gain of 3q correlated with the conversion of tetraploid dysplastic cells to aneuploid carcinoma cells. Although it remains possible that different genetic alterations of 3q occur in different tumors, our data indicate that the i(3q) present in Rh30 rhabdomyosarcoma cells induces aneuploidy. Therefore, the aneuploidy observed in cervical carcinoma cells may be a direct result of increased copy number on 3q resulting in *ATR* overexpression. Taken together, these studies implicate alterations in the *ATR* locus as causing loss of differentiation and cell-cycle abnormalities in several different types of tumors.

9. SUMMARY: CELL-CYCLE CHECKPOINTS AND DIFFERENTIATION

Because both cell-cycle checkpoints and muscle differentiation use the same cyclin-kinase inhibitor, p21, to arrest the cell cycle, and because forced expression of p21 in muscle cells leads to premature differentiation *(37),* a mechanism to protect the myoblast population from premature differentiation induced by DNA damage must exist. We propose that the inhibitory interactions between MDM2 and MyoD and between ATR and MyoD fulfill this role, so that if a myoblast sustains DNA damage, premature differentiation does not occur. A model to integrate these two processes is shown in Fig. 3.

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