

Cancer Susceptibility and the Functions of BRCA1 and BRCA2

Review

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Inherited mutations in *BRCA1* or *BRCA2* predispose to breast, ovarian, and other cancers. Their ubiquitously expressed protein products are implicated in processes fundamental to all cells, including DNA repair and recombination, checkpoint control of cell cycle, and transcription. Here, I examine what is known about the biological functions of the BRCA proteins and ask how their disruption can induce susceptibility to specific types of cancer.

Questions concerning the biological functions of the proteins encoded by the breast cancer susceptibility genes have dominated the field since the genes were identified some six years ago through the analysis of families at high risk from breast and ovarian cancer. *BRCA1* and *BRCA2* encode very large proteins (Figure 1), widely expressed in different tissues during the S and G2 phases, which localize to the cell nucleus. They bear little resemblance to one another or to proteins of known function. Orthologs are not found in the yeast, fly, or worm genomes. Thus, *BRCA1* and *BRCA2* are relative latecomers in evolution, belying their apparently fundamental role in mammalian cells, and hinting at specialized, possibly tissue-specific, functions. These unusual features have at once confounded analyses of the biological function of the BRCA proteins, yet have also enhanced their perceived value to the cancer biology field.

Two major themes have emerged in the recent literature. First, both *BRCA1* and *BRCA2* work to preserve chromosome structure, yet the precise nature of their contribution has proven difficult to define, because both proteins have been implicated in a multitude of different processes including DNA repair and recombination, cell cycle control, and transcription. Second, the similarities between the phenotypes induced by disruption of *BRCA1* or *BRCA2* and their claimed cohabitation in certain macromolecular complexes has prompted speculation that they work together in common cellular pathways.

In this review, I will analyze available information about the biological functions of the BRCA proteins and argue that their role in the maintenance of chromosome structure stems from distinct rather than identical functions in the biological response to DNA damage. A case will be made that spontaneous chromosomal instability in *BRCA*-deficient cells underpins their propensity to undergo neoplastic transformation and at the same time

channels tumor evolution down particular routes. Evolving evidence concerning the participation of BRCA proteins in other cellular processes will also be analyzed, emphasizing the many significant challenges in our current understanding of cancer predisposition induced by *BRCA* gene disruption.

Breast Cancer Genetics

The genetic susceptibility to breast cancer caused by mutations in *BRCA1* or *BRCA2* is just one piece of a larger picture (reviewed in Nathanson et al., 2001). About 10% of breast cancer cases cluster in families; some are due to highly penetrant germline mutations in one or another of a small number of genes, such as *BRCA1* or *BRCA2*, giving rise to high cancer risk. But the majority of cases (“sporadic”) exhibit no clear-cut familial clustering and probably result from the collective effect of multiple, poorly penetrant variations in a much larger group of genes, modified by environmental factors. Of familial cases, germline mutations in *BRCA1* or *BRCA2* account for between 15% and 20% of the observed risk (but are found in over 80% of families where six or more cases occur). Only a small fraction of the remaining risk can currently be attributed to germline mutations in other known genes (for example, the p53 tumor suppressor, the STK11/LKB1 protein kinase, or the PTEN phosphatase). Additional susceptibility alleles for familial breast cancer must therefore exist, but they have so far proven difficult to identify (Easton, 1999).

One defective copy of *BRCA1* or *BRCA2* in the germline is enough to cause cancer predisposition, but the second allele is consistently lost in tumor cells isolated from predisposed individuals. Thus, *BRCA* genes conform at least in part to the classic paradigm for tumor suppressor genes. On the other hand, somatic *BRCA* mutations rarely occur in sporadic cancer cases.

Caretakers of Chromosomal Stability

Cancer susceptibility gene mutations fall into two general classes (Kinzler and Vogelstein, 1997). Genes whose mutation or altered expression relieves normal controls on cell division, death, or lifespan, promoting the outgrowth of cancer cells, have been termed “gatekeepers.” Those whose disruption causes genome instability, increasing the frequency of alterations in gatekeeper genes, work instead as “caretakers.”

A starting point from which to unravel the functions of *BRCA1* and *BRCA2* relevant to cancer predisposition comes from the observation that cells deficient in the murine *BRCA2* homolog sustain spontaneous aberrations in chromosome structure (Figures 2A–2D) that accumulate during division in culture (Patel et al., 1998). Microscopically, the abnormalities not only include broken chromosomes and chromatids, but also triradial and quadriradial structures, markers of defective mitotic recombination that are typical of the human diseases Bloom’s syndrome and Fanconi’s anemia, also associated with increased susceptibility to cancer (including breast cancer). Spectral karyotyping (a chromosome

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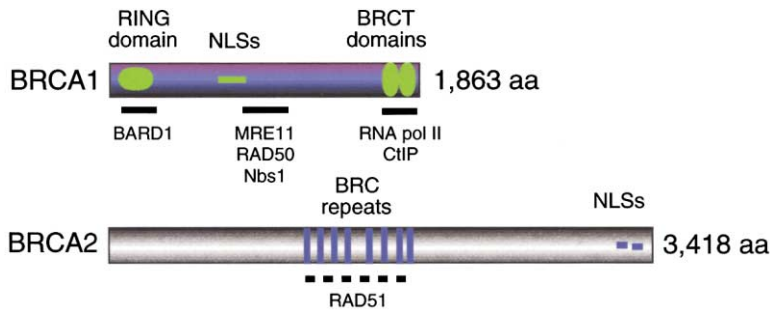


Figure 1. Features of the Human BRCA Proteins

BRCA1 contains an N-terminal RING domain, nuclear localization signals (NLSs), and two C-terminal BRCT domains of ~110 residues (also found in several proteins with functions in DNA repair or cell cycle control). Interacting proteins discussed in the text are shown below approximate regions of binding. BRCA2 contains eight repeats of the ~40 residue BRC motifs. Six of the eight motifs in human BRCA2 can bind directly to RAD51 when expressed in vitro.

“painting” technique) reveals gross chromosomal rearrangements (GCRs) such as translocations or deletions, as well as fusions that encompass multiple, non-homologous chromosomes (Yu et al., 2000). Similar structural aberrations occur in *BRCA1*-deficient mouse cells (Xu et al., 1999a), as well as in *BRCA1*- or *BRCA2*-deficient human cancer cells (Tirkkonen et al., 1997; Gretarsdottir et al., 1998). Collectively, these findings establish that *BRCA* genes are essential for preserving

chromosome structure, suggesting that, in their role as tumor suppressors, they behave as caretakers, suppressing genome instability.

Chromosomal Instability through Inappropriate DNA Double-Strand Break Repair

Studies on yeast mutants that spontaneously accumulate GCRs implicate malfunctions in DNA repair or recombination pathways (Chen et al., 1998a; Chen and

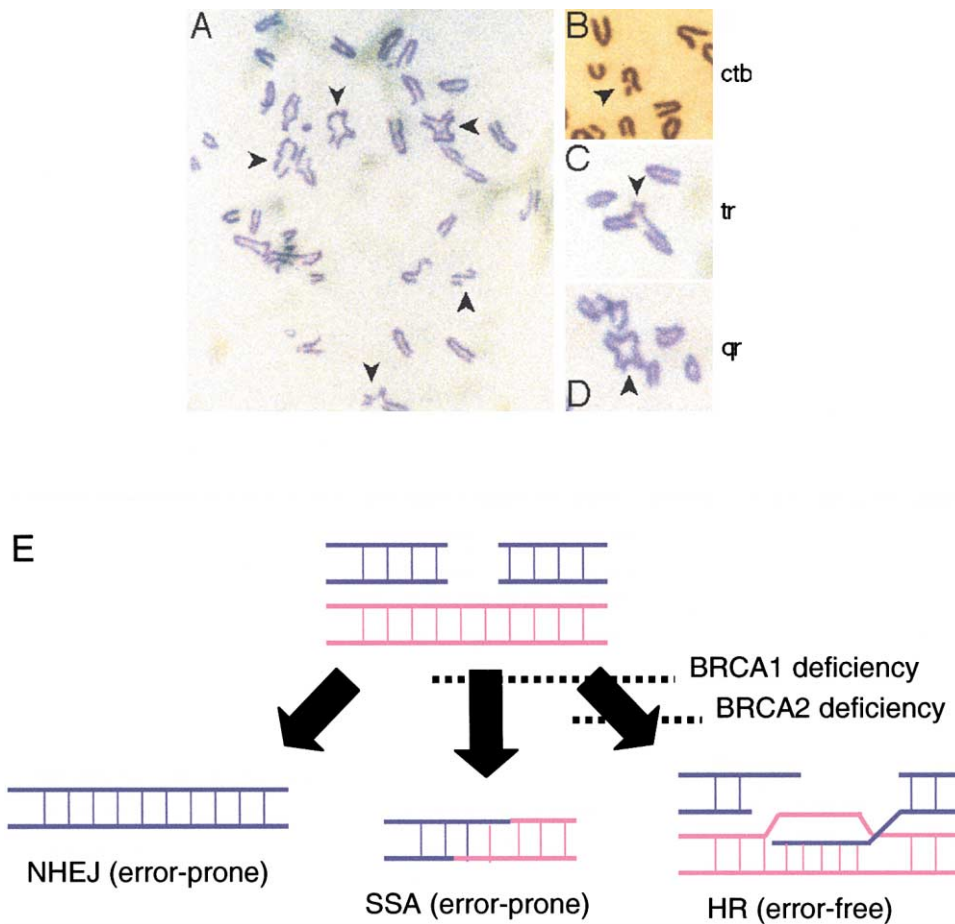


Figure 2. Chromosomal Instability through Inappropriate DNA Double-Strand Break Repair

(A–D) Spontaneous chromosomal instability in *BRCA2*-deficient murine cells. A typical metaphase spread is shown in the largest panel, with aberrations in the U shaped normal mouse chromosomes enlarged on the right. Abbreviations are as follows: Ctb, chromatid break; Tr, triradial; and Qr, quadriradial. Reproduced from Patel et al. (1998) with the permission of Cell Press.

(E) DSB repair in *BRCA*-deficient cells is routed down error-prone pathways because the preferred pathway, error-free HR, is not functional. *BRCA1* deficiency may also compromise SSA.

Kolodner, 1999; Kraus et al., 2001). It emerges from this work that double-strand DNA breaks (DSBs) are an important precursor lesion for GCR formation and may arise when DSBs are repaired in an inappropriate way.

In mammalian cells (Figure 2E), DSBs can be repaired (reviewed in Karran, 2000; Khanna and Jackson, 2001; van Gent et al., 2001) by *nonhomologous end joining* (NHEJ), potentially an error-prone process in which nucleotide alterations are tolerated at the sites of rejoining, or by *recombination* between homologous DNA sequences (HR), error-free when the exchange is between identical sister chromatids (or homologous chromosomes). A third mechanism, *single-strand annealing* (SSA), is also initiated by homologous pairing, except that—unlike HR—the homology is between short stretches of single-stranded DNA (ssDNA) at staggered DSBs, and pairing precedes religation, not strand exchange. SSA is error-prone because sequence information can be lost or rearranged when ends overlapping by as little as ~30 bp are unsuitably joined.

Mammalian experimental models reveal a complex relationship between DSB repair mechanisms and chromosome stability (Khanna and Jackson, 2001; van Gent et al., 2001). On the one hand, error-prone DSB repair can provoke GCR formation. Deletions or translocations may result, for example, when broken ends from different chromosomes or nonadjacent regions of the same chromosome are promiscuously fused together by NHEJ or SSA. On the other hand, deficiency of NHEJ can itself cause chromosomal instability, showing that there are circumstances in which error-free HR alone is insufficient. Thus, different mechanisms are used in mammalian cells to deal with DSBs in different situations, and failure to choose the right repair mechanism can promote—rather than prevent—chromosomal instability.

The mitotic cell cycle is one important determinant in this choice. Repair by HR predominates during S/G2 phases of the mitotic cell cycle, when sister chromatids, the preferred substrate for error-free exchange, are present. The proteins that carry out NHEJ are also present at this time and can contribute to DSB repair, but very little is known about how a choice can be made between these mechanisms (Haber, 1999).

Evidence is emerging that GCRs in *BRCA*-deficient cells result from inappropriate DSB repair during S and G2 (Figure 2E), the phases when the *BRCA* proteins are maximally expressed. Recent work from several groups (Moynahan et al., 1999, 2001; Snouwaert et al., 1999; Tutt et al., 2001; Xia et al., 2001) shows that *BRCA1*- or *BRCA2*-deficient rodent cells or human tumors are specifically deficient in HR, whereas, when measured, NHEJ (and sometimes SSA) remains intact. This gives rise to a scenario wherein spontaneous or induced DSBs in *BRCA*-deficient cells are rerouted for repair by mechanisms that are potentially error-prone, because the preferred mode of (error-free) processing by HR is unavailable. Experimental confirmation that error-prone DSB repair mechanisms predominate in murine *BRCA2*-deficient cells has recently been published (Tutt et al., 2001), and there is some evidence that this also occurs in *BRCA1* deficiency (Moynahan et al., 1999; Snouwaert et al., 1999). Thus, in its simplest conception, chromosomal instability provoked by *BRCA* deficiency is the result of incorrect

routing of DSB processing down inappropriate pathways, rather than the failure of repair per se.

A case can be made from recent data that this common defect in DSB “traffic control” nevertheless arises from distinct roles for *BRCA1* or *BRCA2* in the mechanisms for DSB repair. The differences are best viewed from the standpoint of relevant protein-protein interactions. In the discussion that follows, it will be proposed that the major role of *BRCA2* in DSB repair is through control of the *RAD51* recombinase, while *BRCA1* performs a distinct and more general function as a link between the sensing/signaling and effector components of the mammalian response to DNA damage, helping to ensure that the ensuing response is appropriate to the initiating lesion.

***BRCA2* Controls the *RAD51* Recombinase**

BRCA2 binds directly with *RAD51* (Sharan et al., 1997; Wong et al., 1997), a eukaryotic homolog of bacterial *RecA* essential for DSB repair by HR (but not SSA). The interaction involves a substantial proportion of the total cellular pool of each protein. It occurs primarily through the ~40 amino acid BRC motifs in *BRCA2*, eight repeats of which (Figure 1) are well conserved in sequence and spacing from mammals to birds (Takata et al., 2002).

RAD51 has a catalytic activity central to HR. It coats ssDNA to form a nucleoprotein filament that invades and pairs with a homologous DNA duplex, initiating strand exchange between the paired DNA molecules. Recent evidence suggests that *BRCA2* works directly to regulate the availability and activity of *RAD51* in this key reaction, consistent with the high stoichiometry of their binding (Figure 3).

This model is based on the observation that formation of the *RAD51* nucleoprotein filament *in vitro* is efficiently blocked by peptides encoding any one of several BRC repeats (Davies et al., 2001), the *RAD51* binding motifs within *BRCA2*. Inhibition is evident at a molar ratio of about 3:1 (BRC peptide:*RAD51*) and is equally effective on preformed *RAD51* nucleoprotein filaments. When in the presence of BRC peptides, recombinant *RAD51*—which normally polymerizes spontaneously—is rendered largely monomeric. By analogy to *RecA*, polymerization may be required for nucleoprotein filament formation, and its inhibition suggests a mechanism whereby BRC repeat binding could suppress *RAD51* activity.

Whether or not these *in vitro* biochemical observations based on BRC peptides are relevant to the cellular functions of full-length *BRCA2* is yet to be tested. It is attractive to speculate that sequestration by *BRCA2* of *RAD51*—an avid, abundant DNA binding protein—may be necessary to prevent the unwelcome activation of DNA recombination during normal DNA metabolism (Figure 3). If correct, this predicts that the release of *RAD51* from its sequestered, inactive state must specifically be triggered by DNA damage or replication arrest. Conceivably, the trigger could be phosphorylation of *BRCA2* or *RAD51* by one of the protein kinase cascades known to signal these problems.

Release from sequestration as meant here need not imply release from *BRCA2* binding; it is also possible that *BRCA2* carries active *RAD51* to sites of DSB pro-

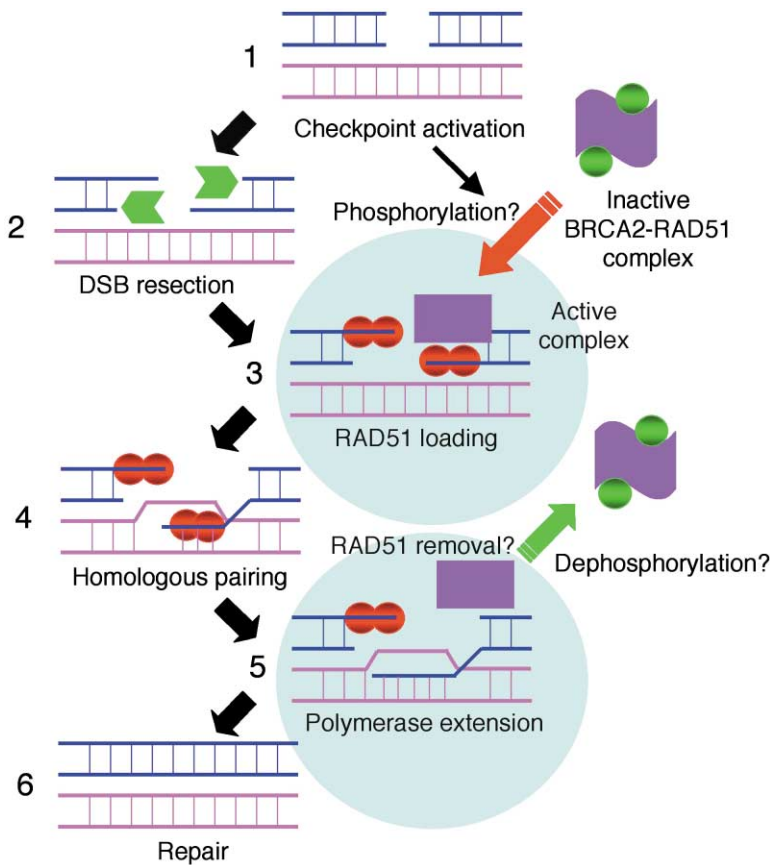


Figure 3. Hypothetical Model for BRCA2 Function in HR

An outline of the HR mechanism is shown: DNA damage or replication arrest cause DSBs that activate signaling mechanisms (1) and are then resected (2) by exonuclease activity to generate ssDNA tracts. RAD51 is loaded onto the ssDNA (3) to form a nucleoprotein filament that mediates homologous pairing (4) followed by strand extension (5), exchange, and repair (6). Putative functions of BRCA2 are highlighted in the gray circles. In this model, phosphorylation by DNA damage-signaling kinases such as ATM or ATR triggers transition of an inactive BRCA2-RAD51 (purple-green) complex to an active complex at the site of damage. At later stages in HR, dephosphorylation may permit the removal of RAD51 from nucleoprotein filaments by BRCA2 binding. Thus, there may be cycling between active and inactive states local to sites of repair. The stoichiometry of BRCA2-RAD51 binding shown here is not meant to be literal.

cessing or replication arrest (Figure 3), consistent with the defects in damage-induced localization of RAD51 that occur after BRCA2 disruption (Yuan et al., 1999; Yu et al., 2000). If this view is correct, the nature of BRCA2-RAD51 binding must change fundamentally in the active state such that nucleoprotein filament formation is no longer suppressed. It is curious in this regard that the spacing between the eight BRC repeat motifs in BRCA2 is conserved from mammals to birds, suggestive, perhaps, of a role in the nucleation of RAD51 filament formation along stretches of ssDNA. Does the shift from inactive to active states involve the “opening up” of a previously constrained BRCA2 structure, with a corresponding change in function from RAD51 sequestration to RAD51 nucleation?

That BRC repeat peptides can dissolve preformed RAD51 nucleoprotein filaments (Davies et al., 2001) provokes speculation about a possible role in the removal of RAD51 from DNA at later stages in the repair process. If phosphorylation serves as a trigger to activate BRCA2-RAD51 for nucleoprotein filament formation, dephosphorylation might suffice to reverse the transition, such that there is cycling between active and inactive states local to sites of repair (Figure 3).

Quantitative considerations raise significant, unanswered questions about these ideas. Given the lower expression of BRCA2 relative to RAD51, how can all (or even most?) nuclear RAD51 remain sequestered in an undamaged cell? Must the proteins come together prior to nuclear entry, such that only inactive RAD51 is deliv-

ered to the nucleus? What fraction of the nuclear BRCA2-RAD51 pool transits from the inactive to the active state following DNA damage? How (and how fast) can the transition be reversed?

A direct role of BRCA2 in the control of RAD51 may explain why both SSA and NHEJ (RAD51-independent processes) are used in BRCA2-deficient cells to repair site-specific DSBs (Tutt et al., 2001). In BRCA1-deficient cells, however, SSA and HR appear to be decreased, and NHEJ predominates as the mechanism for repair (Moynahan et al., 1999). In either scenario, misdirection of DSB repair through error-prone SSA or NHEJ, instead of error-free HR, could account for chromosomal instability through GCR formation at the sites of spontaneous or induced DSBs.

BRCA1 Links DNA Damage Sensing to Biological Responses

In what way does BRCA1 participate in pathways for DSB repair? BRCA1 is also required for efficient HR, but far less is known about how it may work in this reaction. Direct control of RAD51 activity seems unlikely (although the recent discovery that BRCA1 is part of an active ubiquitin ligase enzyme [see later in the review] may in time alter this stance), because the reported BRCA1-RAD51 complex contains no more than 2%–5% of the cellular content of each molecule (Scully et al., 1997b) and is not represented amongst the major BRCA1 pools that can be fractionated biochemically from cells (Wang et al., 2000).

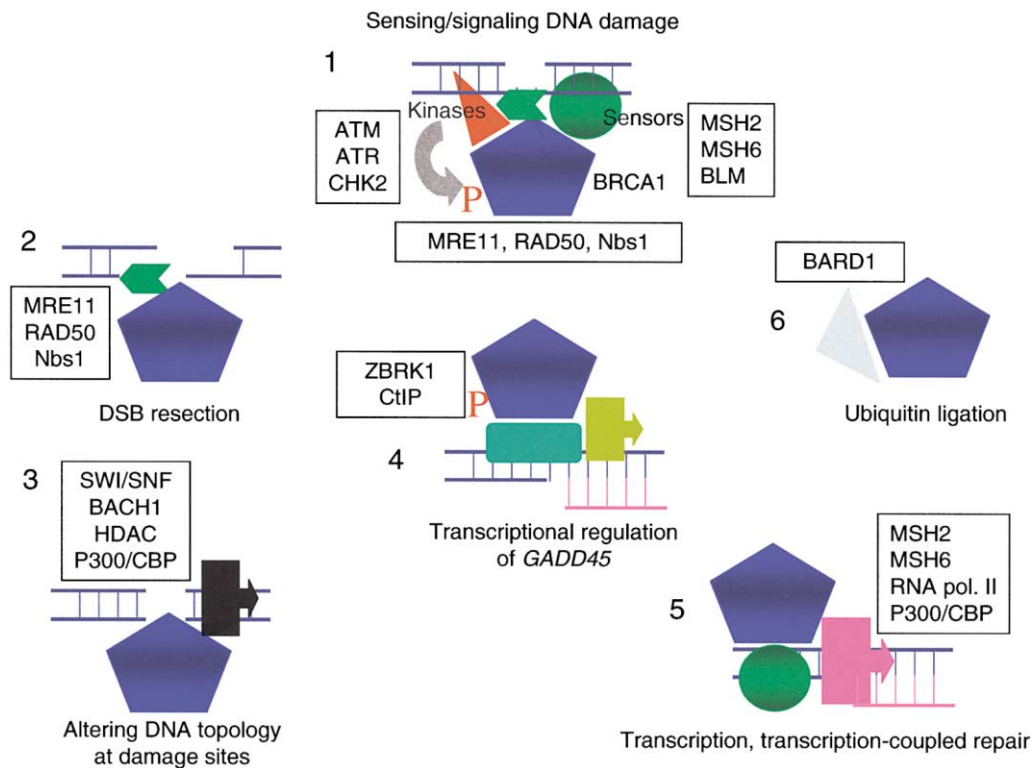


Figure 4. Putative Roles of BRCA1 Based on Reported Interactions

Protein-protein interactions that may mediate particular functions are marked, but the diagrams themselves are naive outlines of a complex (but currently uncertain) regulatory circuitry, and are for illustration only. BRCA1 works as a signal processor (1) during DNA damage responses in complex with proteins that bind to aberrant DNA structures (sensors), and the kinases that signal their presence. Phosphorylation of BRCA1 may be essential for local functions [control of DSB resection (2), altering DNA topology (3)] near a DNA lesion, as well as for distant functions such as transcriptional control of checkpoint genes (4) (e.g., *GADD45*) or targets of estrogen receptor signalling (see Note Added in Proof), or transcription-coupled DNA repair (5). BRCA1 works with BARD1 (6) as a ubiquitin ligase of unknown specificity—this interaction is not dependent on DNA damage and has far-reaching implications for function (see text). These roles need not be mutually exclusive, and will probably involve distinct intracellular pools or modification (by phosphorylation?) of BRCA1 (see text).

Local Activities of BRCA1 at Sites of DNA Damage

Evidence is mounting that BRCA1 plays a more proximal and extensive role in the cellular response to DSBs (Figure 4). Sites of DNA damage are marked within minutes by the phosphorylation of a histone species, H2A-X, which spreads over a region spanning thousands of bases around the lesion, suggesting that chromatin remodeling may occur to facilitate access of the repair machinery. BRCA1 is an early migrant to sites of H2A-X phosphorylation (Paull et al., 2000), consistent with a role in the events that follow at the site of breakage.

Several protein-protein interactions of BRCA1 seem relevant in this light. BRCA1 interacts with the MRE11/RAD50/Nbs1 protein complex (Zhong et al., 1999; Wang et al., 2000), containing the mammalian homologs of yeast molecules known to participate in DSB repair, comigrants to sites marked by phospho-H2A-X. Recent data suggests that the interaction is functional. MRE11 encodes a nuclease activity, which resects flush DSB ends to generate ssDNA tracts (Figure 4). Under certain *in vitro* conditions, BRCA1 can inhibit this activity of MRE11 (Paull et al., 2001), regulating the length and presumably the persistence of ssDNA generation at sites of DNA breakage. ssDNA is a substrate for repair by the HR and SSA mechanisms, and conceivably, a

role for BRCA1 in its generation may be important when directing DSB repair down these routes. Indeed, HR and SSA are defective (but NHEJ is unaffected) in BRCA1-deficient cells.

BRCA1 may also have local activities at DSB sites through its interaction with enzymes that alter chromatin and DNA structure. BRCA1 interacts with SWI/SNF (Bochar et al., 2000) or other proteins (Ye et al., 2001) that remodel chromatin, with regulators of histone acetylation/deacetylation (Pao et al., 2000; Yarden and Brody, 1999), and with DNA helicases, including the RecQ homolog encoded by the Bloom's syndrome gene, BLM (Wang et al., 2000), and the novel helicase BACH1 (Cantor et al., 2001). How these interactions may assist DSB repair is currently speculative. Chromatin changes mediated in part by histone modification could make DNA surrounding a break more accessible to the repair machinery, as could the recruitment of helicase activities.

DNA Damage Sensing and Checkpoint Control of the Cell Cycle

BRCA1 is rapidly phosphorylated after DNA damage in dividing cells, suggesting that it may work downstream of the checkpoint mechanisms that sense and signal DNA damage or problems with DNA replication, during S phase. In yeast, a group of four protein kinases (RAD3p/

MEC1, TEL1p, CHK1p, and CDS1p/RAD53p) are essential—and proximal—components of these mechanisms. There is now good evidence that BRCA1 is phosphorylated by the mammalian homologs of at least three kinases in this group and that the modifications contribute to its function. The kinases include ATM (the RAD3p/TEL1p homolog mutated in the human disease ataxia telangiectasia), the ATM-related MEC1p homolog ATR, and CHK2, homologous to CDS1p/RAD53p. Interestingly, patients with ataxia telangiectasia are susceptible to cancer, and cells deficient in ATM or ATR exhibit a progressive impediment to cell proliferation and spontaneous chromosomal instability, as do BRCA-deficient cells, hinting that the biological functions of these kinases may in part be mediated through BRCA phosphorylation.

BRCA1 as a Signal Processor for DNA Damage Responses

Phosphorylation of BRCA1 (Cortez et al., 1999; Lee et al., 2000; Tibbetts et al., 2000) by each of these kinases is activated by distinct stimuli and is targeted to distinct clusters of serine residues, suggesting that it will serve a distinct purpose in each instance. The chemical nature of the initiating DNA lesion is an important influence, with differences apparent among ionizing radiation (which primarily induces DNA breakage), UV light (nucleotide lesions), or replication arrest induced by hydroxyurea (strand gaps). Thus, ATM and CHK2 phosphorylate BRCA1 after ionizing radiation, whereas ATR is more specifically activated after UV treatment or replication arrest.

One functional consequence is in the checkpoint control of cell cycle progression. BRCA1 has been implicated in several different checkpoint events (Xu et al., 2001a). When exposed to ionizing radiation, BRCA1-deficient cells fail to arrest scheduled DNA synthesis in S phase, a feature also characteristic of ATM deficiency. The G2 arrest induced by radiation requires BRCA1, which must be phosphorylated by ATM for this response to occur. The S and G2 checkpoints are probably activated by DNA breakage, but another checkpoint response mediated by BRCA1, which is postulated to monitor the degree of chromatid decatenation as cells progress from G2 to M, is unresponsive to DNA breakage—and independent of ATM (Deming et al., 2001).

Thus, the scenario that emerges is one where BRCA1 works in multiple pathways that signal cell cycle delays in the presence of different kinds of DNA lesions (Figure 4). Consistent with this idea, BRCA1 cohabits in high-molecular-weight complexes with many different proteins that bind to abnormal DNA structures, as well as with the checkpoint kinases that activate downstream responses (Wang et al., 2000). Complex formation very probably changes dynamically during cell cycle and after different kinds of DNA damage: different multiprotein complexes may be created and dismantled in response to different stimuli. BRCA1's precise role here remains to be clarified. It is attractive to posit, however, that it works as a signal "processor" to coordinate DNA damage-sensing mechanisms with appropriate biological responses. From this viewpoint, it is easy to see how distinct patterns of BRCA1 phosphorylation by different kinases could reflect the circumstances of sensing, and in turn, could dictate the pattern of response.

A key prediction from this model is that the differential phosphorylation of BRCA1, already reported in response to different DNA damaging agents, will affect in a specific way its interaction with different effector pathways. Direct experimental evidence to support this idea is currently limited, and available information suggests it will not be easy to come by. The tumor suppressor p53 (like BRCA1, a protein to which a long list of functions in DNA damage responses has been ascribed) is a case in point. p53, too, undergoes complex patterns of phosphorylation by checkpoint kinases that are predicted to alter downstream functions; but many years after its description, the biological meaning of p53 phosphorylation is yet to be precisely specified.

The likely complexities of the role played by BRCA1 in connecting DNA damage-sensing and response mechanisms are well illustrated by its function in regulating the expression of *GADD45* (Harkin et al., 1999; Li et al., 2000; Zheng et al., 2000), a tumor suppressor gene that is also a downstream target of the p53 pathway. *GADD45* transcription is normally suppressed by a corepressor complex in which BRCA1 associates with the novel KRAB domain transcription factor ZBRK1. After ionizing irradiation, phosphorylation of BRCA1 by ATM relieves *GADD45* repression (although the mechanism by which this may be accomplished is contested [Wu-Baer and Baer, 2001] and remains unclear). In this way, BRCA1 processes signals through ATM to achieve transcriptional regulation of *GADD45* in response to DSBs; however, the specificity of this response and its molecular mechanism remain elusive.

Upstream and Downstream in DNA Damage Responses, Where Is BRCA1?

The portrait presented here gives BRCA1 both upstream and downstream roles in the response to DNA damage (Figure 4). On the one hand, BRCA1 participates in protein complexes that apparently have functions intrinsic to the sensing and signaling of different types of DNA lesions. On the other, it works as a sequence-specific transcriptional regulator of genes whose expression affects checkpoint enforcement and other downstream biological responses. Such diverse functions, while difficult to accommodate in one austere model without selectively ignoring particular swathes of experimental data, are nonetheless not mutually exclusive.

This wider "take" on BRCA1 function does highlight gaps in current knowledge. Notable amongst these is the incomplete information concerning the whereabouts of BRCA1 during division and after DNA damage, or replication arrest. The focal localization of BRCA1 in nuclear "dots" during S and G2 probably reflects only a fraction of the cellular pool, perhaps that which is bound to particular nuclear structures. Visible BRCA1 foci that appear after DNA damage (some of which also contain BRCA2 and RAD51) are likely to represent aggregates of hundreds or more molecules. They do not coincide (Paull et al., 2000) in timing or number with the predicted kinetics of DSB repair, or with the expected number of broken DNA ends. For all these reasons, available data probably provide only a narrow view of where BRCA1 is, or what it is doing. These caveats also apply, by and large, to BRCA2, and similarly restrict exposition of its function.

BRCA2 in Cell Cycle Control?

It is not known if BRCA2 is a target of S and G2 checkpoint kinases, but aspects of its behavior are changed when dividing cells undergo replication arrest or respond to DNA damage (Chen et al., 1998b), and there is reason to believe that the changes can be brought about by phosphorylation. Whether or not BRCA2 participates directly in cell cycle regulation or checkpoint function is more vexed. Where tested, checkpoint function is remarkably well preserved in BRCA2-deficient primary cells (Patel et al., 1998). It has been suggested that BRCA2 participates in G2/M control through its interaction with a novel protein, BRAF35, which preferentially binds in vitro to branched DNA structures (Marmorstein et al., 2001). BRAF35 and BRCA2 colocalize to condensing chromosomes, and the microinjection of antibodies against either molecule delays metaphase progression. It is not straightforward to conclude from these observations that BRCA2 directly regulates mitotic progression. For instance, because BRCA2 is essential for DNA repair, its dysfunction is predicted to cause the appearance of unrepaired DNA lesions, which could signal cell cycle delays through the activation of the known checkpoints that monitor DNA structure, indirectly delaying metaphase progression. Similar caveats apply to interpreting the centrosome amplification that occurs in BRCA2- (or BRCA1-) deficient cells (Tutt et al., 1999; Xu et al., 1999a) as being reflective of a role in mitotic control. It is clear that BRCA-deficient cells are slow to progress through S phase (see later in this review). These delays could—by prolonging the activity of cyclin/cdk complexes operational during S phase (reviewed in Nigg, 2001)—be an indirect cause. Thus, it is difficult to distinguish between the chicken and the egg, and more work will be needed before it becomes clear whether or not BRCA2 is a regulator of cell cycle events, independent of its role in DNA repair.

Fixing Stalled Replication Forks

Why should chromosomal instability occur spontaneously during the division of BRCA-deficient cells? Both BRCA1 and BRCA2 are essential for homologous recombination, and one appealing explanation stems from the notion that an important biological function of recombination systems is to enable the error-free reactivation of DNA replication forks stalled at template lesions (reviewed in Cox et al., 2000). It has been proposed (Venkitaraman, 2000) that the inability to carry out such a function could explain defects in cell proliferation and chromosomal structure observed in BRCA2-deficient cells; aspects of this scheme have also been applied to BRCA1 (Scully and Livingston, 2000; Wang et al., 2000).

Key features of the model come from work on *E. coli*, which shows that many DNA lesions encountered by a replication fork can block its progression, including strand gaps, ultraviolet light-induced nucleotide dimers, and base lesions from the spontaneous oxidation or hydrolysis of DNA. Fork progression in *E. coli* can be reactivated when homologous pairing between the nascent DNA strands enables bypass of the lesion, followed by origin-independent reinitiation of the replication fork from the recombination intermediates.

There is good evidence that recombinational mecha-

nisms for replication restart are frequently used during normal cell division, even where there is no exogenously induced DNA damage. For example, in recombination-defective *E. coli* RecBCD or RecARecD mutants, irreversibly stalled forks break down into unrepaired DSBs (Michel et al., 1997; Seigneur et al., 1998), which spontaneously accumulate to high levels even during normal growth. Moreover, there is limited but provocative evidence that homologous recombination is stimulated by, and necessary for, normal DNA replication in simple eukaryotes. When the progress of replication forks through the yeast ribosomal DNA genes is visualized by two-dimensional gel electrophoresis, X shaped Holliday junction structures are found to accumulate cyclically during normal S phase (Zou and Rothstein, 1997). These structures typically represent the crossovers between DNA strands undergoing recombination; thus, their formation in replicating loci links recombination to normal DNA replication.

The behavior of cells deficient in BRCA proteins in some respects can be related to the paradigms developed in simpler organisms. BRCA-deficient cells suffer a progressive impediment to cell division that worsens with repeated rounds of division in culture (Patel et al., 1998; Xu et al., 1999a), reminiscent of the growth of bacterial mutants deficient in recombination. Chromosomal aberrations accumulate the more often BRCA-deficient cells divide, the frequent occurrence of breaks affecting a single chromatid being consistent with failure to reactivate a replication fork stalled at a gap in the template. BRCA2-deficient cells spontaneously accrue DNA strand breaks during growth (Yu et al., 2000), just as do bacterial RecA pathway mutants, again suggestive of an inability to reactivate stalled replication.

Replication in mammalian cells undoubtedly proceeds in a more complex milieu than in bacteria or yeast, and the high level of cooperativity between replication and recombination implicit in this model cannot yet be explained in molecular terms. Although this needs much further investigation, some of the protein-protein interactions of BRCA proteins suggest their involvement. After hydroxyurea treatment, which stalls replication and induces recombination between sister chromatids, BRCA1 redistributes into discrete nuclear sites (Scully et al., 1997a; Zhong et al., 1999; Wang et al., 2000) that also contain proteins involved in fixing stalled replication (e.g., MRE11/RAD50/Nbs1), in sister chromatid recombination (e.g., BLM helicase), and in DNA polymerase loading (e.g., PCNA, RFC). BRCA2 may also be recruited to such sites (Chen et al., 1998b), where its role in RAD51 control could promote the recombinational events required for replication restart.

Replication “Housekeeping” and Cancer Predisposition

In yeast recombination mutants, replication-associated DSBs are premutagenic lesions. They may become substrates for error-prone repair pathways (Chen et al., 1998a) such as NHEJ or SSA, or they may direct break-induced replication (reviewed in Kraus et al., 2001), generating GCRs such as translocations or deletions. Such a scenario could plausibly explain characteristics of the spontaneous chromosomal instability that occurs in

BRCA-deficient cells, underpinning their mutability and propensity for neoplastic transformation. However, it is still not clear if such abnormalities are measurably enhanced in cells heterozygous for *BRCA* mutations. Studies in murine models have so far revealed no effect of heterozygosity, but it will be important to address this issue in human epithelial cells because inheritance of one defective *BRCA* allele suffices for cancer predisposition.

Transcription, Transcription-Coupled Repair, and RNA Metabolism

The contribution made to tumor suppression by other functions ascribed to BRCA1 and BRCA2 is currently unclear. Connections with transcription and RNA metabolism fall into this category. Reference has already been made, in relation to DNA repair and checkpoint enforcement, to BRCA1's interactions with proteins that modify chromatin structure and to its function as a transcription regulator, and there is limited evidence that BRCA2 may work similarly (Fuks et al., 1998). Moreover, BRCA1 interacts with the RNA polymerase II holoenzyme through a helicase component (Anderson et al., 1998). There is, as yet, no firm evidence that these interactions reflect general roles of BRCA proteins in the control of gene expression, whether tissue-specific or otherwise.

BRCA1-deficient cells are defective in transcription-coupled DNA repair (Gowen et al., 1998; Le Page et al., 2000), a process in which base lesions (following oxidative damage, for example) are removed preferentially from the transcribed DNA strand. Consistent with this idea, BRCA1 associates with the mismatch repair proteins MSH2 and MSH6, known mediators of this process (Wang et al., 2000). A similar, but less severe, defect has been reported in BRCA2-deficient cells, but protein interactions that could contribute to this have not yet been reported (Le Page et al., 2000).

BARD1, a protein which interacts with BRCA1 through the N-terminal RING domain (Figure 1), has been implicated in the control of RNA processing following DNA damage (Kleiman and Manley, 2001). Normally, nascent messenger RNAs must be endonucleolytically cleaved at their 3' end prior to polyadenylation: an *in vitro* assay for this reaction is found to be strongly but transiently inhibited following DNA damage. Inhibition is dependent upon BARD1 and possibly upon complex formation with BRCA1 and the polyadenylation factor CstF50 (Kleiman and Manley, 1999, 2001). The mechanism by which inhibition is brought about is not known, but it is tempting to speculate that the recently identified ubiquitin ligase activity of the BARD1-BRCA1 heterodimer (see later in this review) may be involved, targeting for degradation the proteins that carry out RNA processing. Interestingly, a cancer-associated BARD1 mutant lacks this function (Kleiman and Manley, 2001), a tentative link with tumor suppression.

BRCA1/BARD1 Is a Ubiquitin Ligase

It has recently been demonstrated that, like other RING proteins, the BARD1/BRCA1 complex functions as an E3 ubiquitin ligase of (as yet) undetermined specificity (Hashizume et al., 2001; Ruffner et al., 2001). The heterodimer is far more efficient in this activity than are its

component proteins, and recent studies suggest a structural basis for this, that may also be relevant to target specificity (Brzovic et al., 2001). This poorly characterized function of BRCA1 may have far-reaching implications for its biology. An enzymatic function of this kind could make interactions involving even a very small BRCA1 pool biologically significant, and because the conjugation of ubiquitin or ubiquitin-like molecules has profound and variable consequences for the longevity or activity of substrate proteins, could help to explain the apparent multiplicity of biological roles ascribed to BRCA1. Phosphorylation or other inducible changes in BRCA1 could alter the range of substrate specificities, connecting specific biologic responses to triggering stimuli. Indeed, the central question now pertaining to these findings relates to the nature of the cellular targets for the BRCA1/BARD1 complex (Baer, 2001).

One possible connection is already apparent. The protein encoded by a newly isolated gene *FANCD2* mutated in Fanconi's anemia, a disease characterized by chromosomal instability and cancer predisposition, localizes with BRCA1 to focal sites after DNA damage (Garcia-Higuera et al., 2001). Intriguingly, redistribution is dependent upon monoubiquitination of *FANCD2*, although it is not known if the BRCA1/BARD1 complex can perform this modification.

Checkpoint Inactivation and the Transformation of BRCA-Deficient Cells

Paradoxically in relation to their functions as tumor suppressors, mice deprived of BRCA1 or BRCA2 succumb to early embryonal lethality, accompanied by a severe proliferative defect and signs that p53-dependent cell cycle arrest has been activated (Ludwig et al., 1997; Sharan et al., 1997; Suzuki et al., 1997). Increased understanding of the requirement for BRCA1 and BRCA2 in the maintenance of chromosomal stability makes the basis for these phenotypes more clear, but how do they relate to tumor suppression?

If *BRCA* gene disruption were to occur in an otherwise normal cell, it seems likely that only a limited window of survival would be possible without the accompanying inactivation of cell cycle checkpoints, which would otherwise arrest growth in the face of unrepaired DNA damage and chromosomal aberrations. The chronology of events in the cancer-prone tissues of humans who inherit *BRCA* mutations, therefore, becomes significant. These individuals are heterozygous for the mutations, and since heterozygosity is not itself lethal to cells, the second event in tumorigenesis may involve checkpoint gene inactivation rather than loss of the second *BRCA* allele. Indeed, a study of the sequence of molecular events underlying pancreatic cancer progression in *BRCA2* heterozygotes lends some support to the idea that inactivation of the second *BRCA* allele occurs late (Goggins et al., 2000).

Mouse models have not provided much insight into this issue, because in this setting, heterozygosity alone is insufficient for cancer predisposition. Work in these models (Jonkers et al., 2001; Xu et al., 2001b) does show that coexisting mutations in checkpoint genes such as p53 can accelerate breast cancer development when BRCA1 or BRCA2 is conditionally disrupted in mammary

epithelium and can promote carcinogenesis when *BRCA* heterozygous mice are exposed to the genotoxic effects of ionizing radiation.

Is there evidence that p53 mutations are more frequent in cancers from *BRCA* mutation carriers? This has been hard to ascertain, because p53 mutations occur commonly in sporadic as well as familial cancers. A recent aggregated analysis (Greenblatt et al., 2001) of many prior case-control studies concludes not only that p53 mutations are more frequent in breast cancers that carry *BRCA* alterations, but also that the spectrum of mutations observed is different from sporadic cases. This latter observation lends substance to the idea that mutations inactivating particular functions of p53 may be important for the transformation of *BRCA*-deficient cells. Intriguingly, there is limited evidence that the p53 mutants isolated from *BRCA*-deficient tumors in mice (Lee et al., 1999) or humans (Greenblatt et al., 2001) share some unusual properties. They contain altered residues in the DNA binding domain of p53 outside the traditional mutation “hotspots” and exhibit gain-of-function activities that may be relevant to transformation.

Not all cancers from *BRCA* mutation carriers contain mutant p53, however, suggesting that there will be alternative paths to transformation in this setting. Indeed, the identity of the checkpoints that arrest growth in *BRCA*-deficient cells is not fully clear. Besides checkpoints governed by p53, there is *in vitro* evidence that the inactivation of mitotic checkpoints, such as the spindle assembly checkpoint mediated in mammals by a protein complex containing the Bub1 and BubR1 kinases, is sufficient to overcome growth arrest and promote the transformation of *BRCA2*-deficient murine cells (Lee et al., 1999). Whether mutations affecting this checkpoint mechanism also occur in human *BRCA*-deficient tumors has not yet been ascertained.

Chromosomal Instability Channels Tumor Evolution

In a model where chromosomal instability predisposes to carcinogenesis, it might be predicted that cancers arising in the setting of *BRCA* deficiency would be uniformly early in onset due to an accelerated mutation rate, but also highly variable in their histopathological and molecular features, for the same reason. There is evidence, however, that this is not so, although studies on human *BRCA*-deficient tumors are currently very preliminary. For instance, human *BRCA1*- or *BRCA2*-deficient breast cancers, far from uniform in their histopathology, do share features that distinguish them in a general way from one another and from sporadic cases (Lakhani et al., 1998), even though these are not clear-cut enough to enable diagnosis. Immunohistochemically, *BRCA1*-deficient tumors predominantly fail to express receptors for estrogen, unlike controls. Microarray-based expression profiles comparing a small sample of *BRCA1*-deficient or *BRCA2*-deficient breast cancers with *BRCA*-wild-type controls show a conservation of patterns, possibly robust enough to be of predictive value (Hedenfalk et al., 2001).

Are these findings incompatible with a caretaker role for *BRCA* genes in cancer predisposition? Not necessarily. *BRCA* disruption causes defects in chromosome

structure, cell division, and viability, and so a *BRCA*-deficient cell must acquire alterations during tumor evolution that overcome or at least ameliorate these problems. In molecular terms, this may force tumor evolution down a limited set of routes, limiting the genotypic and phenotypic variability of the end-product. Thus, as previously discussed, particular mutations in checkpoint genes probably offer a selective advantage for the transformation of *BRCA*-deficient cells, and their acquisition may have knock-on effects that further limit diversity. Such constraints may also be set through the effects of *BRCA* disruption on other biological processes, and not just on chromosome stability or checkpoint control. For example, there is emerging evidence that wild-type *BRCA1* inhibits the downstream effects of estrogen receptor signaling (Fan et al., 1999). This pathway could become constitutively active in *BRCA1*-deficient cells, in turn provoking the frequent loss of estrogen receptor expression found in *BRCA1*-deficient breast tumors.

The observed distinctions between *BRCA1*- versus *BRCA2*-deficient tumors emphasize that cancer evolution proceeds down different routes in each group, consistent with the arguments put forward here that the *BRCA* proteins perform distinct functions in overlapping biological processes. Further characterization of these differences at the molecular level will undoubtedly assist understanding of *BRCA* protein function, besides tumorigenesis. Furthermore, the inference that at least some of the molecular changes within each group will be stereotyped holds out hope for improvements in diagnosis and treatment.

Tissue Specificity of Cancer Predisposition

As yet we know of no good reason why cancer predisposition associated with *BRCA* gene mutations should manifest in particular epithelial tissues such as the breast, ovary, pancreas, or prostate. Several hypotheses may be entertained. *BRCA* disruption may have tissue-specific effects that favor transformation. For instance, it may make breast or ovarian cells more sensitive to the effects of local mutagens, such as estrogen metabolites. But, as mentioned previously, tissue-specific effects need not be restricted to a “caretaker” role in genomic stability: *BRCA1* is proposed to work as an inhibitor of estrogen receptor signaling (Fan et al., 1999), and thus, *BRCA* deficiency may promote cell outgrowth or survival, akin to loss of a “gatekeeper” function. Tissue-specific transcriptional regulation mediated in part by homology-dependent chromosome “imprinting” (Scully and Livingston, 2000) has also been invoked in a similar vein. The altered mammary gland development (Xu et al., 1999b) and hormone responsiveness (Bennett et al., 2000) reported in murine models of *BRCA* deficiency could reflect the loss of any such tissue-specific function.

Considered from the perspective that heterozygosity for *BRCA* mutations is sufficient to predispose to cancer, an alternative hypothesis is that the frequency with which loss of the second *BRCA* allele occurs may effectively be higher in certain tissues, perhaps those like the breast or ovary, in which prolonged proliferative quiescence (when *BRCA* gene function may be dispensable for viability) alternates with periodic bursts of proliferative activity.

eration. These conditions may favor the accumulation of cells in which both *BRCA* alleles have been lost, in particular, tissues like the breast and ovary. Having a larger pool of mutant cells from which tumors could eventually evolve may be of particular significance in light of the deleterious effects of homozygosity for *BRCA* mutations.

Whichever of these possibilities has merit (and they are neither exhaustive nor mutually exclusive), it bears emphasis that very little is known about the role of *BRCA* gene products in the biology of epithelial tissues. Without better information, it is unlikely that recent progress in defining the biological functions of *BRCA1* and *BRCA2* can be effectively translated into advances in the prevention or treatment of cancer.

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Note Added in Proof

Recent data suggest that BRCA1 inhibits estrogen receptor signaling through multiple mechanisms that include modulation of coactivator expression (Fan, S., Ma, Y.X., Wang, C., Yuan, R., Meng, Q., Wang, J., Erdos, M., Goldberg, I.D., Webb, P., Kushner, P.J., et al. [2002]. p300 modulates the BRCA1 inhibition of estrogen receptor activity. *Cancer Res.* 62, 141–151) and recruitment of histone deacetylase activity to promoter bound receptors (Zheng, L., Annab, L.A., Afshari, C.A., Lee, W.H., and Boyer, T.G. [2001]. BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor. *Proc. Natl. Acad. Sci. USA* 98, 9587–9592). Models to explain the tissue specificity (or estrogen receptor-negativity) of BRCA1-deficient cancers should be reevaluated in this light.