

Minireview

# Tumour Suppressor Mechanisms in the Control of Chromosome Stability: Insights from *BRCA2*

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**Cancer is unique amongst human diseases in that its cellular manifestations arise and evolve through the acquisition of somatic alterations in the genome. In particular, instability in the number and structure of chromosomes is a near-universal feature of the genomic alterations associated with epithelial cancers, and is triggered by the inactivation of tumour suppressor mechanisms that preserve chromosome integrity in normal cells. The nature of these mechanisms, and how their inactivation promotes carcinogenesis, remains enigmatic. I will review recent work from our laboratory on the tumour suppressor *BRCA2* that addresses these issues, focusing on new insights into cancer pathogenesis and therapy that are emerging from improved understanding of the molecular basis of chromosomal instability in *BRCA2*-deficient cancer cells.**

## INTRODUCTION

It has been clear for many years that somatic alterations of the genome occur at a high frequency in the majority of cells isolated from most common forms of cancer. Recent studies using next-generation sequencing (NGS) have provided a catalogue of such changes at much higher resolution than has previously been possible. Besides small sequence changes (such as point mutations, and small insertions or deletions of nucleotides), NGS studies highlight the abundance of chromosomal aberrations in cancer genomes. These chromosomal aberrations include different forms of structural abnormalities, such as gross chromosomal rearrangements (eg., large insertions or deletions, rearrangement between chromosomes, chromosome fragmentation etc) and regions of amplification, as well as numerical aberrations associated with the gain or loss of whole chromosomes (or aneuploidy). Neither the mechanisms underlying chromosomal instability in cancer cells, nor its potential significance to carcinogenesis and cancer evolution, is well understood.

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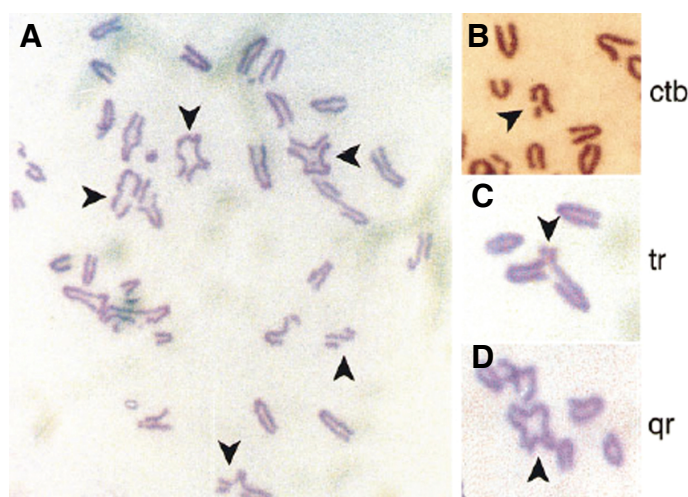
Inherited mutations affecting the *BRCA2* tumour suppressor provide a powerful model in which to study these issues. *BRCA2* was identified in the 1990s through the analysis of families at high risk from breast and ovarian cancer. It encodes a large protein, bearing little resemblance to molecules of known function, which is widely expressed in the dividing cells of many tissues, and localises to the cell nucleus. It has become clear over the past decade that *BRCA2* plays an essential role in preserving the structural and numerical stability of chromosomes during cell division. This review will focus on recent work from our laboratory that provides insights into the pathogenesis and treatment of epithelial cancers associated with *BRCA2* mutations.

## GERMLINE *BRCA2* MUTATIONS AND CANCER SUSCEPTIBILITY

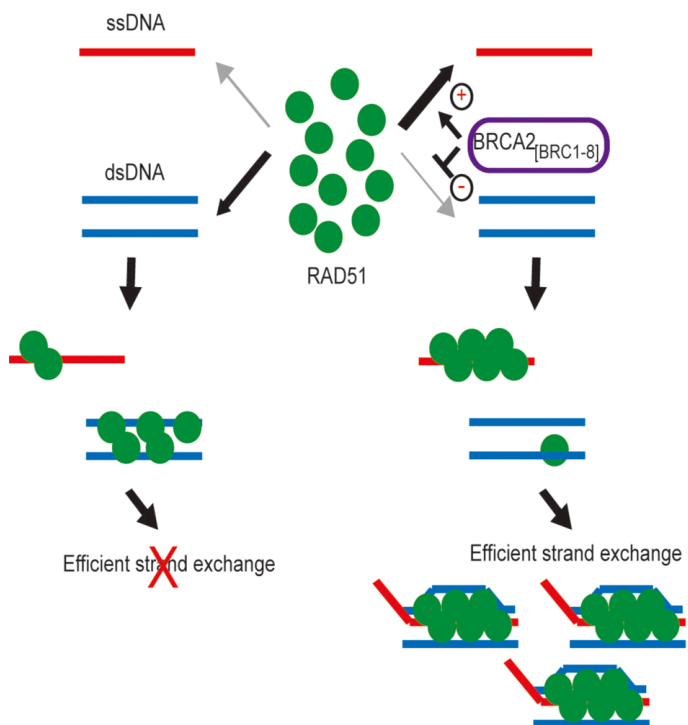
Germline mutations affecting one allele of *BRCA2* were first shown to confer susceptibility to breast and ovarian cancers with a high penetrance, which, by the age of 70 years approaches 40-50% for breast cancer, and 10-15% for ovarian. Individuals who carry *BRCA2* mutations also have an increased risk of cancers of the male breast (~75-fold relative risk), pancreas (4-8-fold), prostate (2-4-fold) or other tissues (Breast Cancer Linkage Consortium, 1999; Thompson and Easton, 2002). It has been proposed that germline heterozygosity for *BRCA2* mutations suffices for cancer predisposition, and there is evidence that the second *BRCA2* allele is inactivated by somatic alterations during tumour progression, consistent with the expectation for a classical 'two hit' tumour suppressor gene described by Knudson.

## *BRCA2* PERFORMS MULTIPLE CELLULAR FUNCTIONS IN TUMOUR SUPPRESSION

A few years after the discovery of the human *BRCA2* gene, we reported that cells carrying a genetically engineered truncation in murine *BRCA2* spontaneously develop instability in chromosome structure and number during division (Fig. 1). These aberrations offer important clues to the cellular functions of *BRCA2* in tumour suppression. For instance, structural aberrations include many chromatid-type lesions, such as breaks that affect a single sister chromatid, as well as tri-radial and quadri-radial structures. Such aberrations are also diagnostic of Fanconi Anaemia, providing a first connection between this syndrome and cancers associated with *BRCA2* deficiency (Patel et al.,



**Fig. 1.** Structural chromosomal aberrations in metaphase spreads from murine embryo fibroblasts homozygous for a targeted truncation in *Brca2*. (A) shows a typical metaphase spread, with arrows marking abnormal chromosomes. Chromatid-type aberrations enlarged in (B-D) show breaks affecting a single sister chromatid, tri-radial and quadri-radial chromosomes respectively. Reproduced with the permission of Elsevier from Patel et al. (1998).

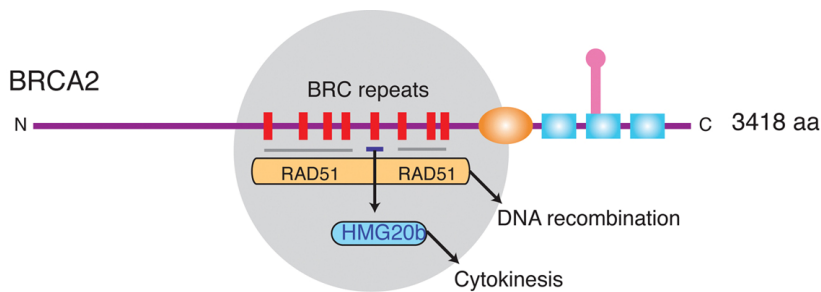


**Fig. 2.** Opposing effects of BRCA2 BRC repeats on RAD51 binding to ssDNA versus dsDNA bolster one another to stimulate strand exchange. When BRCA2 BRC repeats are absent (left-hand side), ssDNA and dsDNA compete for RAD51 binding. Strand exchange is inefficient because premature, stable RAD51 assembly on dsDNA is futile. The BRC repeats of BRCA2 favour the preferential assembly of RAD51 onto ssDNA because their interaction with RAD51 promotes RAD51-ssDNA binding while suppressing RAD51-dsDNA binding (right-hand side). These opposing effects reinforce one another to guide stepwise progression through strand exchange. Reproduced from Shivji et al. (2009).

1998). Similar abnormalities were afterwards shown to occur in murine cells lacking *BRCA1* (Xu et al., 1999) and in cancer cells from *BRCA* mutation carriers (Gretarsdottir et al., 1998; Tirkkonen et al., 1997).

Structural chromosomal aberrations following *BRCA2* inactivation are believed to arise from its role in DNA repair by homologous recombination (HR). *BRCA2* binds directly to the RAD51 recombination enzyme, which has a catalytic activity central to HR. RAD51 coats single-stranded DNA substrates to form a helical nucleoprotein filament that invades and pairs with the homologous DNA duplex, initiating strand exchange between the paired DNA molecules (reviewed in West, 2003). There is

compelling evidence that *BRCA2* works directly to regulate the availability and activity of RAD51 in these reactions, by controlling the strand-specificity of binding to single-stranded (ss) versus double-stranded (ds) DNA substrates. *BRCA2* binds directly to RAD51 through evolutionarily conserved BRC repeat motifs. In human *BRCA2*, 8 BRC motifs are dispersed in an 1127-residue region encoded by exon 11, in which their spacing exhibits conservation amongst mammalian species. We showed (Carreira et al., 2009; Shivji et al., 2009) that the binding of *BRCA2* BRC repeats to RAD51 suffices to promote the stability of RAD51-ssDNA binding, whilst inhibiting the binding of RAD51 to dsDNA substrates. During strand exchange reactions, when



**Fig. 3.** Divergent tumour-suppressive functions of the BRC repeats of BRCA2. The grey circle highlights in a schematic depiction of human BRCA2 the evolutionarily conserved region that contains 8 BRC repeats whose sequence and spacing is preserved amongst mammalian orthologues. Most BRC repeats, including BRC4, are known to bind and control the RAD51 recombinase in reactions that lead to homologous DNA recombination. BRC5 (but not BRC4) binds directly to HMG20b, and this interaction regu-

lates an unrecognized function for HMG20b in the efficient completion of cell division by cytokinesis. Thus, divergent tumour suppressive functions maintaining chromosome segregation as well as chromosome structure may be mediated by the conserved BRC repeats of BRCA2. Reproduced from Lee et al. (2011).

homologous ssDNA and dsDNA are both present, BRCA2 stimulates strand exchange by preventing premature and ultimately sterile engagement of RAD51 with dsDNA before formation of the ssDNA-RAD51 filament and the initiation of strand invasion and homologous pairing. Thus, BRCA2 targets RAD51 to ssDNA while inhibiting dsDNA binding and these contrasting activities together bolster one another to stimulate HR (Fig. 2).

Another role of BRCA2 whose loss may trigger chromosomal breakage is its essential function in the stabilization of stalled DNA replication forks in the late S and G2 phases of the cell cycle. Our laboratory showed several years ago that replication intermediates formed at stalled forks were broken down in BRCA2-deficient cells into dsDNA breaks (Lomonosov et al., 2003), and this has been subsequently attributed to the inhibition of endonuclease activity at stalled forks in a manner that may be independent of RAD51-mediated DNA repair (Schlachter et al., 2011).

Inactivation of *BRCA2* also causes aneuploidy. Murine *BRCA2*-deficient cells accumulate with > 4N DNA content (Patel et al., 1998), and aneuploidy is found in cancer cells from *BRCA2* mutation carriers. The origin of these anomalies is less clear, but may possibly be explained by functions of BRCA2 in mitotic progression at the spindle assembly checkpoint during the transition from metaphase to anaphase (Choi et al., 2012), and/or in the defective completion of cell division by cytokinesis at the stage of daughter cell abscission (Daniels et al., 2004; Lee et al., 2011; Mondal et al., 2012). However, roles for BRCA2 in controlling mitotic entry following activation of the G2 checkpoint for DNA damage have also been proposed (Ayoub et al., 2009; Menzel et al., 2011), although it is currently unclear if their loss contributes to chromosomal instability in *BRCA2*-deficient cells.

Concerning the spindle assembly checkpoint, recent data suggest (Choi et al., 2012) that BRCA2 recruits the PCAF acetyltransferase to the kinetochores of metaphase chromosomes during mitosis, facilitating the acetylation by PCAF of the kinetochore protein BubR1, increasing its stability. Inactivation of BRCA2 decreases BubR1 acetylation and the level of BubR1 during mitosis, leading to weakened checkpoint activity and aneuploidy.

BRCA2 inactivation also causes an increase in the frequency of cells that fail to complete division, but instead form multinucleate structures with  $\geq 4N$  DNA content (Daniels et al., 2004). Although cell cleavage by the actomyosin contractile ring can be initiated, and often reaches near-completion, in many divisions the abscission of the daughter cells does not proceed further. Indeed, BRCA2 is localized to the cytokinetic midbody

by filamin A, where it recruits components of the ESCRT (endosomal sorting complex required for transport) protein complex implicated in cytokinetic abscission (Mondal et al., 2012). Moreover, the cytokinetic role of BRCA2 appears to be separable (Lee et al., 2011) from its function in RAD51 regulation during HR (Fig. 3). Thus, BRCA2 binds to the cytokinetic regulator HMG20b via the BRC repeat BRC5, which binds poorly to RAD51. Conversely, the BRC4 repeat binds strongly to RAD51, but not to HMG20b. In cells, BRC5 overexpression inhibits the BRCA2-HMG20b interaction, triggering defects in cytokinesis. In contrast, BRC4 inhibits the BRCA2-RAD51 interaction and the assembly of RAD51 at sites of DNA damage, but has no effect on cytokinesis.

Thus, in summary, multiple functions for BRCA2 in the preservation of chromosome stability may explain its tumour suppressive function. These include functions in the control of RAD51 during DNA repair by homologous recombination, in mitotic entry at the G2/M transition, and in mitotic progression through functions at the spindle assembly checkpoint and during cytokinesis. The loss of these functions may account for the observed aberrations in chromosome structure and number that occur when BRCA2 is inactivated in cancer cells. The occurrence of chromosomal instability, in turn, plausibly drives mutagenesis and neoplastic transformation in cells lacking *BRCA2* (Venkitaraman, 2002; 2009).

### A GENETICALLY ENGINEERED MOUSE MODEL (GEMM) FOR TISSUE-SPECIFIC CARCINOGENESIS ASSOCIATED WITH *BRCA2* DEFICIENCY

Many genetically engineered mouse models (GEMMs) for the role of tumour suppressors in human carcinogenesis fail to recapitulate the complex combinations of germline and somatic alterations typically associated with human cancers, but instead, focus on the constitutive or conditional inactivation of one or both alleles of the tumour suppressor gene in different mouse tissues. In particular, human diseases associated with tumour suppressors such as *BRCA2* involve germline *BRCA2* alterations in all somatic tissues, with loss of the second allele expected only in the developing cancer cells, where they coexist with a number of additional oncogenic or tumour suppressive alterations. The failure of most available GEMMs to mimic these features means that they are frequently of limited value in modeling human carcinogenesis or cancer therapy.

To address this lacuna, we have created in our laboratory a GEMM for pancreatic cancer associated with inherited *BRCA2* mutations (Skoulidis et al., 2010). Pancreatic ductal adenocar-

cinoma (PDAC) represents the fourth leading cause of cancer mortality worldwide, with an incidence of approximately 217,000 new cases each year associated with ~213,000 deaths (Parkin et al., 2001). The most frequent genetic events underlying the initiation and progression of human pancreatic cancer (Hezel et al., 2006; Maitra and Hruban, 2008) include activating mutations in the KRAS proto-oncogene, in > 90% of PDAC cases (Caldas and Kern, 1995), besides mutations inactivating the TP53 gene in 50-75% of patients (Redston et al., 1994). Moreover, mutations inactivating *BRCA2* are found in an estimated 5-20% of familial PDAC (Couch et al., 2007; Hahn et al., 2003). Conversely, germline inheritance of pathogenic *BRCA2* mutations carries an ~4-8-fold increased lifetime risk of developing PDAC (Breast Cancer Linkage Consortium, 1999).

In patients with germline mutations in *BRCA2*, all somatic tissues contain the mutant allele, whereas the second *BRCA2* allele is wildtype (Wooster et al., 1994). Loss of this second, wild-type *BRCA2* allele in nascent cancer cells (termed 'loss of heterozygosity' or LOH) is widely believed to be essential for carcinogenesis in these patients. The pancreatic cancer model developed in our laboratory (Skoulidis et al., 2010) recapitulates these events (Fig. 4). A truncated allele of murine *Brca2* (*Brca2Tr*), resembling deleterious human germline mutations, which truncates the gene in an evolutionarily conserved and functionally critical region encoded by exon 11, is present in all somatic tissues (Friedman et al., 1998). The second *Brca2* allele, *Brca2F11* (Jonkers et al., 2001) can be conditionally disrupted to remove exon 11 by *loxP*-CRE recombination in the pancreas when PDX1-CRE is expressed there, mimicking LOH in human cancers from mutation carriers. Notably, tissue-specific activation of oncogenic *Kras*<sup>G12D</sup> is driven on a single allele by *loxP*-CRE mediated recombination in the pancreas, mimicking a genetic event that frequently triggers *Kras* activation in human cancers. In addition, in some strains, the mutant p53 allele mutant Tp53<sup>R270H</sup> can also be activated in the pancreas via CRE-mediated recombination. Thus, this mouse model not only faithfully recapitulates several of the key genetic alterations that have been implicated in PDAC pathogenesis, but also mimics the combination of mutant *BRCA2* alleles found in the somatic tissues and cancer tissues from *BRCA2* mutation carriers.

Unexpectedly, our studies reveal that *BRCA2* heterozygosity is sufficient to promote pancreatic cancer development in mice and men; inactivation of the second wild-type allele by LOH is not necessary. Irrespective of p53 mutations, heterozygosity for *Brca2<sup>Tr</sup>* acts with *Kras*<sup>G12D</sup> to accelerate the progression and development of PDAC. Similarly, in a small number of human pancreatic cancer samples from carriers of the Icelandic founder mutation in *BRCA2* (*BRCA2*<sup>999Del5</sup>) (Mikaelsdottir et al., 2004), the majority (75%) of the cases tested do not exhibit LOH. These findings suggest that *BRCA2* - contrary to belief - does not act as a typical Knudson 'two hit' tumour suppressor.

It is not clear how heterozygosity for *Brca2<sup>Tr</sup>* can promote tumorigenesis. One possibility is that this genotype causes defects in DNA repair, but this is not convincingly borne out by studies on cells derived from *Brca2<sup>Tr</sup>*-heterozygous mice (Patel et al., 1998; Yu et al., 2000), or from a similar transgenic model carrying another truncating allele in *Brca2* (Tutt et al., 2002). Thus, the evidence that heterozygosity for the *Brca2<sup>Tr</sup>* mutant allele creates a DNA repair defect is not convincing, although the possibility has not yet been fully excluded.

Our laboratory has recently identified (Jeyasekharan et al., 2013) a protein-targeting mechanism that is disrupted even by heterozygosity for the human mutation *BRCA2*<sup>D2723H</sup>, which is associated with cancer. We find that in the mutant protein

*BRCA2*<sup>D2723H</sup> a nuclear export signal in *BRCA2* that is normally masked by its interaction with a partner protein, DSS1, is exposed, rendering the mutant *BRCA2*<sup>D2723H</sup> into a substrate for binding by the nuclear exportin CRM1. This mislocalizes mutant *BRCA2*<sup>D2723H</sup> into the cytoplasm, in turn decreasing RAD51 nuclear retention even when wild-type *BRCA2* is also present. Thus, these findings suggest that a mechanism regulating the normal nucleo-cytoplasmic distribution of *BRCA2* and RAD51 can be impaired by a heterozygous cancer-associated mutation.

## BRCA2 HETEROZYGOSITY SUFFICES FOR CARCINOGENESIS: IMPLICATIONS FOR CANCER THERAPY

Our results suggesting that *Brca2* heterozygosity is sufficient for pancreatic carcinogenesis driven by mutant *Kras* in mice and men have important implications for cancer therapy. This is because the rationale for the use of targeted agents such as PARP1 inhibitors (PARPi) in *BRCA2*-deficient cancers requires bi-allelic *BRCA2* inactivation in the tumour cells (Bryant et al., 2005; Farmer et al., 2005). Indeed, we have shown in our work (Skoulidis et al., 2010) that PDAC cells that retain a functional *Brca2* allele are resistant to PARPi such as the AstraZeneca compound Olaparib. Our findings argue that in humans, PARP1 inhibitors should only be used in patients when *BRCA2* LOH can be verified in the tumour. Assessment of LOH therefore becomes a critical requirement in the design of human clinical trials for the treatment of *BRCA2*-deficient cancers.

There is mounting evidence that heterozygosity for *BRCA2* is sufficient for human carcinogenesis in tissues other than the pancreas, with the attendant implications for therapy using PARP1i. Lack of LOH has been documented using different methods in up to ~30% of breast cancers (King et al., 2007), by NGS in ~25% high-grade serous ovarian carcinomas (Cancer Genome Atlas, 2011), and in up to 55% of prostatic cancers associated with *BRCA2* mutation carriers (Willems-Jones et al., 2012). Thus, it will be essential in future clinical trial designs to test for LOH in tumour samples to help stratify likely responders to PARP1i or similar targeted therapies.

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