

SURVEY AND SUMMARY

BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution

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ABSTRACT

Germline mutations of the breast cancer associated gene 1 (*BRCA1*) predispose women to breast and ovarian cancers. *BRCA1* is a large protein with multiple functional domains and interacts with numerous proteins that are involved in many important biological processes/pathways. Mounting evidence indicates that *BRCA1* is involved in all phases of the cell cycle and regulates orderly events during cell cycle progression. *BRCA1* deficiency, consequently causes abnormalities in the S-phase checkpoint, the G₂/M checkpoint, the spindle checkpoint and centrosome duplication. The genetic instability caused by *BRCA1* deficiency, however, also triggers cellular responses to DNA damage that blocks cell proliferation and induces apoptosis. Thus *BRCA1* mutant cells cannot develop further into full-grown tumors unless this cellular defense is broken. Functional analysis of *BRCA1* in cell cycle checkpoints, genome integrity, DNA damage response (DDR) and tumor evolution should benefit our understanding of the mechanisms underlying *BRCA1* associated tumorigenesis, as well as the development of therapeutic approaches for this lethal disease.

INTRODUCTION

Breast cancer occurs at the highest frequency and is the second leading cause of cancer mortality in western women. Approximately 90% of breast cancers occur sporadically, without known predisposable genetic alterations. The remaining breast cancer cases are inheritable, which may be caused by mutations of tumor suppressor genes, such as the breast cancer associated gene 1 and 2 (*BRCA1* and *BRCA2*), and other

unidentified tumor suppressor genes (1–4). *BRCA1* was mapped in 1990 (5) and was subsequently cloned in 1994 (6). Germline mutations in *BRCA1* have been detected in approximately half of familial breast cancer cases and most cases of combined familial breast/ovarian cancers [reviewed in (2–4)]. *BRCA1* mutation carriers have 50–80% risk to develop breast cancer by the age of 70 (7–9). *BRCA1* contains 24 exons, encoding a full-length protein of 1863 amino acids in humans and 1812 amino acids in mice (10,11). *BRCA1* also encodes for at least two more protein products of smaller size due to alternative splicing (12–15). One of the variants, *BRCA1-Δ11*, is identical to the full-length form except for the absence of exon 11 (15). The other is *BRCA1-IRIS*, which is a 1399-residue polypeptide encoded by an uninterrupted open reading frame that extends from codon 1 of the known *BRCA1* open reading frame to a termination point 34 triplets into intron 11 (12). *BRCA1* full-length form contains multiple functional domains, including a highly conserved N-terminal RING finger, two nuclear localization signals that are located in the exon 11, an ‘SQ’ cluster between amino acids 1280–1524, and C-terminal BRCT domains (16). *BRCA1* interacts directly or indirectly with numerous molecules, including tumor suppressors, oncogenes, DNA damage repair proteins, cell cycle regulators, transcriptional activators and repressors (Table 1) (17). Consistent with this extensive pattern of interaction, loss-of-function mutations of *BRCA1* result in pleiotrophic phenotypes, including growth retardation, increased apoptosis, defective DNA damage repair, abnormal centrosome duplication, defective G₂/M cell cycle checkpoint, impaired spindle checkpoint and chromosome damage and aneuploidy [reviewed in (18–20)]. These phenotypes are not compatible, at least on the surface, with the tumor suppressor functions assigned to *BRCA1*. It is therefore proposed that mutations in *BRCA1* do not directly result in tumor formation, but instead they cause genetic instability, subjecting cells to a high risk of malignant transformation (21,22).

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Table 1. A list of BRCA1 interacting proteins

Biological functions	Interacting proteins
DDR and repair	MSH2, MSH6, MLH1, ATM, BLM and the RAD50-MRE11-NBS1, DNA replication factor C, RAD51, Fanconi anemia proteins, PCNA, H2AX, c-Abl, MDC1
Tumor suppressors	ATM, ATR, p53, BRCA2, RB, BARD1, BACH1
Oncogenes	c-Myc, casein kinase II, E2F1, E2F4, STK15, AKT
Transcription	RNA polymerase II holoenzyme (RNA helicase A, RPB2, RPB10 α), CBP/p300, HDAC and CtIP, estrogen receptor α , androgen receptor, ZBRK1, ATF1, STAT1, Smad3, BRCT-repeat inhibitor of hTERT expression (BRIT1)
Cell cycle related	Ayclin A, Cyclin D1, Cyclin D1, CDC2, Cdk2, Ckd4, γ -tubulin, p21, p27
Stress response, apoptosis	MEKK3, IFI16, X-linked inhibitor of apoptosis protein (XIAP)
Others	BAP1, BIP1, BRAP2, importin α

GENETIC INSTABILITY ASSOCIATED WITH BRCA1 MUTATIONS

One of the important features of BRCA1 associated breast cancer is that it contains a higher degree of aneuploidy than tumors without BRCA1 mutations (23). A cell line, HCC1937, which was derived from a homozygous BRCA1 deficient breast tumor, exhibits a high number of chromosomal gains and losses, homozygous deletion of tumor suppressors p53 and PTEN, and loss of heterozygosity at multiple loci known to be involved in the pathogenesis of breast cancer (24). Furthermore, mammary tumors developed from a mouse model carrying a mammary specific disruption of *Brca1* exhibit extensive chromosome aneuploidy (25). Using comparative genomic hybridization (CGH), and spectral karyotyping (SKY), Weaver *et al.* (26) demonstrated that the genomic instability in these tumors is similar to the pattern of chromosomal gains and losses, and structural abnormalities found in human BRCA1 mutation associated breast cancers. These alterations include the loss of all or a part of chromosome 14, including 14D3, where Rb1 is mapped, loss of p53 and over-expression of ErbB2, c-Myc, p27, cyclin E and cyclin D1, in the majority of tumors, although they were virtually ER-alpha and p16 negative (26,27).

The genomic abnormality found in BRCA1 mutant tumors is likely a direct consequence of BRCA1 deficiency, as the chromosome abnormalities also occurred in mouse embryos homozygous for a loss-of-function mutation in *Brca1* (28), in the mouse embryonic fibroblast (MEF) cells homozygous for a targeted deletion of *Brca1* full-length form (*Brca1* ^{$\Delta 11/\Delta 11$}) (29), and in cultured cells upon acute deletion of *Brca1* through Cre-LoxP mediated approaches (30,31). *Brca1* deficient cells also exhibited impaired DNA damage repair, centrosome amplification and abnormalities in all major cell cycle checkpoints (19,32). These defects should be responsible for the genetic instability found in BRCA1 mutant cells. As many of important aspects of BRCA1, including its structural feature and mutation spectrum in breast/ovarian cancers, the subcellular and intranuclear localization patterns, its hypothesized role in DNA double- or single-strand break repair, its assembly into DNA repair foci with multiple factors, and ubiquitination-ligase activity of BRCA1-BARD1 heterodimer, have been discussed in depth in a number of recent reviews (1,17–19,33–37) (<http://research.nhgri.nih.gov/bic/>), here I would like to focus on the current advances regarding the role of BRCA1 in cell cycle progression and its impact on genome integrity and cancer evolution.

FUNCTIONS OF BRCA1 IN CELL CYCLE CHECKPOINTS

The ability to control precisely the ordering and timing of cell cycle events is essential for maintaining genome integrity and preventing mutations that can disrupt normal growth control. Cells treated with DNA damaging agents, such as γ radiation, ultraviolet (UV) radiation, adriamycin or cisplatin, coordinately arrest their cell cycle progression at the G₁–S phase, the S phase and the G₂–M phase to allow times for repairing the damage. Cellular machineries that mediate cell cycle arrest are called cell cycle checkpoints, which monitor DNA status and ensure the completion of the previous phase in the cell cycle before advancing to the next phase (38–40). The *Brca1* transcripts are induced in late G₁ and become maximal after the G₁–S checkpoint (41). BRCA1 protein undergoes hyperphosphorylation during late G₁ and S, and is transiently dephosphorylated early after M phase (42). BRCA1 is also associated with numerous proteins that may play important functions in all phases of the cell cycle (Table 1). These observations suggest a putative role of BRCA1 in cell cycle regulation. Indeed, recent findings have indicated that BRCA1 is involved in all phases of the cell cycle and plays an important role in coordinating cell cycle progression, which is essential for maintaining genome integrity.

G₁/S cell cycle checkpoint

BRCA1–RB interaction. Shortly after the cloning of *BRCA1*, it was found that inhibition of BRCA1 expression with antisense oligonucleotides enhanced growth of normal and malignant mammary cells (43), and that introduction of wild-type BRCA1 into tumor cells inhibited cell proliferation (44). Interestingly, the growth-inhibitory activity associated with BRCA1 expression was only detected in a subset of cell lines tested, including breast and ovarian but not lung or colon cancer cells (44). Although this observation is consistent with the fact that human BRCA1 mutation carriers mainly develop breast and ovarian tumors, a study attributed this inhibitory activity to the presence of RB protein (45). Aprelikova *et al.* (45) found that only cells carrying wild-type RB were sensitive to BRCA1 induced G₁ arrest, while RB–/– cells were not. To provide a molecular basis for this observation, they further showed that BRCA1 interacts with hypophosphorylated RB. Since hypophosphorylated RB interacts with E2F to prevent transcription of downstream genes and inhibits cell proliferation, it is conceivable that binding to BRCA1 keeps RB in the hypophosphorylated state to achieve

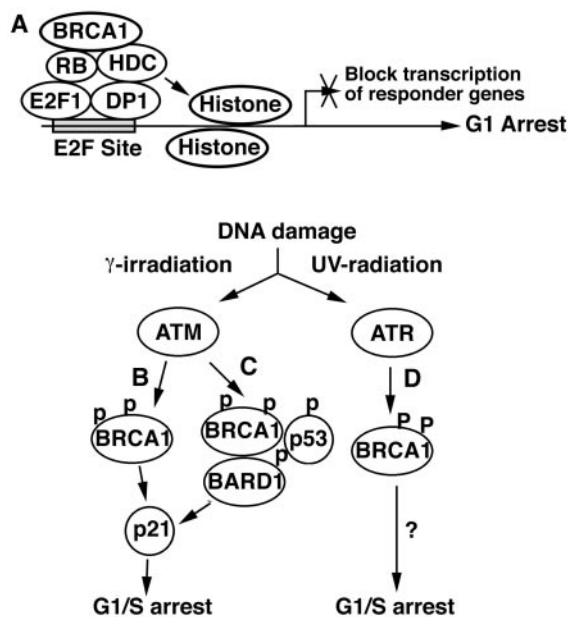


Figure 1. Current views of BRCA1 functions in G_1 and G_1/S cell cycle checkpoints. (A) A model illustrating a negative role for BRCA1 in G_1 arrest. BRCA1 binds to hypophosphorylated RB, which interacts with E2F to form an active complex that blocks transcription. BRCA1 and RB also recruit HDC, which deacetylates histones associated with the promoter, thereby promoting formation of nucleosomes that inhibit transcription. (B and C) Expression of BRCA1 may cause G_1/S arrest through a p53-independent (B), and p53 dependent (C) mechanisms. (D) UV radiation may also cause BRCA1-mediated G_1/S arrest, although it is not clear whether p21 is involved.

growth arrest. BRCA1 C-terminal domains (BRCT) also form a complex with two RB-binding proteins, RbAp46 and RbAp48, and histone deacetylases 1 and 2 (HDAC1 and HDAC2) (46). The RB and histone deacetylase complex (HDC) is thought to suppress transcription of E2F-responsive genes, providing additional evidence for growth inhibition through RB (Figure 1A).

BRCA1-p21 induction. Of note, an earlier investigation attributed the growth-inhibitory effect of BRCA1 to p21WAF1/CIP1, a well-known cyclin-dependent kinase inhibitor (47–49). Somasundaram *et al.* (50) showed that ectopic over-expression of a wild-type, but not a mutant form, of BRCA1 up-regulated p21 expression and prevented cell cycle progression into the S phase. Such an inhibition effect was not observed in p21 $^{-/-}$ cells. Because p53 is a major regulator of p21 expression, they also transfected BRCA1 into p53 $^{-/-}$ cells and found that the induction of p21 and inhibition of cell growth by BRCA1 did not require wild-type p53 (Figure 1B).

ATM-BRCA1/BARD1-p53-p21 axis. Contrasted to the above finding, a recent study provided evidence that p53 is involved in the BRCA1 induced G_1/S cell cycle checkpoint. It was shown that BRCA1 always forms a heterodimer with BARD1, a BRCA1 associated RING domain protein, *in vivo*, and the heterodimerization is required to maintain their stability (37,51). Using small interfering RNA (siRNA) to deplete BRCA1 or BARD1, Fabbro *et al.* (52) demonstrated that the BRCA1–BARD1 complex is required for ATM/ATR (ataxia-telangiectasia-mutated and

Rad3-related)-mediated phosphorylation of p53 at Ser-15 following IR or UV radiation-induced DNA damage. This study had several interesting findings. First, the dependence of p53(Ser-15) phosphorylation on the BRCA1–BARD1 complex is quite unique, as phosphorylation of a number of other ATM/ATR targets including H2AX, Chk2, Chk1 and c-jun does not depend on the presence of BRCA1–BARD1 complex. Second, although both IR and UV radiation-induced p53(Ser-15) phosphorylation, only IR radiation could induce G_1/S arrest, while UV radiation did not. Because rapid IR-induced phosphorylation of downstream targets is catalyzed by ATM, while ATR mediates rapid UV induced phosphorylation, this observation suggests that the BRCA1–BARD1 complex mediates ATM, but not ATR, catalyzed p53(Ser-15) phosphorylation. Lastly, they demonstrated that the inhibition of p53(Ser-15) phosphorylation by BRCA1–BARD1 acute suppression compromises p21 induction and G_1/S checkpoint arrest. Thus, this study reveals an important role of p21 in mediating BRCA1 function in G_1/S arrest by IR radiation, and the induction of p21 transcription requires p53 activation (Figure 1C).

A prediction from these observations is that the absence of BRCA1 might decrease p21 expression and impair the G_1/S cell cycle checkpoint. However, inconsistently, BRCA1-null embryos exhibited increased levels of p21 and cellular proliferation defects, and died at early postgastrulation stages (28). This is perhaps due to p53 activation triggered by un-repaired DNA damage and genetic instability. Consistently, BRCA1-null embryos survived 1–2 days longer in a p53-null or p21-null genetic background (28,29,53,54). Thus, the activation of p53 triggered by genetic instability in BRCA1 $^{-/-}$ embryos obscures the functions of BRCA1 in the G_1/S cell cycle checkpoint. It was shown previously that MEF cells carrying double mutations of BRCA1 and p53 were defective at the G_1/S cell cycle checkpoint (29). However, this defect cannot be attributed to BRCA1 deficiency, as the absence of p53 alone could impair the G_1/S cell cycle checkpoint (49,55).

Interestingly, a recent study using primary fibroblasts from human BRCA1 heterozygotes (BRCA1 $+/-$) and wild-type (BRCA1 $+/+$) indicated that UV radiation could induce G_1/S cell cycle arrest in BRCA1 $+/+$ cells, while BRCA1 $+/-$ cells displayed a moderate impaired G_1/S cell cycle checkpoint (56). Because UV radiation does not activate the p53 mediated G_1/S cell cycle checkpoint (52), this study provides an example that reduced dose of BRCA1 could attenuate the G_1/S arrest in cells where p53 is not activated. UV induced phosphorylation is primarily mediated by ATR, which has been shown to interact with BRCA1 (57). However, it remains unclear how BRCA1 induces the G_1/S arrest upon UV radiation (Figure 1D).

In summary, BRCA1 induced G_1/S arrest may occur through a number of distinct pathways that involve many important BRCA1 interacting proteins, including ATM, ATR, BARD1, RB, p53 and p21 and their downstream effectors (Figure 1).

S phase cell cycle checkpoint

Another cell cycle checkpoint induced by ionizing radiation is the S-phase checkpoint, which primarily represents an inhibition of replication initiation upon the DNA damage. A lack of

an IR-induced S-phase checkpoint results in persistent DNA synthesis [radioresistant DNA synthesis (RDS)] at early time points after IR. Studies indicated that cells isolated from A-T patients and Nijmegen breakage syndrome (NBS) patients were defective in this checkpoint (58). However, conflicting results were reported regarding whether BRCA1 plays a role in the S-phase checkpoint. One study reported a normal S-phase checkpoint in the BRCA1^{-/-} HCC1937 cells, but no experimental details were provided to support this claim (59). In contrast, Xu *et al.* (60,61) found that HCC1937 cells were actually defective in S-phase checkpoint, exhibiting a RDS similar to that displayed by AT mutant and NBS mutant cells. Furthermore, their data indicated that the defective IR-induced S-phase checkpoint in HCC1937 cells could be overcome by reconstitution of a wild-type BRCA1, which confirms a role of BRCA1 in this checkpoint.

ATM-BRCA1 interaction. BRCA1 is phosphorylated at several serine sites, including Ser-988, -1387, -1423, -1497 and -1524 upon IR radiation (62,63). To explore whether one of these sites was an important determinant of the IR-induced S-phase checkpoint, mutations of some of these sites were introduced into BRCA1 and tested in HCC1937 cells. Data indicated that wild-type BRCA1 and BRCA1 constructs mutated at either Ser-1423 or both Ser-1423 and Ser-1524 all complemented the defective IR-induced S-phase checkpoint in HCC1937 cells. In contrast, transfection of a construct bearing a mutation at Ser-1387 of BRCA1 failed to complement this defect (61). Because the Ser-1387 in BRCA1 is a known target of ATM phosphorylation, this finding indicates that the phosphorylation of Ser-1387 in BRCA1 is specifically required for the ATM-mediated S-phase checkpoint after ionizing irradiation.

ATR-BRCA1 interaction. Evidence has also been presented that BRCA1 is involved in the S-phase checkpoint activated by stalled replication forks, which can be induced by treatment of cells with UV or hydroxyurea (HU). It has been shown that ATR and ATM phosphorylate BRCA1 on several distinct and overlap Ser/Thr residues, including Ser-1423 (62–64). Tibbetts *et al.* (64) demonstrated that increased expression of ATR enhanced the phosphorylation of BRCA1 on Ser-1423 following cellular exposure to HU or UV light, whereas doxycycline-induced expression of a kinase-inactive ATR mutant protein inhibited HU- or UV light-induced Ser-1423 phosphorylation. Furthermore, the dramatic relocalization of ATR nuclear foci in response to DNA damage overlaps with the nuclear foci formed by BRCA1. Thus, ATR directly phosphorylates BRCA1 in response to damaged DNA or stalled DNA replication, and both proteins are likely the components of the same genotoxic stress-responsive pathway. These results suggest that ATR activates the intra-S checkpoints in response to stalled replication forks in a manner analogous to that of ATM-dependent induction of these checkpoints after exposure to IR.

BRCA1 also interacts with several other proteins that play an essential role in the S-phase checkpoint. These include the mediator of DNA damage checkpoint protein 1 (MDC1), H2AX, p53 binding protein 1 (53BP1) and MRE11/RAD50/NBS1 (MRN), which form nuclear foci upon IR radiation and arrest cells in the S phase (65). In addition, Durant

and Nickoloff (66) have also proposed a cell-cycle-dependent model in which DNA-PK inhibits replication protein A in the S phase of the cell cycle, while BRCA1 inhibits the exonuclease activity of the MRN complex and facilitates DNA repair and S phase arrest.

G₂/M cell cycle checkpoint

In addition to the G₁/S cell cycle checkpoint and the S phase cell cycle checkpoint, IR radiation also activates the G₂/M cell cycle checkpoint, which rapidly delays movement of G₂ cells into the mitosis (M) phase. Loss of this checkpoint allows cells with damaged DNA to proceed into the M phase, increasing the likelihood of abnormal chromosomes being passed to the daughter cells. Using MEF cells derived from *Brcal*^{Δ11/Δ11} embryos, Xu *et al.* (15) investigated whether the absence of BRCA1 would affect this cell cycle checkpoint. Their data indicated that BRCA1 wild-type cells exhibited a sharp reduction in mitotic index within 1–4 h after IR radiation. In contrast, *Brcal*^{Δ11/Δ11} cells showed no reduction in mitotic index upon IR radiation during the same period of the time. The lack of an immediate mitotic delay following γ-irradiation indicates that elimination of full-length BRCA1 abolishes this checkpoint. They also treated cells with UV radiation and methyl methanesulfonate (MMS). Their data indicated that UV treated *Brcal*^{Δ11/Δ11} cells showed a dramatic reduction in mitotic index, while the MMS treated mutant cells were largely defective in the G₂/M cell cycle checkpoint. Taken together, these observations suggest that the defect in the G₂/M cell cycle checkpoint is specific to certain types of DNA damage.

BRCA1-Chk1 interaction. BRCA1 interacts with many proteins that play a role in cell cycle progression (Table 1). It is important to determine whether any BRCA1 interacting proteins are involved in this process, and how they affect the G₂/M cell cycle checkpoint in relation to BRCA1. Yarden *et al.* (67) provided a significant advance on this issue, showing that BRCA1 regulates the expression, phosphorylation and cellular localization of Chk1, a known regulator of the G₂-M cell cycle checkpoint. Their data also indicated that BRCA1 affects the expression of both Wee1 kinase, an inhibitor of Cdc2/cyclin B kinase, and the 14-3-3 family of proteins that sequesters phosphorylated Cdc25C and Cdc2/cyclin B kinase in the cytoplasm (68,69). Thus BRCA1 regulates key effectors that control the G₂/M checkpoint and is therefore involved in regulating the onset of mitosis (Figure 2).

BRCA1-ATM interaction. The chain of the DNA damage response (DDR) starts with upstream kinases, ATM and ATR, which phosphorylate DNA damage sensors, Chk1 and Chk2, and play important roles in the G₂/M cell cycle checkpoint (70). ATM, ATR and Chk2 also interact with and phosphorylate BRCA1 (57,62–64). It was revealed that the phosphorylation of BRCA1 by ATM is required for activation of the G₂/M checkpoint using the HCC1937 cells, which are defective in both S phase and G₂/M cell cycle checkpoints (60,61). In their effort to distinguish the role BRCA1 Ser-1387 and Ser-1423 in these cell cycle checkpoints, they found that mutation of BRCA1 Ser-1423 abolished the ability of BRCA1 to mediate the G₂/M checkpoint, while mutation of BRCA1 Ser-1387 affected S phase function of BRCA1 upon IR

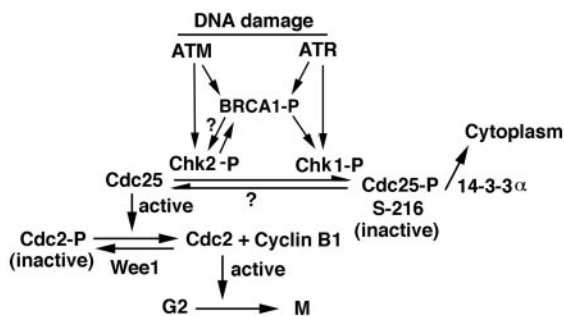


Figure 2. A model illustrating a role of BRCA1 in the G₂/M cell cycle checkpoint. BRCA1 can be phosphorylated by ATR, ATM and Chk2. BRCA1 regulates expression and cellular localization of Chk1, although it is not clear whether Chk1 can phosphorylate BRCA1. Both Chk1 and Chk2 inactivate Cdc25 by phosphorylate it at Ser-216. The phosphorylation of Cdc25 at Ser-216 not only inactivates this protein but also allows it to bind to 14-3-3 α , leading to its exporting from the nucleus. This results in the decrease of the active form of Cdc25, which is a phosphatase involved in dephosphorylation of Cdc2. The dephosphorylated form of Cdc2 is the active form that promotes cell progression from G₂ to M phase. Therefore, the reduced amount of Cdc25 results in the decrease of active form of Cdc2, which prevents G₂ to M transition. Another two factors are also involved in this pathway. One is Cyclin B1, which forms an active complex with Cdc2 to promote cell progression from G₂ to M phase, and the other is Wee1, a kinase that puts phosphate on an inhibitory site of Cdc2, thereby inhibiting function of Cdc2. Thus, expression changes of these genes could theoretically affect G₂/M cell cycle checkpoint.

radiation (60,61). These results clarify the cell cycle checkpoint roles for each of these phosphorylation products.

BRCA1-Chk2 interaction. Chk2, which is an immediate phosphorylation target of ATM, phosphorylates and co-localizes with BRCA1 within discrete nuclear foci prior to DNA damage by γ -irradiation (71). Phosphorylation of BRCA1 at serine 988 is required for the release of BRCA1 from Chk2. This phosphorylation is also important for the ability of BRCA1 to restore survival after DNA damage in the BRCA1-mutated cell line HCC1937. To study the impact of Ser-988 mutation on BRCA1 function, Kim *et al.* (72) mutated the equivalent serine (Ser-971) in mouse *Brcal* using a knock-in approach. The *Brcal*^{S971A/S971A} mice were developmentally normal, however they suffered a moderately increased risk of spontaneous tumor formation, with a majority of females developing uterus hyperplasia and ovarian abnormalities at two years of age. After treatment with DNA damaging agents, i.e. γ -irradiation and MNNG, *Brcal*^{S971A/S971A} mice exhibited several abnormalities, including increased body weight, abnormal hair growth pattern, lymphoma, mammary tumor and endometrial tumor. In addition, the onset of tumor formation became accelerated with 80% of mutant mice developing tumors at 1 year of age. These observations suggest that Chk2 phosphorylation of Ser-971 is involved in *Brcal* function in modulating DDR and repressing tumor formation. The *Brcal*^{S971A/S971A} mutant cells also exhibited a partially loss of the G₂/M cell cycle checkpoint upon IR radiation. This study suggests that G₂/M checkpoint regulation of BRCA is partly modulated by Chk2 phosphorylation in addition to other factors, such as ATM and Chk1 as reviewed earlier (Figure 2).

BRCA1-Aurora interaction. Aurora-A is one of three serine/threonine kinases (A, B and C) and is known to regulate mitotic progression in various organisms [reviewed in (73–76)]. Aurora-A is frequently amplified in several human

cancers and over-expression of Aurora-A has been detected in 94% of invasive duct adenocarcinomas of the breast (77–83). It was recently shown that Aurora-A physically binds and phosphorylates BRCA1 at S308, and the phosphorylation is correlated with impaired function of BRCA1 in regulating G₂/M transition (84). This finding suggests a link between Aurora-A over-expression and impaired BRCA1 function in genetic instability and tumorigenesis. However, it was recently reported that transgenic mice carrying *Aurora-A* over-expression in the mammary gland did not develop mammary tumors although they all exhibited mammary hyperplasia (85). In that study, chicken β -actin promoter was linked to the *Aurora-A* gene through a stopper sequence. The transgene is not expressed unless the stopper is deleted by WAP-Cre, which is activated by pregnancy hormones. Because all the mice only went through one full-cycle of pregnancy, it is possible that the deletion of the stopper, which is required for the expression of the *Aurora-A* transgene, was incomplete. To test this, we have generated a transgenic mouse strain that over expresses the *Aurora-A* in the mammary epithelium using the promoter of the mouse mammary tumor virus (MMTV). We found that the *MMTV-Aurora* transgene is expressed at high levels during the mammary cycle of development and induces mammary tumor formation in about 50% of mice with an average latency of 20 months (X. Wang and C. X. Deng, unpublished data). Further investigations are required to determine whether or not the tumorigenesis in the *MMTV-Aurora* transgenic mice is entirely or partly due to the impaired BRCA1 function in regulating the G₂/M cell cycle checkpoint.

Other BRCA1 interacting proteins. Upon UV radiation, BRCA1 is phosphorylated on Ser-1423 and Ser-1524 by ATR (86). It was shown previously that BRCA1 mutant MEF cells exhibited a normal G₂/M cell cycle checkpoint upon UV radiation (15), suggesting that phosphorylation of BRCA1 by ATR may not have a direct impact on this checkpoint. This observation, however, does not rule out a possibility that ATR may play a role in the BRCA1-mediated G₂/M checkpoint through other mechanisms. For example, the genetic interplay between BRCA1 and ATR in the G₂/M cell cycle checkpoint could occur in their common downstream genes, such as Chk1. In addition, it was shown that BRIT1 (BRCT-repeat inhibitor of hTERT expression), a repressor of human telomerase function that is implicated in cellular immortalization (87,88), is required for the expression of BRCA1, NBS1 and Chk1 (89). Lin *et al.* (89) found that when BRIT1 expression is depleted by RNAi, cells exhibited defects in both the S phase and G₂/M cell cycle checkpoints and become IR radiation sensitive. Thus, the checkpoint defects in the absence of BRIT1 are likely to result from its regulation of Nbs1, BRCA1 and Chk1.

The spindle checkpoint

During the mitotic phase, duplicated DNA is first condensed and packed to form sister chromatids, which are then equally separated into newly formed daughter cells. Any premature or mis-separation of sister chromatids will lead to the loss or gain of chromosomes in daughter cells, leading to aneuploidy, which is a prevalent form of genetic instability of human cancers (40). The spindle checkpoint ensures the astonishing accuracy of chromosome segregation by preventing cells with

unaligned chromosomes from exiting mitosis. Molecular components of the spindle checkpoint include two evolutionarily conserved protein families, Mad (Mad1, Mad2, Mad3/BubR1) and Bub (Bub1, Bub2, Bub3), as well as other components [reviewed in (90–93)]. In the metaphase, the sister chromatids attach to the mitotic spindle at kinetochores that consist of protein complexes associated with centromeric DNA. After all sister chromatids have attached to the bipolar spindle and their kinetochores are under tension, a large ubiquitin ligase called the anaphase-promoting complex (APC) and its associated substrate-binding co-factor, Cdc20, are activated. The activated APC-Cdc20 tags the securin protein with polyubiquitin chains and promotes its degradation. This, in turn, activates the separate and results in a proteolytic cleavage of the cohesion complex between the sister chromatids and triggers the onset of the anaphase. The spindle checkpoint monitors the attachment of sister chromatids to the spindle and is activated by the lack of microtubule occupancy and tension at the kinetochores (Figure 3A). During the process of spindle checkpoint activation, transient interaction between BubR1-Bub3-Cdc20 and

Mad2-Cdc2 complex leads to the formation of the BubR1-Bub3-Mad2-Cdc20 complex (the MCC), which is a more efficient inhibitor of APC (Figure 3B). A number of investigations revealed an essential role of Mad2 in the MCC [reviewed in (94–96)]. Consistently, microinjection of Mad2 antibodies yields premature anaphase onset and chromosome mis-segregation (97). Absence of Mad2 in mouse embryos resulted in accumulation of mitotic errors and apoptosis, leading to early lethality at E5-E6, while haploinsufficiency of Mad2, which results in about 30% less of Mad2 protein, provokes lung tumors after a long latency period (98,99).

Studying *Brca1*^{Δ11/Δ11} cells during the mitotic phase, Wang *et al.* (31) found that about 30% of these cells displayed abnormal chromosomes, including chromosome bridging, lagging chromosomes in metaphase, anaphase and telophase (Figure 3C and D). It has been shown that a single unattached kinetochore is sufficient to activate the spindle checkpoint and arrest the cell at metaphase (100). Thus, the observations that *Brca1*^{Δ11/Δ11} cells exhibited abnormal chromosome behavior and could advance to the anaphase and telophase suggest that

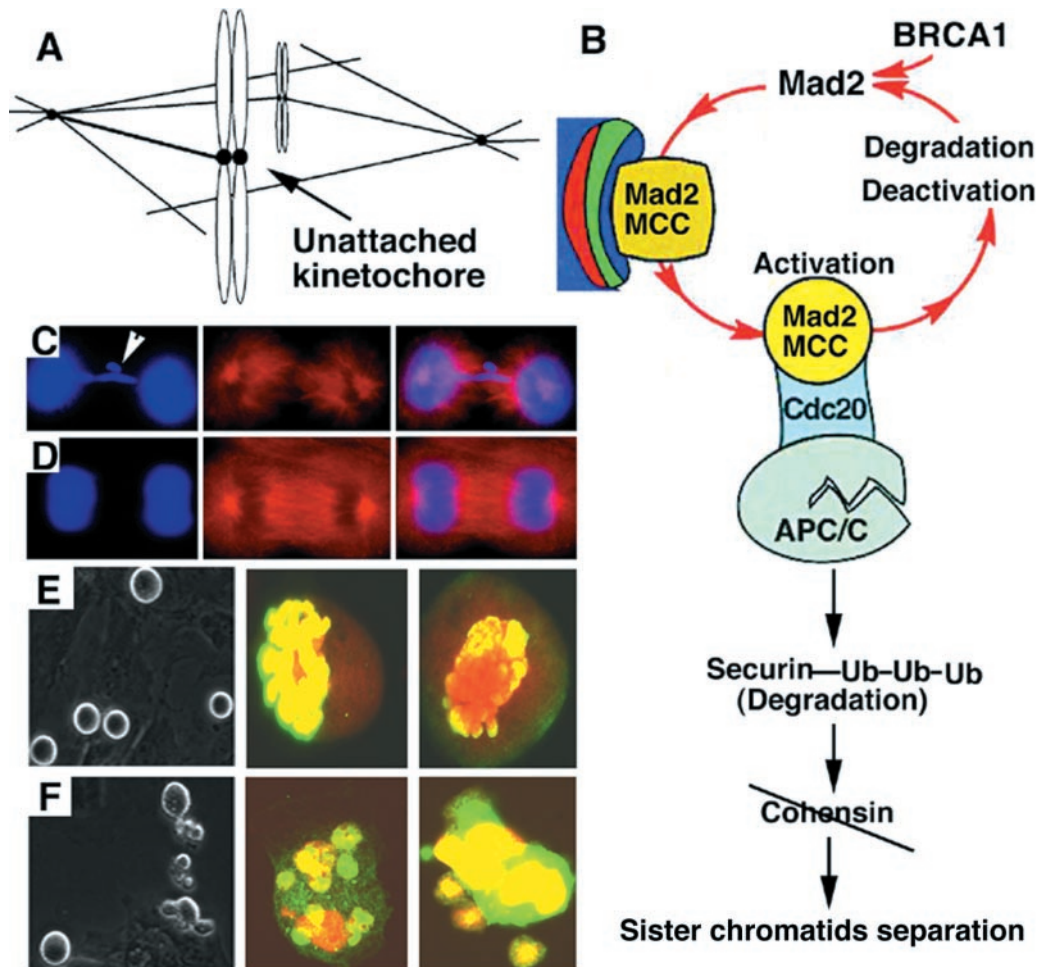


Figure 3. The spindle checkpoint, activation and deficiency. (A) A bipolar spindle showing an unattached kinetochore (arrow). (B) The activation of the spindle checkpoint at the kinetochore leads to the formation of the BubR1-Bub3-Mad2-Cdc20 complex (MCC). The MCC binds and inhibits activity of APC, preventing sister chromatids from separation. It is shown that Brca1 interacts with Mad2 promoter and positively regulates its expression. (C and D) Images of *Brca1*^{Δ11/Δ11} (C) and wild-type (D) MEF cells stained with Dapi and an anti- α -tubulin antibody. Arrows pointed to lagging chromosomes. (E and F) Morphology difference between wild-type (E) and *Brca1*^{Δ11/Δ11} MEF (F) cells in responding to nocodazole treatment. *Brca1*^{Δ11/Δ11} MEF cells at mitotic phase (as revealed by both round up and positive for phosphorylated histone H3 antibody staining) exhibited significantly more fragmented cells. Phosphorylated histone H3 antibody staining also indicated that many mutant cells contained fragmented chromosomes.

the spindle checkpoint is defective. This notion was confirmed by their further experiment, in which the *Brcal*^{Δ11/Δ11} cells were treated with nocodazole, a reagent that depolymerizes microtubules and activates the spindle checkpoint. Their data indicated that about 60% *Brcal* wild-type cells were arrested at metaphase 12 h after the nocodazole treatment (Figure 3E), while *Brcal* mutant cells failed to undergo metaphase arrest (Figure 3F), and died due to apoptosis.

Accompanied by these defects, *Brcal*^{Δ11/Δ11} cells also displayed decreased expression of a number of genes that are involved in the spindle checkpoint, including Mad2, Polo-like-Kinase, Bub1, BubR1 and ZW-10. Given the critical role of Mad2 in the spindle checkpoint (94,97,101), Wang *et al.* (31) further addressed the possible interaction between *Brcal* and Mad2. Using a tetracycline-regulated system to express BRCA1 in UBR60 cells (102), they demonstrated that BRCA1 positively regulates Mad2 by interacting with its promoter. Furthermore, over-expression of Mad2 in mutant cells partially overcame the spindle checkpoint defects. These observations provide strong evidence that *Brcal* plays an important role in the spindle checkpoint through maintaining Mad2 expression.

A similar finding was made in BRCA1 wild-type human prostate (DU-145) and breast (MCF-7) cancer cells upon acute suppression of BRCA1 using small interfering RNA that is specific to BRCA1. In these cells, Bae *et al.* (103) demonstrated that attenuation of the mitotic cell cycle checkpoint was accompanied by down-regulation of multiple genes implicated in the mitotic spindle checkpoint (e.g. Bub1, Bub1b, He, Stk6 and Birc5). Interestingly, their data also revealed expression changes of many other genes that are involved in chromosome segregation (Esp11, Nek2, Pttg1, multiple kinesin and kinesin-like proteins), centrosome function (ASP), cytokinesis (Prc1, Plk, Mphosph1 and Kns12), and the transition into and progression through mitosis (B-type cyclins, Cdc2 and Cdc20). BRCA1 knockdown also caused the accumulation of binucleated and multinucleated cells, suggesting a defect in the coordination of cytokinesis and karyokinesis. These findings suggest that BRCA1 transcriptionally regulates gene expression for orderly mitotic progression.

DDR AND CANCER EVOLUTION

Early attempts to create animal models for BRCA1 associated breast cancer were not successful, as BRCA1 deficiency invariably results in embryonic lethality, primarily due to elevated cell death and growth retardation (28,29). An animal model carrying mammary specific disruption of BRCA1 (*BRCA1*^{Col/Col}; *MMTV-Cre*) also exhibited increased apoptosis and abnormal mammary branch morphogenesis before tumor formation at low frequency with long latency (25). Why do BRCA1 deficient cells die? Mounting experimental evidence indicates that organisms have acquired anti-proliferative and cell death-inducing mechanisms that prevent clonal expansion of mutant cells (104). A key mechanism is the DDR, which can be activated not only by DNA damage but also by some other conditions, such as oncogene expression, loss of tumor suppressors (105). Recently, it was shown that during the early cancerous development of lung or bladder tumors, oncogene induced aberrations in DNA replication activates DDR, as

evidenced by the phosphorylation of H2AX (γH2AX) followed by the activation of ATM-Chk2-p53 signaling in the tumor tissues (106,107). The progression to malignant transformation requires the inactivation of DDR, which would in turn create genetic instability and accelerate cancer evolution.

Does BRCA1 deficiency activate DDR and thereby cause growth arrest and/or apoptosis? Several lines of evidence indicate that it may be the case. It was shown that the death of BRCA1 mutant cells is directly linked to the activation of p53, as deletion of p53 and/or its downstream mediator p21 partially rescues BRCA1-null embryos (28,29,53,54). Moreover, it was shown that elimination of either one or both wild-type *p53* alleles completely overcame embryonic lethality caused by the targeted deletion of full-length *Brcal* and allowed *Brcal*^{Δ11/Δ11} mutant mice to survive to adulthood (29). Further analysis indicated that haploid loss or complete loss of p53 resulted in attenuated apoptosis and G₁-S checkpoint control, allowing *Brcal*^{Δ11/Δ11} cells to proliferate (29). Interestingly, most *Brcal*^{Δ11/Δ11}; *p53*^{+/-} male mice suffered premature aging, exhibiting higher expression levels of p53 compared with controls (108). This suggests that remaining wild-type p53 is activated, which may be a cause for premature aging.

It has been reported that over 90% of human BRCA1 deficient breast cancers also bear p53 mutations, while p53 alterations are only found in about 40% sporadic breast cancers (109). Furthermore, most of mammary tumors developed in the *BRCA1*^{Col/Col}; *MMTV-Cre* mouse model also spontaneously mutated their p53 (25), suggesting that the loss of p53 may be responsible for tumorigenesis. To directly test the role of p53 in BRCA1 associated tumorigenesis, a p53-null mutation allele was introduced into BRCA1 conditional mutant model, and the data indicated that introduction of a p53-null mutation into these mice can significantly accelerate mammary tumor formation (25). This data indicated that inactivation of p53 and *Brcal* deficiency synergistically induce mammary tumor formation. However the factors responsible for p53 activation in the absence of *Brcal* are poorly understood. To investigate this, we employed a genetic test by crossing *Brcal*^{Δ11/+} mice with mutant mice carrying targeted mutations of genes in the DDR pathway, including ATM, Chk1, Chk2, p19, Pten, Parp-1, p21 and Gadd45. Our data indicated that ATM or Chk2 inactivation is equivalent to p53 inactivation in that it allows *Brcal*^{Δ11/Δ11} embryos to survive to adulthood (L. Cao and C. X. Deng unpublished data). An earlier investigation also revealed that *Brcal* deficiency resulted in Chk2 phosphorylation and the Chk2-dependent activation of p53 (110). These observations support a model indicating that BRCA1 deficiency results in genetic instability, leading to the activation of ATM-Chk2-p53 DDR signaling, which, in turn, serves as a natural barrier against malignant transformation of BRCA1 mutant cell (Figure 4).

Experimental data also indicate that the inactivation of DDR is not sufficient for *Brcal* deficient cells to undergo malignant transformation. *Brcal* mutant mice in either a p53 or Chk2 mutant genetic background developed tumors in a stochastic fashion, suggesting additional factor(s) is needed to tumorigenesis. Consistent with this notion, it has been demonstrated that BRCA1 associated tumors are frequently associated with increased expression of oncogenes, such as cyclin D1, c-Myc and ErbB2, and loss of heterozygosity of tumor suppressor genes (23–27).

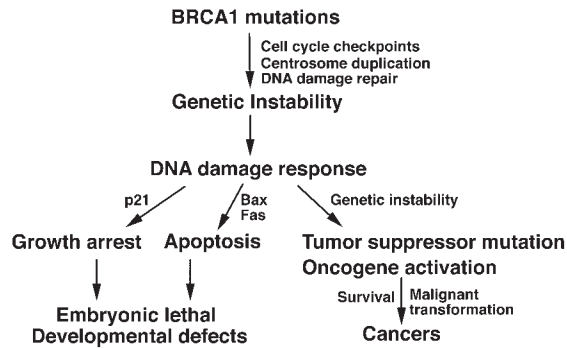


Figure 4. A model illustrating connections among cell cycle checkpoints, centrosome duplication, DNA damage repair, genetic instability, DNA damage response, developmental abnormalities and tumorigenesis caused by BRCA1 deficiency.

CONCLUSIONS AND FUTURE DIRECTIONS

In this review, I have examined experimental evidence regarding the role of BRCA1 in cell cycle checkpoints, genetic stability and tumorigenesis. BRCA1 deficiency results in defective S phase, G₂/M and spindle checkpoints. The defects in these cell cycle checkpoints, combined with abnormal centrosome duplication and defective DNA damage repair could cause genetic instability in BRCA1 deficient cells. The genetic instability, in turn, triggers a series of physiological responses, most promptly the DNA damage response, i.e. activation of ATM-Chk2-p53 signals as manifested by G₁/S arrest due to up-regulation of p21, and apoptosis due to the activation of pro-apoptotic signals. On the other hand, the absence of BRCA1 allows further genetic alterations, including further tumor suppressor mutations and activation of oncogenes, which overcomes growth defects and ultimately results in breast cancer formation (Figure 4).

The examination of the literature also reveals many unanswered questions. First, the role of BRCA1 in the G₁/S cell cycle checkpoint needs further scrutiny, as BRCA1 deficient cells exhibit an intact G₁/S cell cycle checkpoint. p53 activation has obscured the role of BRCA1 in this checkpoint and further mechanistic studies should overcome this barrier. Second, it has been shown that expression of many genes that are critical for the spindle checkpoint is down regulated in cells carrying a targeted disruption (31) or RNAi mediated acute suppression of BRCA1 (103). However, it is not clear about the potential relationship between BRCA1 and expression of these genes. Even in the most well studied case, the *Mad2* gene, whose expression is regulated by BRCA1 in a number of experimental systems, the published data was ambiguous about whether the interaction of BRCA1 with the promoter of *Mad2* was direct or indirect (31). Until this is done, the regulatory role of BRCA1 in the spindle checkpoint remains un-established. Third, acute suppression of BRCA1 also causes altered expression of many genes that are involved in chromosome segregation, centrosome function or cytokinesis (103). Although this observation is interesting, the claim that BRCA1 regulates gene expression for orderly mitotic progression is premature unless solid evidence is provided. Fourth, recent investigations demonstrated that some major functions of BRCA1 could be attributed to the heterodimer formed between BRCA1 and BARD1 (35,37). In addition,

germline mutations of BARD1 were also found in breast and ovarian cancers (111,112). Thus potential roles of the BRCA1/BARD1 heterodimer in tumor suppression, DNA damage repair, cell cycle checkpoints and regulation of centrosome duplication should be interesting topics for future studies. Finally, current chemoprevention and therapy are suboptimal (113). The knowledge regarding functions of BRCA1 in cell cycle checkpoints, genome integrity, DDR and cancer evolution may facilitate drug screening and better design of therapeutic approaches. Reagents that cause reactivation of cell cycle checkpoints, cell death, defective DNA damage repair and/or promote mutant cells through an lethal mitosis should be favorable in the treatment of BRCA1 associated breast cancer. Some promising data attacking weakness of BRCA1/2 tumors has been provided by a number of recent publications (114–116).

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