The Role of Homologous Recombination Genes in Dealing with Replication Stress

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Abstract

Every organism needs to duplicate its DNA, in a process called DNA replication. DNA replication is an essential feature of every living organism, since it accounts for sustaining the organism. The replication process needs to be performed very accurately, since faults in replication or DNA damage can lead to mutations and various kinds of diseases, such as cancer. Damage to the DNA will lead to replication stress, which will lead to the slow progression or stalling of replication forks. This in turn can lead to fork degradation and DNA damage, so it is very important that replication stress is repaired accurately and with speed. There are different damaging factors which lead to replication stress such as IR or UV radiation, depleted dNTP pools other damaging agents, or naturally occurring stress such is torsional stress and double-strand DNA breaks. There are various ways in which the cell can deal with replication stress, one of them is homologous recombination, which is performed primarily during double-strand breaks. Homologous recombination makes use of BRCA genes, which are often mutated in heredity breast cancers and ovarian cancers. These genes might also play another role during replication stress although this role is yet poorly understood. This role of BRCA genes could be promising in finding new potential targets for cancer therapy. That is why this review will focus on the role of homologous recombination genes during replication stress and if this is a potential target for future therapy.

Keywords: Homologous recombination, replication stress, BRCA1, BRCA2

Introduction

DNA replication is an important feature of organisms, because it is essential for the duplication of the organism. It also is a very error-prone process and replication is therefore coupled to various protective mechanism to maintain the integrity of the genome. Constantly renewing almost all cells of the organism can lead to accumulations of mutations which can be induced by factors such as IR or UV radiation, depleted dNTP pools[1,2] other damaging agents[3], or naturally occurring stress such is torsional stress[4] and double-strand breaks.

As mentioned above, many different factors, however, potentially form threats for successful replication. These damaging agents can lead to replication stress. Replication stress is defined as inefficient DNA replication that causes DNA replication forks to progress slowly or stall[5]. The cell needs to cope with these problems before the replication fork degrades, or else it can lead to mutations or cell death. The cell has different mechanisms to cope with all these kinds of replication stress. Recent data suggests that one of these mechanisms involves homologous recombination (HR) components, which are used primarily to repair double-strand breaks (DSB). In its role to repair DSBs, homologous recombination uses the sister chromatid as a template to make an error-free repair of the DSB. This pathway involves the homologous recombination genes BRCA1 and BRCA2, which are two genes often found mutated in hereditary breast cancers and ovarian cancers[6]. Furthermore BRCA1 and BRCA2 have recently been found to also fulfill other functions independently of homologous recombination during replication stress[7]. How exactly, HR genes are involved during replication stress is still poorly understood, that is why I asked the following question: ‘’What is the role of homologous replication

genes during replication stress and could these genes be a potential target for future cancer therapy?''

This review therefore focuses on the question of what the role is of homologous recombination genes during replication stress and if these genes or pathways involving these genes can be used as targets for new cancer treatments. In this review a number of ways in which the cell can cope with replication stress or DNA damage will be discussed and what the role of BRCA1 and BRCA2 is in the repair. Furthermore potential targets of therapy will be discussed such as PARP inhibitors in BRCA1 and BRCA2 deficient cells, as found in breast and ovarian cancers[8,9].

DNA replication

Each DNA molecule consists of two strands of nucleotides which are complementary to one and another. Each strand can act as a template for the synthesis of a new complementary strand. The two strands can separate from each other so that new nucleotides can bind to the template, creating two exact copies of the DNA molecule. This process is called *DNA replication*. The copying must be carried out with accuracy, because errors can lead to mutations that can alter the function of the gene, which in turn can lead to different kinds of diseases such as cancer. DNA replication is tightly monitored to ensure that the genome is replicated just once per cell cycle and that DNA replication is complete before mitosis begins, this is monitored by the cell cycle checkpoints. DNA replication is performed by a set of proteins that together make up the replication machine.

The DNA double helix is a very stable molecule. The two DNA strands are held together by a large number of hydrogen bonds between the nucleotides. The two strands of DNA first need to be opened before the strands can be used as a template. DNA replication is started by initiator proteins that bind to the DNA and separates the strands by breaking the hydrogen bonds between the nucleotides. The positions at which the DNA is first opened are called *replication origins.* All organisms studied so far seem to have an excess of origins[10]. Indeed, there are two types of replication origins, namely early and late origins. The chromatin is packaged in a way that makes it possible for some origins to be accessed at the beginning of the S-phase (early), and normally late replication origins are not needed and are replicated silently. When the cell encounters replication stress such as a stalled fork, the late origins are made accessible to continue DNA replication[11]. These replication origins are distributed along the chromosomes, marked by a particular sequence of nucleotides. This sequence often consists of A-T-rich stretches, which are easier to pull a part due to the fewer number of hydrogen bonds. The origins are marked by the formation of a pre-replicative complex (preRC) in the G1 phase, before DNA replication, through the binding of the origin recognition complex (ORC) and the recruitment of additional replication factors, such as cell division control protein 6 (Cdc6), chromatin licensing and DNA replication factor 1 (Cdt1) and the minichromosome maintenance (MCM) helicase complex, which contains the six subunits Mcm2–Mcm7[12], to these sites. Because the preRC cannot be assembled later in the cell cycle, because of to the inhibitory activity of the S, G2 and M phase cyclin-dependent kinases (CDKs), the maximum number of origins available for an S phase is determined during G1 when the preRC is formed. Furthermore, replication through an origin must remove or inhibit preRCs in order to prevent re-replication[10,13]. This feature limits replication to once per cell cycle.

At the replication forks, the replication machine is moving along the DNA, which opens up the two strands of the double helix. These strands can then be used as a template to produce the daughter strands. At each fired origin, two sister replication forks are formed from one replication origin, that move away from the origin in opposite direction. An important enzyme in the replication machine is DNA polymerase, which catalyzes the addition of nucleotides to the 3' end of a growing DNA strand.

DNA polymerase can catalyze the synthesis of a DNA chain in only one direction, namely at the 3’ end. This causes a problem for the strand that needs to grow at the 5’ end. To overcome this problem, the growth of the 5’ end is made discontinuously, in separate small pieces, called *Okazaki fragments*. with the DNA polymerase working backward from the replication fork in the 5’ to 3’ direction for each piece. The pieces are later put together to form a continuous new strand. The DNA strand that is synthesized discontinuously is called *the lagging strand* and the strand that is synthesized continuously is called the *leading strand*.

DNA polymerase makes only about one mistake every 10^7 nucleotide pairs replicated, making it very accurate. This is accomplished by the error-correcting activity of DNA polymerase, called *proofreading*. Before the enzyme adds a nucleotide to the daughter strand, it checks whether the previous nucleotide added is correctly base-paired to the template strand. If it is not added correctly, DNA polymerase removes the mispaired nucleotide and tries again.

DNA polymerase can only add a nucleotide to a base-paired nucleotide in a DNA double helix and thus cannot start a completely new DNA strand. For this a different enzyme is needed, that can join two nucleotides together without the need for a base-paired end. This enzyme is called *primase*. Primase makes a short length of RNA on the DNA template which is used as a primer for DNA synthesis. This strand of RNA of around 10 nucleotides long, is base-paired to the template strand and provides a base-paired 3’ end as a starting point for DNA polymerase. On the leading strand only one primer is needed as the formation of this strand is continuous. But for the lagging strand multiple primers are needed to replicate the small DNA fragments which are later joined together. To join the separate Okazaki fragments together three additional enzymes are needed. A nuclease (RNase H) breaks apart the RNA primer, than a DNA polymerase called a repair polymerase (Polδ) replaces the RNA with DNA, and the enzyme DNA ligase I (LigI) joins the 5’ end of the fragment to the 3’ end of the following fragment.

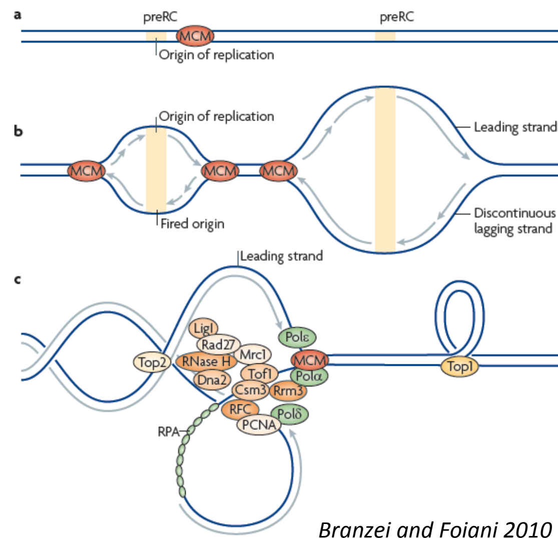


Figure 1. ***The replication machine.*** *(a) DNA replication is initiated from multiple replication origins, marked by the pre-replicative complex (preRC). (b) MCM helicase unwinds the DNA duplex and two replication forks are formed in opposite directions, starting from the replication origins. On the leading strand a continuous DNA strand is formed and on the lagging strand the discontinuous Okazaki fragments are formed. (c) There are different proteins present at the replication fork during replication, such as the MCM helicase which unwinds the DNA duplex, which grants access to DNA polymerase-α (Polα) primase, replicative polymerase-δ (Polδ) and polymerase-ε (Polε) which elongate the primers. Another protein, called proliferating cell nuclear antigen (PCNA; also known as Pol30) which is loaded by the clamp loader, the replication factor c (RFC) complex keeps the DNA polymerase firmly attached to the DNA template.*

One of the most important proteins of the replication machine is the MCM helicase complex, a protein that uses the energy of ATP hydrolysis to speed along DNA, unwinding the double helix as it moves. The unwinding of the double helix also leads to torsional stress in the rest of the DNA molecule. This stress is relieved by the topoisomerases TOP1 and TOP2. To keep the DNA helix opened continuously to provide exposed templates for the polymerase another component of the replication machine, replication protein A (RPA), clings to the single stranded DNA exposed by the helicase and prevents it from re-forming base pairs. It than allows access to the polymerase DNA polymerase-α (Polα)

primase, replicative polymerase-δ (Polδ) and polymerase-ε (Polε) which elongate the primers. Another protein, called *proliferating cell nuclear antigen* (PCNA; also known as Pol30) which is loaded by the clamp loader, the replication factor c (RFC) complex keeps the DNA polymerase firmly attached to the DNA template[12]. On the lagging strand, the sliding clamp releases the polymerase from the DNA each time an Okazaki fragment is completed.

Replication stress

Replication is important for the survival of the organism, but could also be a dangerous feature. It is important because it leads to duplication of the DNA, which is essential for keeping the organism healthy. On the other hand replication can be dangerous for the organism, because it can lead hazardous situations, such as nucleotide mismatches and double-strand DNA-breaks, which need to be repaired accurately to prevent deletions or insertions of nucleotides or lager segments of DNA. As mentioned above DNA replication stress is defined as inefficient DNA replication that causes DNA replication forks to progress slowly or stall[5]. There are several factors that cause replication stress and replication stress-induced DNA damage. These factors include alterations in pools of dNTP precursors needed for DNA synthesis, changes in the expression of proteins required for synthesis of dNTPs or other components of DNA synthesis, decreased frequency in which DNA replication initiates at origins of replication (producing larger replicons), hyper-DNA replication, which is caused by the activation of origins more than once per S phase, DNA damage lesions that block replication forks, and inhibition of DNA replication by drugs such as: hydroxyurea (HU) which depletes nucleotide pools, the topoisomerase inhibitors (including the Topoisomerase I inhibitor camptothecin and the Topoisomerase IIa (TopoIIa) inhibitor etoposide), or the DNA polymerase inhibitor aphidicolin[14]. Replication stress also occurs in regions of DNA that are intrinsically difficult to replicate due to secondary structures or that are difficult to unwind during DNA replication. Proteins bound to DNA can also cause replication forks to pause, and thus causing replication stress[15].

Alterations in dNTP pools
Changes in dNTP pool sizes can lead to replication anomalies, mutations and fork stalling[1,2]. This can be caused by agents that reduce dNTP pools, or by a diminished expression of ribonucleotide reductase (RNR), which is responsible for dNTP synthesis. Another cause is an insufficient expression of the nrdAB gene, which encodes for RNR[16]. Both these examples lead to an shortage of nucleotides, which makes it impossible to synthesize new DNA.

Torsional stress
Unwinding of the duplex DNA changes the topology and conformation of the neighboring DNA molecule, leading to increased helical tension. The linking number describes the intertwining of the complementary strands in any DNA molecule. This measures the number of times that one strand crosses the other strand in the DNA helix, and the number of times that one segment of double helix crosses another in higher-order superhelical structures[4]. This linking number decreases during DNA replication and thus creates stress on the DNA molecule. This stress is called *torsional stress*. To relieve this tension DNA breakage and reunion reactions are needed, which are made possible by DNA nucleases called *topoisomerases*[17]. When the DNA molecule intertwines during DNA replication two different kinds of torsional stress can be induced, namely supercoiling ahead of the replication fork or precatenanes, which is an intertwinement of the newly created sister duplexes behind the replication fork.

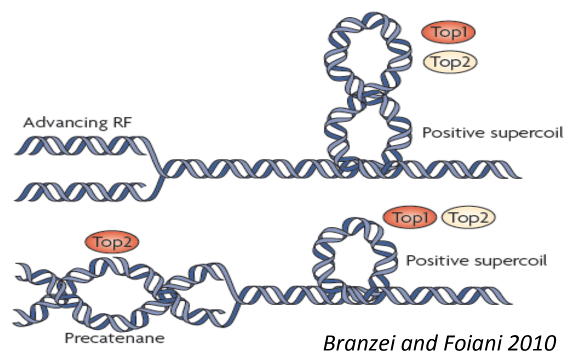


Figure 2. **Dealing with torsional stress.** Unwinding of the DNA duplex creates a positive supercoil ahead of the replication fork, which can be removed by TOP1 and TOP2. This process also creates precatenanes behind the replication fork which can be removed by TOP2.

Another way in which a replication fork can be stalled is by covalent links between complementary DNA strands. These covalent bonds completely block RF progression by preventing strand separation. These links are called *interstrand cross links* (ICLs). These links are formed, for instance, by speficic cancer therapeutics such as cisplatin, which are intended by these therapies. However, interstrand cross links can also be formed not intendedly by cellular metabolites[18].

Dealing with DNA damage

As described above, DNA damage such as replication stress, single stranded breaks, double-stranded breaks are detected by checkpoints. When DNA damage is detected, the cell responds to the damage by ensuring cell cycle arrest, replication fork stabilization and DNA repair.

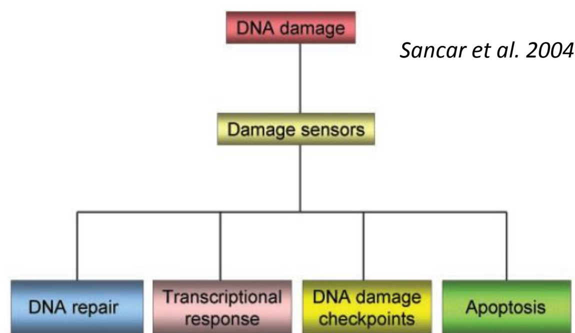


Figure 3. **Response to DNA damage.** DNA damage is detected by multiple damage sensors, which in turn trigger DNA repair, transcriptional repsponse, DNA damage checkpoint response or apoptosis, according the severity of the damage.

Cell cycle arrest

The first thing that needs to be performed, when a cell encounters DNA damage, is cell cycle delay or arrest, so that the DNA replication does not proceed. DNA damage checkpoints are responsible for the delay or arrest of cell cycle progression[19]. There are three checkpoints during the duplication of the cell, namely the G1/S, intra-S, and G2/M checkpoint. Here we will only discuss the intra-S checkpoint, which is the checkpoint which is responsible for responding to DNA replication stress. As mentioned, the intra-S checkpoint is activated by damage encountered during the S-phase[20]. The main feature of S-phase arrest is the inhibition of firing of late origins of replication[21]. There are different damage sensors for the intra-S checkpoint, which consists of checkpoint and repair proteins. In case of damage such as DNA damage by UV, ATR is the main damage sensor, which in cooperation with ATRIP forms the ATR-ATRIP heterodimer[22]. ATR can bind on different places such as the chromatin[23], UV-induced lesions[3], or to RPA-coated single-stranded DNA[24] and become activated. After activation of ATR it phosphorylates CHK1, which phosphorylates and thus downregulates CDC25A, which leads to inactivation of Cdk2/Cyclin E complex. This inhibits the firing of replication origins leading to cell cycle arrest[25]. In the case of single-stranded DNA coated with RPA, the ATR-ATRIP complex and the checkpoint clamp loader Rad17, which is required to load proliferating cell nuclear antigen (PCNA)-like checkpoint clamp RAD9–RAD1–HUS1 (also known as 9-1-1) onto ssDNA, are recruited. ATR than phosphorylates the 9-1-1 complex, which leads to activation of the checkpoint through the CHK1/CDC25A/Cyclin E transduction[12].

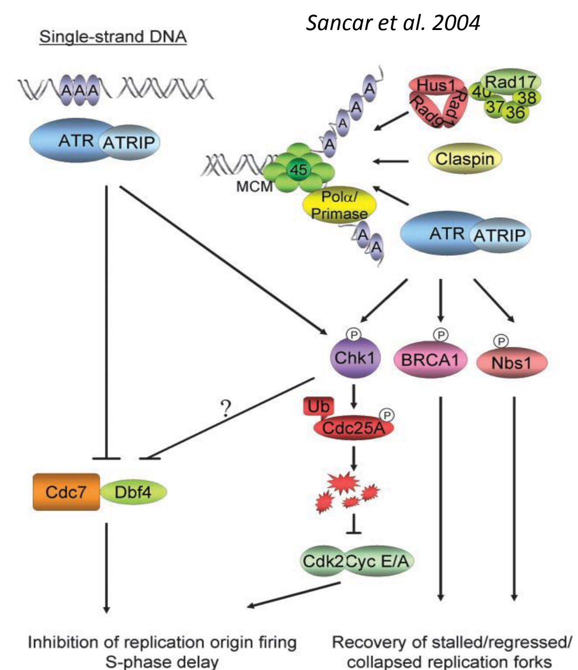


Figure 4. **The ATR-ATRIP checkpoint.** When a replication fork stalls, the ATR-ATRIP complex, Rad17-RFC, the 9-1-1 complex, and Claspin are recruited to RPA-coated single strand DNA at the replication fork. ATR phosphorylates CHK1 and activated CHK1 phosporylates CDC25A. This leads to inactivation of CDK2/Cyclin E complex, which inhibits the firing of replication origins leading to cell cycle arrest.

case of special branched DNA stuctures, (the M/R/N complex, BRCA1, BRCA2)[26,27] binds to the DNA. In addition to actively contributing to DNA repair, these proteins also activate the intra-S checkpoint by a kinase signaling pathway. It seems that the S-phase checkpoint initiated by double-strand breaks proceeds through two pathways. The well-understood ATM-Chk2-Cdc25A-Cdk2 pathway is strictly a checkpoint response. A second pathway, which depends on phosphorylation of SMC1 by ATM with the aid of BRCA1, FANCD2, and NBS1, likely also plays a role in cell-cycle arrest[28,29].

Replication fork stabilization

When an individual replication fork is stopped for one of the previous mentioned reasons it needs to be stabilized in order to prevent collapse. The proteins responsible for the stabilization of the stalled fork are the checkpoint kinases: ATR also called mitosis entry checkpoint protein 1 and CHK1. These proteins stabilize RFs and prevent dis assembly of the replisome[30,31], thus preventing collapse. This happens when single-strand DNA coated with RPA at stalled forks recruits ATR through the ATRIP-RPA interaction. Than Rad17 loads the 9-1-1 complex through a Rad9-RPA interaction, which is used to activate ATR. Rad9 also recruits TopBP1, which is essential for ATR activation. ATR is than able to phosphorylate Rad17, which can then recruit Claspin. Claspin is then also phosphorylated which leads to a phosphorylated Rad17-Claspin complex. This complex activates CHK1 which effector proteins which stabilize stalled forks and repair collapsed forks[32,33].

The role of HR genes in DNA damage repair

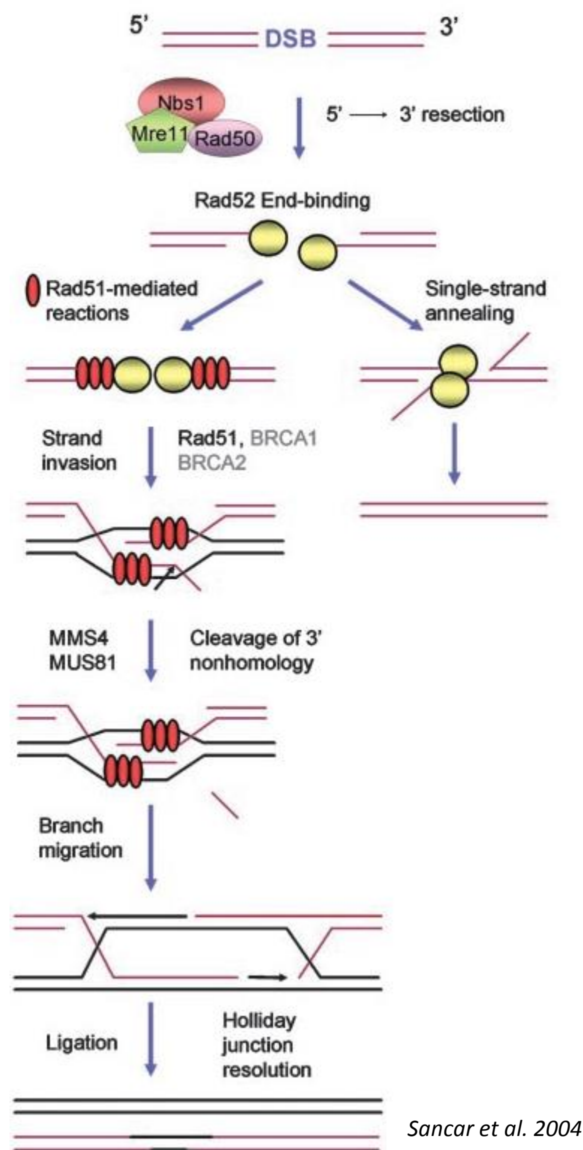
There are different ways in which a cell can cope with the consequences of replication stress, one of them involves homologous recombination. Homologous recombination is primarily used for the repair of DSBs, but also plays a role in the repair of interstrand cross links.

DNA double-strand breaks

There are several ways in which a double-strand break can occur, namely exposure to ionizing radiation, or specific chemotherapeutics such as bleomycin or collapse of replication forks. Double-strand breaks can be very problematic because both strands of a DNA helix are broken, and therefore the complementary strand is not available as a template for repair as is seen in a single stranded break. An accurate repair of DNA damage is needed for the maintenance of genomic stability. As mentioned before, there are several ways in which the cell can do this.

Double-strand breaks can be repaired by non-homologous end-joining (NHEJ) or by homologous recombination. Non-homologous end-joining repairs the DSB by attaching the broken strands to each other, which potentially gives rise to eventual deletions or insertions. With HR this is a very different story. For example with the synthesis-dependent strand annealing pathway. After a DSB, the MRE11, Rad50, Nbs1 complex (MRN complex) binds to the DNA on both sides of the break. Then a piece of both 5' ends is cut to create short 3' overhangs of single strand DNA in a process called *DNA end resection*[34]. Than the RPA protein, which is used to protect the exposed single-strands of DNA, as mentioned above, binds the 3' overhangs[35]. Rad51 subsequently forms a filament of nucleic acid and protein on the single strand of DNA coated with RPA, called a *nucleoprotein filament*. This filament than searches for sequences that are similar to the 3' overhang. When the filament finds that similar sequence, the single-stranded nucleoprotein filament moves into the similar recipient DNA duplex, which is generally a sister chromatid. This process is called *strand invasion*. The strand invasion leads to the forming of a displacement loop (D-loop) between de invading 3'overhang strand and the sister chromatid. DNA polymerase than extends the 3' end strand by synthesizing new DNA. This changes the D-loop to a cross-shaped structure, called a *Holiday Junction*[36]. During extension of the invading 3' strand the Holiday Junction between the donor en

recipient DNA slides in a process called *branch migration*, which releases the newly formed 3' end strand. This DNA end is now able to anneal to the other 3' overhang in the damaged chromosome trough complementary base pairing[37].



Sancar et al. 2004

Figure 5. **Homologous recombination after a DSB.** After a DSB the MRN-complex binds to the DNA to both sides of the break. It than cleaves a part of the 5' end to create 3' end overhangs. RPA than coats the 3' end overhangs and forms a nucleoprotein filament with Rad51, with the help of BRCA1 and BRCA2. The nucleoprotein filament than searches for sequences that are similar to the 3'overhang, generally on a sister chromatic, and invades it creating a D-loop. DNA polymerase can now extend the 3' ends, which changes the D-loop to a Holiday Junction. The newly formed 3' strand is than released by branch migration and can be annealed to the other 3' overhang in the damaged chromosome.

The role of BRCA1 in homologous recombination

BRCA1 is involved in DNA repair in several ways. The main function of BRCA1 is to regulate DNA end resection. Through this function, BRCA1 is required for the formation of RAD51 foci[38].Also BRCA1 is found in several complexes which are involved in the response to, or repair of DNA damage. One of these complexes is the BRCA1-BRCA2-Containging Complex, which also consists of Rad51[39]. This complex has an E3 ubiquitin ligase activity, of which it is speculated that it regulates factors involved in DNA repair. Ultimately it is thought, that within this complex, BRCA1 can recruit BRCA2 to sites of recombination.

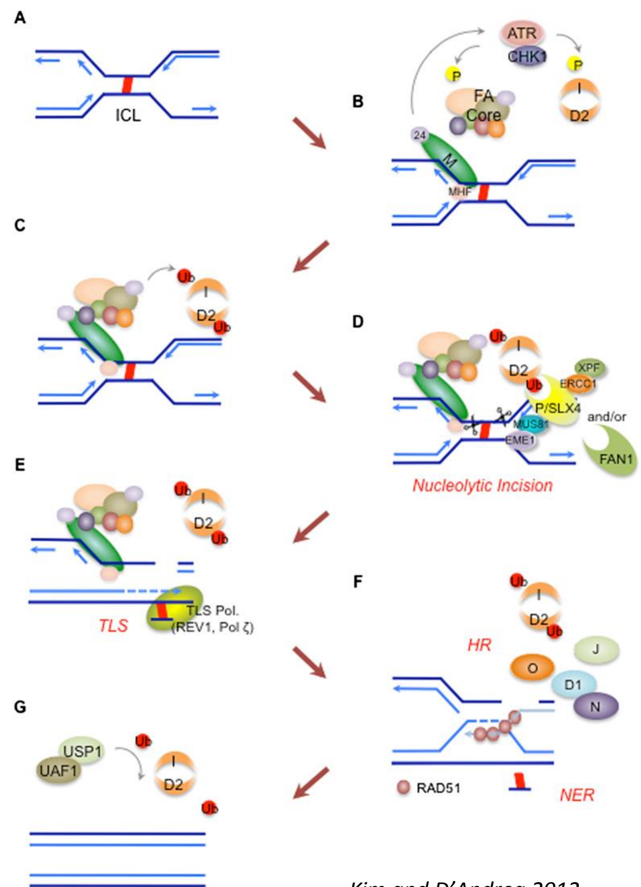
Another complex in which BRCA1 is present is the BRCA1-Associated Genome Surveillance complex (BASC)[40]. This complex can bind abnormal DNA structures and thus has the ability to act as a DNA damage sensor. It also functions directly in DNA replication and repair. This gives BRCA a sort of coordinating role in DNA replication and repair. BRCA1 is mainly thought to be required early during HR, where is it essential for end resection. There is some evidence that BRCA1 plays a role in the activation of the intra-S phase checkpoint by stalled replication forks[41]. First of all there is evidence that after damage by IR the activation of the intra-S checkpoint requires phosphorylation of BRCA1[42]. And secondly it was reported that BRCA1 stimulates the transcription of p27, which induces intra-S phase arrest[43].

The Role of BRCA2 in homologous recombination

BRCA2 plays a variety of roles in HR. It plays an important role in the repair of DSBs by HR, which is dependent on Rad51[44]. It appears that BRCA2 regulates the function of Rad51 in different ways. First of all BRCA2 brings Rad51 to the DSBS. BRCA can bind directly to Rad51 through its C-terminus and the BRC repeats located in the protein[45]. BRCA2 is responsible for the transport of Rad51 into the nucleus and to the DSB. Here Rad51 is released and forms the nucleoprotein filament required for HR[46]. It is suggested that a fraction of Rad51 is bound to BRCA2 at all time, but is immobile and that this immobile fraction is mobilized

upon replication stress[47]. This could mean that BRCA2 holds RAD51 in a state of readiness until replication stress occurs, so that the complex can easily be localized to the sites of the DSB.
Secondly, BRCA is able to prevent the degradation of nascent strands at stalled forks by MRE11. Rad51 attached to BRCA2 at the C-terminus is required for this protection of stalled forks. Although this function is independent of HR, it still plays a critical role in maintaining genomic stability and probably suppressing tumor genesis[12].

Interstrand cross link repair
Repair of interstrand cross links is mediated by the Fanconi anaemia pathway, TLS polymerases and homologues recombination[48]. First of all there is the Fanconi anaemia pathway which consists of around 15 FA gene products that make up the ICL repair pathway. Eight of these proteins, FANC A/B/C/E/F/G/L/M, make up for a multisubunit ubiquitin E3 ligase complex, also known as the core complex. This complex activates the monoubiquitination of FANCD2 and FANCI after genotoxic stress[49]. The FANCM subunit is the initiator of the pathway by binding to FA-associated protein 24kDA (FAAP24). This complex recognizes DNA lesion and recruits the rest of the FA core complex, thus stabilizing the stalled replication fork[50,51,52]. After this FANCD2 and FANCI are ubiquinated by the FA core complex, which is responsible for the regulation of this pathway[53]. The core complex also ubiquinates the FANCD2-1 heterodimeric comples, which coordinates crosslink repair activities in cooperation with other FA proteins such as D1, also known as BRCA2/J/N, also known as PALB2/O/P. FANCD2 than recruits different nucleases to the lesion to make a nucleolytic incision on both sides of the cross-link[54]. Than the nucleases promote cross-link unhooking, which is a process that converts a stalled replication fork into a DSB[55]. The translesion DNA synthesis (TLS) pathway than turns the cross-linked part of the DNA duplex, mediated by TLS polymerases such as REV1 and Pol z, so that it can be bypassed for the restoration of the strands. The DSB can then be repaired by HR as mentioned above. Nucleotide excision repair (NER) removes remaining adducts and fills up the gap.



Kim and D'Andrea 2012

Figure 6. (a) After detection of an interstrand cross-link the replication fork stalls. (b) The ubiquitin E3 ligase complex is than recruited to the ICL. (c) The ubiquitin E3 ligase complex monoubiquitinates FANCD2 and FANCI. (d) FANCD2 than recruits different nucleases to the lesion to make a nucleolytic insicion on both sides of the cross-link. (e) The nucleases promote cross-link unhooking, which is mediated by REV1 and Pol z, so that it can be bypassed for restoration of the new strands. (f) The DSB that remains can then be repaired by homologous recombination.

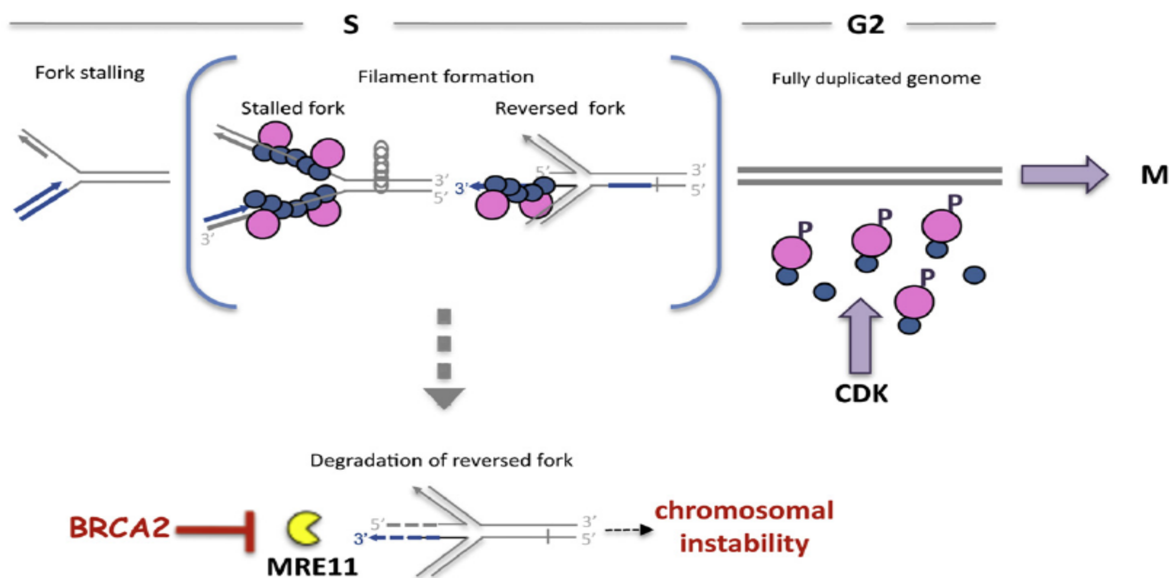
The role of BRCA2 during replication stress

Because of the extensive function of BRCA2 during HDR it is presumed that BRCA2 is also required during replication stress[56,57]. This subject has been thoroughly investigated by Jasin and colleagues, which researched the double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11[7]. After a replication fork stalls, it will start to degrade when it is not protected. This causes a problem because

it stops the DNA replication. This problem is caused by MRE11[7]. Fork degradation starts in the 3'-5' direction on the leading strand and to the 5'-3' direction on the lagging strand, which is promoted by MRE11. MRE11 however is not only negative as it also plays a role in the DSB repair. In the DSB repair MRE11 plays a role in the end processing, in which it has nuclease activity that promotes 5'-3' resection of DNA ends which is crucial in the HDR process. Jasin et al. showed that BRCA2 prevents degradation of nascent strands by MRE11 at stalled forks. This is due to the stabilizing function of BRCA2 to Rad51 filaments, which is necessary for protection of stalled forks but not for DSB repair through HDR. This provides evidence that BRCA2 not only plays a role in HR, but also in other processes. Thus these results show a role for BRCA2 in maintaining genomic stability, and possibly in suppressing tumorigenesis, independent of HR. Furthermore they showed that BRCA2 prevents rather than repairs nucleolytic lesions at stalled RFs, which is distinct from HR[7]. Another possibility in which BRCA2 independently of HDR could play a role during replication stress, is in the cell-cycle progression. When a replication fork stalls, CHK1 inhibits CDK, which prevents phosphorylation of BRCA2. When the lesion is restored and the genome is fully duplicated Rad51 filaments are no longer needed for protection of the strands. CDK can now phosphorylate BRCA2 again which leads to RAD51 disassembly, which promotes entry into the M-phase[58]. In summary BRCA2 plays an important role during replication stress by preventing degradation of stalled replications forks and progression of the cell-cycle towards mitosis. As shown BRCA2 deficiency will lead to more nucleolytic lesions and destabilization of the replication forks during replication stress, which in turn can cause fork degradation and further DNA damage.

Potential targets for cancer therapy

Targeting BRCA1 and BRCA2
As shown above, BRCA1 and BRCA2 are important for the repair of DSBs by HR. When a mutation occurs in BRCA1 or BRCA2 which leads to a failure of the HR mechanism, the DSBs needs to be repaired by alternative mechanisms which are more error-prone. When BRCA2 is deficient, the normal HR repair pathway is impaired and the repair of



Jasin et al. 2011

Figure 7. BRCA2 (pink circles) is responsible for stabilization of Rad51 filaments (blue circles), preventing the stalled replication fork from reversing. On the other hand BRCA2 also prevents MRE11 from degrading reversed forks, which improves chromosomal stability. When the cause of the stalled replication fork is repaired, genome duplication can proceed. CDK than phosphorylates the BRCA2 C-terminal, which allows Rad51 filaments to dissociate from the replication fork, promoting progression into the M-phase.

DSBs will go through the homology-directed repair pathway, called *single strand annealing* (SSA)[59]. This pathway only uses one DNA duplex, so it is not able to use the other DNA duplex as a template, which makes it more error-prone[60]. In the case of BRCA1 deficiency, DSBs are repaired by an error-prone microhomology-mediated sub-pathway of NHEJ[59,61]. This pathway leads to deletion around the DSBs and is therefore less preferable. This use of more error-proned pathways could be an explanation for the observed genomic instability in these cells. These cells are therefore more sensitive to DNA damaging agents that cross-link DNA and to platinum drugs, such as cisplatin and carboplatin[38,62,63]. This increased sensitivity could be exploited for treatment involving BRCA deficiency[64]. Recent studies show that there is also another therapeutic approach for BRCA-deficient cells. In this approach PARP is inhibited, which is a DNA repair protein. By inhibiting PARP DSBs are created which need BRCA1 or BRCA2 for their repair. This will result in *synthetic lethality*[8,9]. Synthetic lethality is a concept in which a mutation in two genes leads to cell death, while a mutation in one of the two genes is viable. PARP-1 is an enzyme that is involved in the repair of single-strand breaks. When a cell has inactivated PARP-1 it is unable to repair these breaks, which can lead to DSBs when encountered by a replication fork DSBs[65]. This would normally be repaired by HR, but in BRCA1/2-deficient cells, this is performed by the more error-prone mechanisms causing chromosome aberrations and loss of viability[66]. Recent studies have shown that PARP inhibitors are synthetically lethal with defects in other HR proteins, such as ATM, ATR and Fanconi Anemia genes, suggesting that this can be applied to a wider therapeutic spectrum than just BRCA deficient cells[67].

Targeting the ATR and CHK1 checkpoints

Research has shown that other possible targets of therapy are components of the ATR pathway. Knockdown of CHK1 by siRNA induced G2 arrest and increased cytotoxicity induced by radiation and cisplatin in HCT116 cells (human colon cancer cells)[68]. Other studies have shown that this induced cell-cycle arrest is most efficient when combined with agents that cause replications stress. For example the agent AZD7762 alone does not affect cell proliferation[69] but it enhances the camptothecin-induced G2 arrest[70]. Furthermore CHK1 is upregulated in Myc-overexpressing lymphomas and inhibition of CHK1 is cytotoxic in Myc-amplified cells[71,72]. At last CHK1 inhibition also showed to be synthetically lethal with various inhibitors of the PARP[73]. In contrast to CHK1, knockdown of ATR sensitizes cells to platinum drugs[74]. A new prospect for the future is the synthetic lethality between ATR and CHK1 inhibitors in combination with PARP inhibitors[73,75]. As mentioned above this could be a viable approach in tumors associated with Myc amplification[71,72], or with defects in the Fanconi pathway[76].

Targeting the Fanconi pathway

Cancer cells can become resistant to chemotherapy, due to certain DNA repair pathways. Inhibiting these repair pathways may provide new targets for cancer therapy. One of these repair pathways is the FA pathway. By inhibiting TLS polymerases, which play an important role in the FA pathway, they might be able to increase the effectiveness of cross-linking chemotherapeutic drugs. Inhibition of the TLS polymerase Rev1 leads to an increased sensitivity to cisplatin in lymphoma and non-small-cell lung cancer (NSCLC) and partially prevents the acquisition of drug resistance[77,78]. Other studies have shown that use of the anti-cancer agent Bortezomib[79] reduces intracellular ubiquitin, which leads to an insufficient FANCD2 ubiquination[80]. This leads to a decreased function of the FA pathway which again increases the effectiveness of cisplatin as mentioned above.

Final conclusions and discussion

This review shows that BRCA1 and BRCA2 play an important role in the repair of the double-strand breaks and to inter-strand cross-links with support of the Fanconi anaemia pathway. But this is not only role of BRCA1 and BRCA2 as shown in this review. They also play a part in the cell-cycle arrest[28,29], in stabilization of

replication forks and in progression to the M-phase which are independent of homologous recombination[7]. As shown, BRCA1 mainly had a coordinating role in DNA replication and repair[38], in contrast to BRCA2 which is responsible for the transport of Rad51[46] and plays a role in the protection of stalled forks[12]. They both seem to play a role in the cell cycle arrest in which BRCA1 plays a role in the activation of the intra-S phase checkpoint by stalled replication forks[41] and BRCA2 by disassembling Rad51 after repair of DNA damage so that the cell can progress to the M-phase[58]. In the case of inter-strand cross-links the Fanconi pathway in cooperation with TLS polymerases induce a DSB, which can be resolved by HR[48].

As mentioned BRCA1 and BRCA2 are often involved in breast and ovarian cancers[6] making them potential targets for therapies due to synthetic lethality[8,9]. As shown in this review PARP inhibitors for example lead to DSBs, which are harder to repair in BRCA deficient cells, since they only have acces to more error-proned patwhays. This makes it a good target for therapy[8,9]. The same goes for TLS polymerase inhibitors, which when inhibited are unable to remove ICLs, thus making them more susceptible for cross-linking chemotherapeutic drugs, such as cisplatin[77,78]. At last CHK1 and ATR also show to be very promising therapeutic targets, especially in Myc-amplified tumor cells[71,72].

This review shows that homologous recombination does play an important role during replications stress. As well as for their role in homologous recombination, as for their role independent of homologous recombination. This also makes them a potential future therapeutic target, for example in the concept of synthetic lethality.

In summary, BRCA1 and BRCA2 fulfill a variety of functions in response to DNA damage and Fork degradation, making them very important in the case of replication stress. Deficiency of these genes can lead to more nucleolytic lesions and destabilization of the replication forks during replication stress, which in turn can cause fork degradation and further DNA damage. This can in turn lead to various forms of cancer. That is why research for potential therapeutic targets in BRCA deficient cells is needed and thus far very promising.

References

1. Stubbe J. Ribonucleotide reductases: the link between an RNA and a DNA world? *Curr Opin Struct Biol* **10:**731–736 (2000).
2. Wheeler LJ, Rajagopal I & Mathews CK. Stimulation of mutagenesis by proportional deoxyribonucleoside triphosphate accumulation in *Escherichia coli*. *DNA Repair (Amst)* **4:** 1450–1456 (2005).
3. Unsal-Kacmaz K, Makhov AM, Griffith JD, Sancar A. Preferential binding of ATR protein to UV-damaged DNA. *Proc. Natl. Acad.Sci. USA* **99**:6673–78 (2002).
4. Postow L, Crisona NJ, Peter BJ, Hardy CD & Cozzarelli NR. Topological challenges to DNA replication: conformations at the fork. *Proc. Natl Acad. Sci. USA* **98**: 8219–8226 (2001).
5. Burhans WC & Weinberger M. DNA replication stress, genome instability and aging. *Nucl Acids Res* **35**:7545-7556 (2007).
6. Gudmundsdottir K and Ashworth A. The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. *Oncogene* **25**:5864–5874 (2006).
7. Schlacher K, Christ N, Siaud N, Egashira A, Wu H, Jasin M. Double-Strand Break Repair-Independent Role for BRCA2 in Blocking Stalled Replication Fork Degradation by MRE11. *Cell* **145**:529-542 (2011).
8. Hartwell L. Theoretical biology. A robust view of biochemical pathways. *Nature* **387**: 855–857 (1997).
9. Kaelin WG. The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer* **5**:689–698 (2005).
10. Zegerman P & Diffley JF. DNA replication as a target of the DNA damage checkpoint. *DNA Repair (Amst.)* **8**:1077–1088 (2009).
11. Farkash-Amar S, Simon I. Genome-wide analysis of the replication program in mammals. *Chromosome Res* 18:115-125 (2010).
12. Branzei D & Foiani M. Maintaining genome stability at the replication fork. *Nat Rev* **11**:208-219 (2010).
13. Laskey RA & Harland RM. Replication origins in the eukaryotic chromosome. *Cell* **24**:283–284 (1981).
14. Allen C, Ashley AK, Hromas R and Nickoloff JA. More forks on the road to replication stress recovery. *J Mol Cell Biol* **4**:4-12 (2011)
15. Ibarra A, Schwob E & Mendez J. Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication. *Proc. Natl Acad.Sci. USA* **105**, 8956–8961 (2008).

16. Nordlund P and Reichard P. Ribonucleotide reductases. *Annu Rev Biochem* **75:** 681–706 (2000).
17. Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. *Nature Rev. Mol. Cell. Biol.* **3**:430–440 (2002).
18. Scharer OD. DNA interstrand crosslinks: natural and drug‐induced DNA adducts that induce unique cellular responses. *Chembiochem* **6**:27–32 (2005).
19. Nyberg KA, Michelson RJ, Putnam CW, Weinert TA. Toward maintaining the genome: DNA damage and replication checkpoints. *Annu. Rev. Genet.* **36**:617–56 (2002).
20. Paulovich AG & Hartwell LH. A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. *Cell* **82**:841–47 (1995).
21. Painter RB, Young BR. Radiosensitivity in ataxia-telangiectasia: a new explanation. *Proc. Natl. Acad. Sci. USA* **77**:7315–17 (1980).
22. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev.***15:**2177–96 (2001).
23. Hekmat-Nejad M, You ZS, Yee MC, Newport JW, Cimprich KA. Xenopus ATR is a replication-dependent chromatin-binding protein required for the DNA replication checkpoint. *Curr. Biol.* **10**:1565–73 (2000).
24. Zou LZ, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* **300**:1542–48 (2003).
25. Heffernan TP, Simpson DA, Frank AR, Heinloth AN, Paules RS, et al. An ATR- and Chk1-dependent S checkpoint inhibits replicon initiation following UVC-induced DNA damage. *Mol. Cell. Biol.* **22**:8552–61 (2002).
26. D'Amours D, Jackson SP. The Mre11 complex: at the crossroads of dna repair and checkpoint signalling. *Nat. Rev. Mol. Cell. Biol.* **3**:317–27 (2002).
27. D'Andrea AD, Grompe M. The Fanconi anaemia/BRCA pathway. *Nat. Rev. Cancer* **3**:23–34 (2003).
28. Kim ST, Xu B, Kastan MB. Involvement of the cohesin protein, Smc1, in Atm-dependent and independent responses to DNA damage. *Genes Dev.* **16**:560–70 (2002).
29. Yazdi PT, Wang Y, Zhao S, Patel N, Lee EYHP, Qin J. SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. *Genes Dev.* **16**:571–82 (2002).
30. Cobb JA, Bjergbaek L, Shimada K, Frei C & Gasser SM. DNA polymerase stabilization at stalled replication forks requires Mec1 and the RecQ helicase Sgs1. *EMBO J.* **22**:4325–4336 (2003).
31. Lucca C *et al.* Checkpoint‐mediated control of replisome‐fork association and signalling in response to replication pausing. *Oncogene* **23**:1206–1213 (2004).
32. Ciccia A and Elledge SJ. The DNA damage response: making it safe to play with knives. *Mol. Cell* **40**:179–204 (2010).
33. Kemp MG, Akan Z, Yilmaz S et al. Tipin-replication protein A interaction mediates Chk1 phosphorylation by ATR in response to genotoxic stress. *J. Biol. Chem.* **285**:16562–16571 (2010).
34. Mimitou EP, Symington LS. Nucleases and helicases take center stage in homologous recombination. *Trends in Biochemical Science* **34**:264–272 (2009).
35. Wold MS. Replication protein A: heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. *Annual Review of Biochemistry* **66**:61–92 (1997).
36. Sung P, Klein H. Mechanism of homologous recombination: mediators and helicases take on regulatory functions. *Nature Reviews Molecular Cell Biology* **7**:739–750 (2006).
37. Helleday T, Lo J, Van Gent DC, Engelward BP. DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA Repair (Amst.)* **6**:923–935 (2007).
38. Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR, Bishop DK. The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. *J Biol Chem* **275**:23899–23903 (2000).
39. Dong Y, Hakimi MA, Chen X, Kumaraswamy E, Cooch NS, Godwin AK et al. Regulation of BRCC, a holoenzyme complex containing BRCA1 and BRCA2, by a signalosome-like subunit and its role in DNA repair. *Mol Cell* **12**:1087–1099 (2003).
40. Wang Y, Cortez D, Yazdi P, Neff N, Elledge SJ, Qin J. BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev* **14**:927–939 (2000).
41. Tibbetts RS, Cortez D, Brumbaugh KM, Scully R, Livingston D, Elledge SJ et al. Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. *Genes Dev* **14**:2989–3002 (2000).
42. Williamson EA, Dadmanesh F, Koeffler HP. BRCA1 transactivates the cyclin-dependent kinase inhibitor p27(Kip1). *Oncogene* **21**:3199–3206 (2002).
43. Xu B, O'Donnell AH, Kim ST, Kastan MB. Phosphorylation of serine 1387 in Brca1 is specifically required for the Atm-mediated S-phase checkpoint after ionizing irradiation. *Cancer Res* **62**:4588–4591 (2002).
44. Moynahan ME, Cui TY, Jasin M. Homology-directed dna repair, mitomycin-c resistance, and chromosome stability is restored with correction of a Brca1 mutation. *Cancer Res* **61**:4842–4850 (2001).
45. Bignell G, Micklem G, Stratton MR, Ashworth A, Wooster R. The BRC repeats are conserved in mammalian BRCA2 proteins. *Hum Mol Genet* **6**:53–58 (1997).
46. Spain BH, Larson CJ, Shihabuddin LS, Gage FH, Verma IM. Truncated BRCA2 is cytoplasmic: implications for cancer-linked mutations. *Proc Natl Acad Sci USA* **96**:13920–13925 (1999).
47. Yu DS, Sonoda E, Takeda S, Huang CL, Pellegrini L, Blundell TL et al. Dynamic control of Rad51 recombinase by self-association and interaction with BRCA2. *Mol Cell* **12**:1029–1041 (2003).
48. Niedzwiedz W *et al.* The Fanconi anaemia gene *FANCC* promotes homologous recombination and error‐prone DNA repair. *Mol. Cell* **15**:607–620 (2004).
49. Wang W. Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. *Nat Rev Genet* **8**:735–748 (2007).
50. Ciccia A, Ling C, Coulthard R, Yan Z, Xue Y, Meetei AR, Laghmani EH, Joenje H, McDonald N, de Winter JP et al. Identification of FAAP24, a Fanconi anemia core complex

protein that interacts with FANCM. *Mol Cell* **25**:331–343 (2007).
51. Collis SJ, Ciccia A, Deans AJ, Horejsi Z, Martin JS, Maslen SL, Skehel JM, Elledge SJ, West SC, Boulton SJ. FANCM
and FAAP24 function in ATR-mediated checkpoint signaling independently of the Fanconi anemia core complex. *Mol Cell* **32**:313–324 (2008).
52. Schwab RA, Blackford AN, Niedzwiedz W. ATR activation and replication fork restart are defective in FANCMdeficient
cells. *EMBO J* **29**:806–818 (2010).
53. Cole AR, Lewis LPC, Walden H. The structure of the catalytic subunit FANCL of the Fanconi anemia core complex. *Nat Struct Mol Biol* **17**:294–298 (2010).
54. Crossan GP, Patel KJ. The Fanconi anaemia pathway orchestrates incisions at sites of crosslinked DNA. *J Pathol*
226:326–337(2012).
55. Ciccia A, McDonald N, West SC. Structural and functional relationships of the XPF/MUS81 family of proteins. *Annu Rev Biochem* **77**:259–287 (2008).
56. Budzowska M and Kanaar R. Mechanisms of dealing with DNA damage-induced replication problems. *Cell Biochem. Biophys.* **53**:17–31 (2009).
57. Nagaraju G and Scully R. Minding the gap: the underground functions of BRCA1 and BRCA2 at stalled replication forks. *DNA Repair (Amst.)* **6**:1018–1031 (2007).
58. Ayoub N, Rajendra E, Su X, Jeyasekharan AD, Mahen R and Venkitaraman AR. The carboxyl terminus of Brca2 links the disassembly of Rad51 complexes to mitotic entry. *Curr. Biol*. **19**:1075–1085 (2009).
59. Stark JM, Pierce AJ, Oh J, Pastink A, Jasin M. *Mol Cell Biol* **24**:9305–9316 (2004).
60. Lyndaker AM, Alani E. A tale of tails: insights into the coordination of 3 end processing during homologous recombination. *BioEssays* **31**:315–321 (2009).
61. Moynahan ME, Chiu JW, Koller BH, Jasin M. Brca1 controls homology-directed DNA repair. *Mol Cell* **4**:511–518 (1999).
62. Yu VP, Koehler M, Steinlein C, Schmid M, Hanakahi LA, van Gool AJ et al. Gross chromosomal rearrangements and genetic exchange between nonhomologous chromosomes following BRCA2 inactivation. *Genes Dev* **14**:1400–1406 (2000).
63. Tutt A, Bertwistle D, Valentine J, Gabriel A, Swift S, Ross G et al. Mutation in Brca2 stimulates error-prone homology-directed repair of DNA double-strand breaks occurring between repeated sequences. *EMBO J* **20**:4704–4716 (2001).
64. Tutt A, Lord CJ, McCabe N, Farmer H, Turner N, Jackson SP et al. Molecular Approaches to Controlling Cancer: Cold Spring Harbour Symposia Quantitative Biology LXX. Stillman B, Stewart D (ed). *CSHL Press: NY,* pp. 139–148 (2005).
65. Dantzer F, de La Rubia G, Menissier-De Murcia J, Hostomsky Z, de Murcia G, Schreiber V. Base excision repair is impaired in mammalian cells lacking Poly(ADP-ribose) polymerase-1. *Biochemistry* **39**:7559–7569 (2000).
66. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* **434**:913–917 (2005).
67. McCabe N, Turner NC, Lord CJ, Kluzed K, Biakowska A, Swift S et al. Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. *Cancer Res* **66**:8109-8115 (2006).
68. Carrassa L et al. Chk1, but not Chk2, is involved in the cellular response to DNA damaging agents: differential activity in cells expressing or not p53. *Cell Cycle* **3**:1177–1181 (2004).
69. McNeely S et al. Chk1 inhibition after replicative stress activates a double strand break response mediated by ATM and DNA-dependent protein kinase. *Cell Cycle* **9**:995–1004 (2010).
70. Zabludoff SD et al. AZD7762, a novel checkpoint kinase inhibitor, drives checkpoint abrogation and potentiates DNA-targeted therapies. *Mol. Cancer Ther*. **7**:2955–2966 (2008).
71. Ferrao PT et al. Efficacy of CHK inhibitors as single agents in MYC-driven lymphoma cells. *Oncogene 10.1038/onc.2011.358* (2011).
72. Hoglund A et al. Therapeutic implications for the induced levels of chk1 in myc-expressing cancer cells. *Clin. Cancer Res.* **17**:7067–7079 (2011)
73. Mitchell C et al Poly(ADP-ribose) polymerase 1 modulates the lethality of CHK1 inhibitors in carcinoma cells. *Mol. Pharmacol.* **78**:909–917 (2010).
74. Wagner JM and Karnitz LM. Cisplatin-induced DNA damage activates replication checkpoint signaling components that differentially affect tumor cell survival. *Mol. Pharmacol.* **76**:208–214 (2009).
75. Peasland A et al. Identification and evaluation of a potent novel ATR inhibitor, NU6027, in breast and ovarian cancer cell lines. *Br. J. Cancer* **105**:372–381 (2011).
76. Chen CC et al. CHK1 inhibition as a strategy for targeting Fanconi Anemia (FA) DNA repair pathway deficient tumors. Mol. Cancer **8**:24 (2009).
77. Doles J, Oliver TG, Cameron ER, Hsu G, Jacks T, Walker GC, Hemann MT. Suppression of Rev3, the catalytic subunit of Polz, sensitizes drug-resistant lung tumors to chemotherapy. *Proc Natl Acad Sci* **107**:20786–20791 (2010).
78. Xie K, Doles J, Hemann MT, Walker GC. Error-prone translesion synthesis mediates acquired chemoresistance. *Proc Natl Acad Sci* **107**:20792–20797 (2010).
79. Orlowski RZ, Kuhn DJ. Proteasome inhibitors in cancer therapy: Lessons from the first decade. *Clin Cancer Res* **14**:1649–1657 (2008).
80. Jacquemont C, Taniguchi T. Proteasome function is required for DNA damage response and Fanconi anemia pathway activation. *Cancer Res* **67**:7395–7405 (2007).