Defects in homologous recombination repair behind the human diseases: FA and HBOC

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Abstract
Hereditary breast and ovarian cancer (HBOC) syndrome and a rare childhood disorder Fanconi anemia (FA) are caused by homologous recombination (HR) defects, and some of the causative genes overlap. Recent studies in this field have led to the exciting development of PARP inhibitors as novel cancer therapeutics and have clarified important mechanisms underlying genome instability and tumor suppression in HR-defective disorders. In this review, we provide an overview of the basic molecular mechanisms governing HR and DNA crosslink repair, highlighting BRCA2, and the intriguing relationship between HBOC and FA.

Introduction
Breast or ovarian cancer is common, and one of the leading causes of mortality in women worldwide (Ferlay et al. 2015, Torre et al. 2015). Although hereditary forms of breast and ovarian cancer (HBOC) are estimated to account for only 5–20% of all cases of breast or ovarian cancer (Nielsen & van Overeem Hansen 2016), HBOC has been recently attracting considerable public attention in many parts of the world, including Japan. A fraction (~25%) of HBOC cases are caused by monoallelic, that is dominant, mutations in either the BRCA1 or BRCA2 genes (Nielsen & van Overeem Hansen 2016), the well-known tumor suppressors that were identified by linkage analysis in the mid-1990s (Miki et al. 1994, Wooster et al. 1995). The lifetime risk of developing breast or ovarian cancer can be up to ~80% in these affected individuals. Mutations in BRCA1/2 also predispose individuals to cancer in other organs such as prostate or pancreas.

The BRCA genes are considered to be ‘caretakers’, and they function in homologous recombination (HR) repair, thereby protecting our genome from carcinogenic alterations. Furthermore, cancer genome sequences have revealed an unexpectedly high frequency of HR gene mutations in sporadic cases of high-grade serous ovarian cancer (Cancer Genome Atlas Research Network 2011), highlighting an important role of HR in cancer prevention via genome maintenance.

As the loss of HR activities by the biallelic disruption of either the Brca1 or Brca2 genes in mice results in early embryonic lethality (for example see Ludwig et al. 1997), it was a real surprise to the scientific community that the D’Andrea lab at Harvard University discovered biallelic mutations in BRCA2 in a subset of patients with the rare childhood hematological disorder, Fanconi anemia (FA) (FA-D1 subgroup) in 2002 (Howlett et al. 2002).
The combined effects of these biallelic BRCA2/FANCD1 mutations appear to be hypomorphic and somehow compatible with life.

FA is very rare, but still the most prevalent among the inherited forms of bone marrow failure syndrome (Auerbach 2009). It is primarily an autosomal recessive disorder that is clinically characterized by congenital malformations, progressive development of hypoplastic anemia and cancer predisposition that often results in hematological malignancies such as acute myelogenous leukemia (AML) or myelodysplasia (MDS) as well as various solid tumors, especially head and neck squamous carcinoma (Alter 2014). FA was first described in 1927 by the Swiss pediatrician Guido Fanconi (Lobitz & Velleuer 2006). Traute Schroeder and coworkers reported spontaneous chromosomal breakage in FA in 1964 (Schroeder et al. 1964). Ten years later, Sasaki and Tonomura discovered that FA cells are extremely sensitive to ICL-inducing agents such as mitomycin C (MMC) (Sasaki & Tonomura 1973), resulting from defective interstrand crosslink (ICL) repair. It is now generally accepted that an ICL is repaired through consecutive steps of multiple DNA repair activities including the HR mechanism (Fig. 1) (Duxin & Walter 2015).

More recently, it has been recognized that mutation carriers of some of the FA genes (e.g., parents of the FA patients) may actually develop HBOC and that the other HBOC genes (i.e., BRCA1 (Domchek et al. 2013, Sawyer et al. 2015)) can cause an FA-like disorder when biallelically mutated (Bogliolo & Surrallés 2015). Thus, HBOC and the FA genes do overlap to some extent (Tables 1 and 2). In a simplified view, it could be said that near-total loss of (or hypomorphic) HR repair activities causes the FA phenotype (or sometimes FA-like, see the ‘The core HR genes in the FA pathway’ section below), whereas breast and ovarian cancer without constitutive symptoms (i.e., HBOC) is caused when the HR repair activities are disabled to certain levels. However, HR is severely disabled in the HBOC tumors, by loss of heterozygosity (LOH), promoter methylation or other mechanisms. In this review, we provide a brief overview of the current understanding of the molecular mechanism of HR and ICL repairs, highlighting BRCA2, and their relationship with two important human diseases, HBOC and FA.

**Genome integrity is maintained by DNA repair**

Each day, every cell incurs a large number of DNA lesions that threaten the integrity of the genome. These lesions originate either from an exogenous source (e.g., X-rays or ultraviolet light) or are created endogenously by metabolic pathways (i.e., free radicals or aldehydes) or by programmed cellular activities (i.e., VDJ recombination in developing lymphocytes or meiotic recombination in germ cells) (Hoeijmakers 2001). Alternatively, it is known that replication stress provoked by oncogene activation or fork collision with transcription machineries can induce stalled replication forks that may result in fork collapse in genomic regions such as common fragile sites (Debatisse et al. 2012).

A DNA double-strand break (DSB) is among the most severe insults to the genome, and it can be repaired by two basic mechanisms termed HR or nonhomologous end joining (NHEJ). HR and NHEJ function in a cooperative and overlapping manner, or perhaps paradoxically, can compete with each other (Takata et al. 1998, Prakash et al. 2015). Although NHEJ can function throughout the cell cycle, the HR pathway only functions during S/G2 phase. In essence, NHEJ unites two DNA ends by ligation without any requirement of homology, often after processing (i.e., removal or addition of short stretches of nucleotides) of the ends. Therefore, the repair process can be error-prone. On the other hand, HR functions by a ‘copy-and-paste’ mechanism of genetic information transfer from an intact homologous template to the damaged DNA, and therefore it occurs without sequence alteration. HR normally requires replicated DNA (sister chromatid) as a template, and this is one of the reasons for HR to be restricted to the S and G2 phases of the cell cycle.

In addition to their role in DSB repair, HR proteins have an important role during S phase, where they function in restarting stalled replication forks or protecting stalled replication forks from collapsing due to nucleolytic digestion (Hashimoto et al. 2012, Schlacher et al. 2012). For example, loss of Rad51 in mice (Tsuzuki et al. 1996) or chicken DT40 cells (Sonoda et al. 1998) causes cell lethality, which is accompanied by chromosomal breaks likely due to replication fork collapse. HR is also an integral step during ICL repair, as illustrated in Fig. 1.

**Basic molecular mechanisms of HR repair**

The mechanisms of HR can be best explained in the context of DSB repair (Fig. 2). To initiate DSB repair through HR, the DSB end needs to be nucleolytically resected to generate 3′ single-stranded DNA (ssDNA). This is quickly coated by the trimeric ssDNA-binding protein complex – replication protein A (RPA). The RPA complex is displaced by RAD51, which is the central player in mammalian HR.
repair, resulting in the formation of RAD51 nucleoprotein filaments. This reaction is facilitated by mediator proteins including BRCA2 and RAD51 paralogs and can be monitored as the formation of subnuclear small dots (RAD51 foci) by immunohistochemical detection.

RAD51 is a homolog of *Escherichia coli* RecA, which mediates the core enzymatic reactions in HR (West 2003). It catalyzes (1) searching for a homologous HR template (homology search) and then (2) pairing of the ssDNA-RAD51 filament with the template DNA (strand invasion and homologous pairing) once the filament encounters the appropriate homologous double-stranded DNA. These reactions result in the formation of a D-loop that consists of heteroduplex DNA coated with RAD51 and displaced ssDNA. The next step is DNA repair synthesis initiated from the invading 3′ ssDNA end. In Figure 1

An overview of the ICL repair pathway. When a replisome collides with an ICL (I), the leading strand initially stalls ~20 nucleotides away from the lesion (II). After a second fork converges at the ICL, BRCA1 facilitates the dissociation of the CMG complex (consisting of Cdc45, MCM2-7 and GINS) from chromatin at the stalled fork, allowing the leading strand to approach to the −1 nucleotide adjacent to the ICL (III). In the next step, the SLX4-XPF-ERCC1 complex incises the DNA and unhooks the lesion in a FA pathway-dependent manner (IV). Translesion synthesis (TLS) polymerase (Polζ, or possibly Polκ, Polη, or Poli) extends the leading strand synthesis past the unhooked, ICL-associated nucleotide (V), and HR and the nucleotide excision repair (NER) pathway repair the remaining lesion (VI).
most instances in mitotic cells, the extended ssDNA is displaced from the template strand and is then annealed by the other processed single-stranded DNA end (i.e., the other end of the DSB). This mechanism is termed ‘synthesis-dependent strand annealing (SDSA) pathway’, and the final product does not contain crossover events (non-crossover). In some cases, the recombination intermediates are converted into a ‘double Holliday junction’ and subsequently resolved by Holliday junction resolvases (e.g., GEN1 or the SLX4 complex) (Garner et al. 2013, Wyatt et al. 2013) (with or without crossover) or the BLM helicase complex (without crossover) (Wu & Hickson 2003). For more complete discussion of HR mechanisms such as their relationship with competing NHEJ (the pathway choice) or Holliday junction resolvases, readers should refer to the recent excellent reviews (Chapman et al. 2012, Sarbajna & West 2014).

### Basic molecular mechanisms of ICL repair

An ICL covalently bridges two nucleotides on opposite DNA strands and hampers critical DNA transactions such as DNA replication and transcription. Recent studies from Walter’s lab using Xenopus egg extracts and plasmid DNA harboring an ICL provided a comprehensive and persuasive view on the detailed mechanisms of ICL repair (Fig. 1) (Räschle et al. 2008, Knipscheer et al. 2009, Long et al. 2011, Duxin & Walter 2015).

According to this model, two converging replication forks from opposite directions first stall ~20bp away (~20) from the ICL, and then leading strand synthesis progresses to the −1 position with respect to the ICL, perhaps after the removal of the CMG helicase. Next, the ICL and ssDNA regions are recognized, leading to the activation of the checkpoint kinase and the FA pathway. The exact mechanism by which these events are accomplished still remains unclear. DNA strands on both sides of the ICL are incised by recruited nucleases, resulting in DSB formation in one of the sister chromatids (unhooking). The DNA replication occurs over the incised ICL by a bypass DNA polymerase specialized in translesion synthesis (TLS), such as REV1 or REV3, perhaps after PCNA monoubiquitination by the RAD18 ubiquitin ligase. The DSB is resected, and the core HR reaction is initiated with the formation of RAD51 filaments. Finally, the short nucleotide fragment that contains the remnant of the ICL is recognized and removed by nucleotide excision repair (NER). How each component of the FA pathway is involved in the ICL repair is described in the following section.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Syndrome with germline mutations</th>
<th>Functions</th>
<th>Cancer type</th>
<th>Penetrance</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA1</td>
<td>HBOC</td>
<td>Homologous recombination</td>
<td>Breast and ovarian cancer</td>
<td>High</td>
</tr>
<tr>
<td>BRCA2</td>
<td>HBOC</td>
<td>Homologous recombination</td>
<td>Breast and ovarian cancer</td>
<td>High</td>
</tr>
<tr>
<td>PTEN</td>
<td>Cowden syndrome, PTEN hamartoma</td>
<td>Phosphatidylinositol 3-phosphate, suppresses AKT signaling</td>
<td>Breast cancer</td>
<td>High</td>
</tr>
<tr>
<td>TP53</td>
<td>Li–Fraumeni syndrome</td>
<td>Transcription factor, regulates cell cycle, apoptosis, senescence</td>
<td>Breast and ovarian cancer</td>
<td>High</td>
</tr>
<tr>
<td>CDH1</td>
<td>Hereditary diffuse gastric cancer syndrome</td>
<td>E-cadherin gene, maintains cell adhesion</td>
<td>Breast and ovarian cancer</td>
<td>High</td>
</tr>
<tr>
<td>STK11</td>
<td>Peutz–Jeghers syndrome</td>
<td>Serine/threonine kinase, regulates cell polarity</td>
<td>Breast and ovarian cancer</td>
<td>High</td>
</tr>
<tr>
<td>NBS1</td>
<td>Nijmegen breakage syndrome</td>
<td>Cell cycle checkpoint after DNA damage, member of the MRN complex</td>
<td>Breast cancer</td>
<td>High</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromatosis type I</td>
<td>Negative regulator of Ras signaling</td>
<td>Breast cancer</td>
<td>High</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia</td>
<td>P13 kinase-related kinase, cell cycle checkpoint and DSB repair</td>
<td>Breast cancer</td>
<td>High</td>
</tr>
<tr>
<td>CHK2</td>
<td>Li–Fraumeni syndrome</td>
<td>Activation of cell cycle checkpoint after DNA damage</td>
<td>Breast and ovarian cancer</td>
<td>Moderate</td>
</tr>
<tr>
<td>FANCJ</td>
<td>Fanconi anemia</td>
<td>Interstrand crosslink repair</td>
<td>Breast and ovarian cancer</td>
<td>Moderate</td>
</tr>
<tr>
<td>FANCM</td>
<td>Fanconi anemia</td>
<td>Interstrand crosslink repair</td>
<td>Breast and ovarian cancer</td>
<td>Moderate</td>
</tr>
<tr>
<td>PALB2</td>
<td>Fanconi anemia</td>
<td>Interstrand crosslink repair, homologous recombination</td>
<td>Breast and ovarian cancer</td>
<td>Moderate</td>
</tr>
<tr>
<td>RAD51C</td>
<td>FA-like syndrome</td>
<td>Interstrand crosslink repair, homologous recombination</td>
<td>Breast and ovarian cancer</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

**Table 1** Hereditary breast and ovarian cancer (HBOC) risk genes.
genes are classified into three subgroups by their functional roles in the ICL repair as explained below (Fig. 3).

The FA core complex and the key downstream complex consisting of FANCD2 and FANCI

The first FA group is the ‘ubiquitination module,’ which comprises the E3 ubiquitin ligase complex (termed FA core complex) and its substrates, the FANCD2–FANCI (D2-I) complex (Fig. 3). These genes have been shown to function in HR, mainly based on Jasin’s recombination assay. In this assay, a chromosomal DSB is induced within an integrated recombination substrate by a plasmid-encoded rare restriction enzyme I-SceI, which recognizes a specific 18bp sequence (Rouet et al. 1994). The HR repair pathway then uses homologous DNA segments placed either upstream or downstream of the DSB, resulting in

Table 2  Fanconi anemia (FA) and FA-like syndrome genes. There are already 20 distinct genes identified in these syndromes, and all proteins are required for interstrand crosslink (ICL) repair. Heterozygous germline mutations in several genes are also related to HBOC.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Synonym</th>
<th>Functions</th>
<th>Symptoms</th>
<th>Heterozygous germline mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FANCA</td>
<td></td>
<td>Component of the FA core complex</td>
<td>FA pathologies</td>
<td></td>
</tr>
<tr>
<td>FANCB</td>
<td></td>
<td>Component of the FA core complex</td>
<td>FA pathologies</td>
<td></td>
</tr>
<tr>
<td>FANCC</td>
<td></td>
<td>Component of the FA core complex</td>
<td>FA pathologies</td>
<td></td>
</tr>
<tr>
<td>FANCD1</td>
<td>BRCA2</td>
<td>HR repair, recruits RAD51 onto DNA, interacts with FANCN, Stalled replication fork protection</td>
<td>FA pathologies, not all patients show bone marrow failure</td>
<td>HBOC</td>
</tr>
<tr>
<td>FANCD2</td>
<td></td>
<td>Ubiquitinated after DNA damage, Stalled replication fork protection</td>
<td>FA pathologies</td>
<td></td>
</tr>
<tr>
<td>FANCE</td>
<td></td>
<td>Component of the FA core complex</td>
<td>FA pathologies</td>
<td></td>
</tr>
<tr>
<td>FANCF</td>
<td></td>
<td>Component of the FA core complex</td>
<td>FA pathologies</td>
<td></td>
</tr>
<tr>
<td>FANCG</td>
<td>XRCC9</td>
<td>Component of the FA core complex</td>
<td>FA pathologies</td>
<td></td>
</tr>
<tr>
<td>FANCI</td>
<td></td>
<td>Ubiquitinated after DNA damage, required for FA core complex activation</td>
<td>FA pathologies</td>
<td></td>
</tr>
<tr>
<td>FANCIJ</td>
<td>BACH1, BRIP1</td>
<td>ICL repair, HR repair, 3’ to 5’ helicase, interacts with BRCA1</td>
<td>FA pathologies</td>
<td>HBOC</td>
</tr>
<tr>
<td>FANCL</td>
<td>PHF9</td>
<td>Component of the FA core complex, E3 ubiquitin ligase</td>
<td>FA pathologies but no cancers</td>
<td></td>
</tr>
<tr>
<td>FANCM</td>
<td>Hef</td>
<td>DNA translocase, required for FANCI-D2 ubiquitination</td>
<td>Unknown, the only known patient also has a FANCA mutation</td>
<td>HBOC</td>
</tr>
<tr>
<td>FANCN</td>
<td>PALB2</td>
<td>HR repair, interacts with BRCA1 and BRCA2, facilitates BRCA2 function</td>
<td>FA pathologies</td>
<td></td>
</tr>
<tr>
<td>FANCO</td>
<td>RAD51C</td>
<td>RAD51 paralog, HR repair, RAD51 nucleoprotein filament stability</td>
<td>FA-like syndrome, no bone marrow failure and cancer</td>
<td></td>
</tr>
<tr>
<td>FANCN</td>
<td>SLX4</td>
<td>Coordinates XPF-ERCC1, interacts with MUS81-EME1 and SLX1 nucleases</td>
<td>FA pathologies</td>
<td></td>
</tr>
<tr>
<td>FANCQ</td>
<td>XPF, ERCC4</td>
<td>Endonucleases, associates with ERCC1, ICL unhooking</td>
<td>FA pathologies</td>
<td>HBOC</td>
</tr>
<tr>
<td>FANCR</td>
<td>RAD51</td>
<td>HR repair, stalled fork protection</td>
<td>FA-like syndrome, no bone marrow failure and cancer</td>
<td></td>
</tr>
<tr>
<td>FANCS</td>
<td>BRCA1</td>
<td>HR repair, promotes RAD51 recruitment, interacts with FANCN</td>
<td>FA-like syndrome, no bone marrow failure</td>
<td>HBOC</td>
</tr>
<tr>
<td>FANCT</td>
<td>UBE2T</td>
<td>E2 ubiquitin-conjugating enzyme for FANCD2 complex, interacts with FANCL</td>
<td>FA pathologies</td>
<td></td>
</tr>
<tr>
<td>FANCU</td>
<td>XRCC2</td>
<td>RAD51 paralog, HR repair, RAD51 nucleoprotein filament stability</td>
<td>FA-like syndrome, no bone marrow failure</td>
<td></td>
</tr>
</tbody>
</table>
the expression of the neomycin resistance gene or GFP. In cells lacking these FA genes, the efficiency of HR repair is substantially decreased in chicken DT40 cells (Yamamoto et al. 2003, 2004) or mildly decreased in human cells (Nakanishi et al. 2005). How these proteins function in HR is still under investigation; however, milder HR defects in human cells may indicate that these ‘ubiquitination module’ FA genes do not provide ‘core’ HR functions. It seems more likely that they modulate the function of the core HR machineries (such as BRCA2 or CtIP, see the ‘The nuclease in the FA pathway’ section below) or TLS polymerase (Kim et al. 2012) or they may regulate histone dynamics (Sato et al. 2012). This module is reported to be required for incision/unhooking of the crosslink during ICL repair (Knipscheer et al. 2009, Klein Douwel et al. 2014, Duxin & Walter 2015), and thus functions in the conversion of an ICL to a DSB.

The FA core complex includes FA proteins FANCA, B, C, E, F, G, J, L, M and FA-associated proteins, such as FAAP24, FAAP20 and FAAP100 (Kottemann & Smogorzewska 2013, Bogliolo & Surrallès 2015, Ceccaldi et al. 2016). In response to an ICL and/or a stalled replication fork, the FA core complex is somehow activated downstream of the checkpoint kinase ATR-ATRIP through multiple phosphorylation of FANCI (Ishiai et al. 2008), FANCM (Singh et al. 2013) or FANCA (Collins et al. 2009) and monoubiquitinates FANCD2 at lysine 561, which is a critical activating event in the FA pathway (Garcia-Higuera et al. 2001, Matsushita et al. 2005). Recent studies indicate that ubiquitin-like with PHD and RING finger domain 1 (UHRF1) protein functions as an ICL recognition factor and may participate in these steps (Liang et al. 2015, Tian et al. 2015). Recently, we and two different groups identified UBE2T, which encodes an E2 ubiquitin-conjugating enzyme, as a causative gene for FA (Hira et al. 2015, Rickman et al. 2015, Virts et al. 2015). UBE2T/FANCT is essential for this monoubiquitination event to proceed. FANCI is a paralog of FANCD2 and its binding partner and also undergoes monoubiquitination dependent on the core complex and monoubiquitination of FANCD2 (Smogorzewska et al. 2007, Ishiai et al. 2008).

Figure 2
Schematic of HR pathway. When a double-strand break (DSB) is generated after DNA replication during S and G2 phase, both strands are resected in the 5’ to 3’ direction to generate 3’ overhangs. Almost immediately, replication protein A (RPA) is loaded onto the single-stranded (ss) DNA, and then replaced by a RADS1 nucleoprotein filament in a process requiring BRCA1–PALB2–BRCA2. RAD51 carries out strand invasion of the sister chromatid by the ssDNA tail and extends the resulting D-loop formation. In synthesis-dependent strand annealing (SDSA), the D-loop structure quickly dissociates from the ssDNA after synthesis of a complementary single strand, and then another strand anneals with a processed ssDNA. An alternate pathway forms a double Holliday junction (dHJ). After second end capture and fill-in synthesis, the Holliday junction is dissociated by the TopII–BLM complex or resolved by resolvase complexes that contain SLX4, MUS81 and GEN1.
However, the mechanism by which it orchestrates the repair machinery is still not entirely clear.

*FANCM* is a human homolog of the archael helicase/nuclease Hef gene (*Komori et al. 2004*), which encodes a DNA translocase (*Meetei et al. 2005*). It is necessary for chromatin loading of the FA core complex (*Kim et al. 2008*) and checkpoint activation (*Huang et al. 2010*), and it plays a distinct role in bypass replication past an ICL by promoting ‘traverse’ of the lesion (*Huang et al. 2013*). Biallelic *FANCM* mutations were identified in a single patient, but the defects in cells from the patient could not be reversed by the expression of wild-type *FANCM* (*Meetei et al. 2005*). This was later found to be due to the presence of simultaneous mutations in *FANCA* (*Singh et al. 2009*). Thus, to date, there have been no human FA patients identified with causative mutations solely in *FANCM*. Furthermore, in the Finnish population, individuals with homozygous loss-of-function *FANCM* mutations do not show any FA phenotype (*Lim et al. 2014*), suggesting that *FANCM* is not a *bona fide* FA gene, though it clearly contributes to the function of the FA pathway and is a candidate HBOC gene (*Kiiiski et al. 2014, Peterlongo et al. 2015*).

As FANCD2 and FANCI form a dimeric complex that seems quite stable (*Sato et al. 2012*), and they are mutually dependent on each other for activating monoubiquitination (*Smogorzewska et al. 2007, Ishiai et al. 2008*), it has been assumed that these proteins should function together. However, it was discovered recently that FANCI, not FANCD2, has an upstream role for foci formation of the core complex components, like FANCA (*Castellà et al. 2015*). It would be interesting to determine which molecule (FANCM vs FANCI) is furthest upstream in localizing the core complex. Further, ATR-phosphorylated FANCI regulates the replicative helicase MCM complex, thereby suppressing dormant origin firing as a distinct function outside of the FA pathway (*Chen et al. 2015*). FANCD2 was also found to interact with MCM helicase independently of its monoubiquitination, where it restrains DNA synthesis in stressed cells, attenuating cell proliferation and carcinogenesis (*Lossaint et al. 2013*).

### The nucleases in the FA pathway

One of the essential events in ICL repair is an incision of the crosslink, which is called ‘unhooking’. In the
current understanding, this event depends on the monoubiquitinated D2-I complex and is carried out by the nuclease complex SLX4-XPF (Knipscheer et al. 2009, Long et al. 2011, Kim et al. 2013, Hodkinson et al. 2014, Klein Douwel et al. 2014, Duxin & Walter 2015). SLX4/FANCP is a large protein that itself can function as a scaffold and is mutated in FA-P patients (Kottemann & Smogorzewska 2013, Bogliolo & Surrallés 2015, Ceccaldi et al. 2016). SLX4 accumulates in the chromatin containing DNA damage via its tandem UBD domains. It has been reported that SLX4 is recruited by monoubiquitinated FANCD2 (Yamamoto et al. 2011). However, there is a conflicting report (Lachaud et al. 2014), and the UBD domain is generally considered to bind to K63-linked polyubiquitin (Lachaud et al. 2014). How SLX4 is tethered to the sites of damage and how the D2-I complex affects unhooking are important issues that need to be resolved.

Of note, biallelic mutations in XPF that specifically affect the cellular sensitivity to ICLs but not UV were identified among unclassified FA patients (Bogliolo et al. 2013, Kashiyama et al. 2013). Now this group of patients is termed FA-Q. XPF was originally identified as one of the causative genes for a UV-sensitive disorder – xeroderma pigmentosum (XP). This is an interesting example of distinct phenotypes due to specific mutations affecting different features of a single protein.

Another nuclease FAN1, which associates with monoubiquitinated FANCD2, was also thought to function for ICL repair in an FA pathway-dependent manner. However, quite recently, Lachaud and coworkers demonstrated that FANCD2 monoubiquitination-dependent FAN1 recruitment is dispensable for ICL repair function of FAN1 but is required for DNA replication fork progression and the prevention of chromosome abnormalities (Lachaud et al. 2016).

CtIP is an important nuclease required for end resection of DSBs and has been shown to interact with BRCA1 as well as the MRN complex. We and others have identified CtIP as a novel interactor of FANCD2 (Murina et al. 2014, Unno et al. 2014). MMC-induced CtIP recruitment to damage foci is dependent on the interaction with FANCD2, and this recruitment appears to be required for end resection of the DSB generated after unhooking of the ICL. CtIP depletion mildly sensitizes cells to MMC treatment, consistent with the role of CtIP downstream of FANCD2 (Murina et al. 2014, Unno et al. 2014).

The group of HR/FA genes that operate in the repair ICLs includes BRCA2/FANCD1, Brip1/FANCJ, PALB2/FANCN, RAD51C/FANCO and XRCC2/FANCU (Kottemann & Smogorzewska 2013, Bogliolo & Surrallés 2015, Ceccaldi et al. 2016). BRCA1/FANCN (Domchek et al. 2013, Sawyer et al. 2015) and RAD51/FANCN (Ameziane et al. 2015, Wang et al. 2015) genes are recent and surprising additions to this group. Many of these are HBOC genes (Table 1) and RAD51 mediators. Thus, they function as the core HR machinery.

BRCA2/FANCD1 was identified as the first core HR gene implicated in FA (Howlett et al. 2002). As it is a well-known tumor suppressor and HBOC gene, this led to the exciting possibility that mutations in other FA genes would also cause HBOC. However, this prediction turned out to be a bit too simplistic. Genes encoding the core complex components and FANCD2/FANCI are unlikely to be a high-penetrance HBOC gene (Seal et al. 2003, Berwick et al. 2007), although there are some reports indicating FANCM (Kisiki et al. 2014, Peterlongo et al. 2015) or FANCC (Berwick et al. 2007, Thompson et al. 2012) could be considered as candidate HBOC genes.

PALB2/FANCN is the partner and localizer of BRCA2 (Xia et al. 2006), and also binds BRCA1, linking the two critical HR proteins BRCA1 and BRCA2 (Sy et al. 2009, Zhang et al. 2009). Interaction with BRCA1 facilitates the recruitment of PALB2–BRCA2 complex to the DNA damage sites. PALB2 stabilizes BRCA2 and critically regulates the functions of BRCA2 as a mediator for RAD51. A recent study revealed that PALB2–BRCA1 interaction is a regulatory point during cell cycle progression (see the ‘Additional regulators of HR and RAD51 function’ section below) (Orthwein et al. 2015). Strikingly, FA-D1 and FA-N patients develop leukemia and kidney or brain tumors at a very early age, with much higher frequency than other FA complementation groups (Hirsch et al. 2004, Wagner et al. 2004, Reid et al. 2007).

Five RAD51 paralogs (e.g., RAD51B, RAD51C, RAD51D, XRCC2, XRCC3) interact in two distinct complexes (Masson et al. 2001) – RAD51B/C/D/XRCC2 (BCDX2) and RAD51C/XRCC3 (CX3). In addition to BRCA2, they also function as RAD51 mediators. It was confirmed that these two complexes are functionally different from each other (Yonetani et al. 2005). Furthermore, analysis of the C. elegans Rad51 homolog revealed that RAD51 paralogs remodel presynaptic filaments of RAD51 into
a stabilized and flexible conformation, which prevents ssDNA degradation by nucleases and RAD51 dissociation (Taylor et al. 2015). Among these paralog genes, RAD51C or XRCC2 mutations were reported in FA-like patients with physical characteristics and chromosome breakage test results similar to FA. Thus, these patients are classified as FA-O (Vaz et al. 2010) or FA-U group (Shamseldin et al. 2012). Cells lacking RAD51 paralog genes generally exhibit a similar phenotype and deficiency in HR (Takata et al. 2000, 2001); therefore, it is possible that humans defective in any of the paralog genes may display a similar FA-like phenotype.

Recently, biallelic mutations in the BRCA1 gene have finally been identified in two patients with early-onset ovarian or breast cancer (Domchek et al. 2013, Sawyer et al. 2015). Diepoxybutane (DEB) induced chromosome breakage test was performed in one of them and found to be positive (Domchek et al. 2013, Sawyer et al. 2015), leading to the designation of FANCs. BRCA1/FANCS is known to target BRCA2/FANCD1 to the DSB site via interaction with PALB2/FANCN (Zhang et al. 2009). It has been reported that one of the BRCA1 interactors, Brip1 helicase, is responsible for the FA-J subgroup. These patients display a typical FA phenotype, albeit not a particularly severe one (Kitao & Takata 2011, Kottemann & Smogorzewska 2013, Bogliolo & Surrallés 2015, Ceccaldi et al. 2016). It was also recently discovered that a monoallelic mutation in RAD51 can give rise to FA-like symptoms by a dominant negative mechanism (termed FA-R subgroup) (Ameziane et al. 2015, Wang et al. 2015).

Interestingly, although FA-D1/N/J patients display the usual constellation of FA symptoms, the patients belonging to FA-O/R/S subgroups do not appear to develop BMF (Bogliolo & Surrallés 2015). Although it is possible that they eventually develop BMF in long-term follow-up, it seems inappropriate to classify these patients as having FA, and at the moment, they should be called FA-like. It will be highly interesting to clarify why these patients do not (or tend not to) develop hematopoietic stem cell failure because patients belonging to FA-D1/N/J/O/R/S all seem to have a similar pathophysiology due to HR deficiency. Of note, a recent study demonstrated that BRCA1 deficiency, specifically in mouse bone marrow, causes hematopoietic defects (Vasanthakumar et al. 2016).

**Structure and function of BRCA2 in the HR pathway**

**General features**

The BRCA2 primary structure is depicted in Fig. 4. BRCA2 is a huge protein that encompasses 3418 amino acids in humans. Shahid and coworkers recently revealed the structure of full-length BRCA2 as being a dimer, using cryo-EM 3D reconstruction (Shahid et al. 2014). The sequence conservation among BRCA2 orthologs among various species is mostly limited to the N-terminal, the middle part, and the C-terminal regions; therefore, these regions may be more important for genome maintenance (Takata et al. 2002).

**N-terminus**

In the BRCA2 N-terminus, there is a region that binds to PALB2/FANCN (Buisson et al. 2010, Menzel et al. 2011). PALB2 facilitates BRCA2 localization and RAD51
chromatin loading at the damage site. This region of BRCA2 was also reported to bind to EMSY (Hughes-Davies et al. 2003). The EMSY-binding region of BRCA2 is encoded by exon 3, which is known to be deleted in cancer (Hughes-Davies et al. 2003, Cousineau & Belmaaza 2011). The EMSY locus is amplified in sporadic breast cancer (13%) and higher-grade ovarian cancer (17%). At the cellular level, EMSY overexpression leads to defective HR (Cousineau & Belmaaza 2011), consistent with the notion that the EMSY-binding region of BRCA2 is likely to coincide or overlap with the PALB2-interacting region.

BRC repeats
In the middle part (the residues between 990 and 2100), BRCA2 harbors eight tandem BRC repeats, each consisting of about 30 amino acids, which are well conserved across human, mouse, rat and chicken (Takata et al. 2002, Yang et al. 2002). Not only the sequences themselves but also the spacing between them is well conserved across species. These individual repeats are the primary motifs through which BRCA2 binds to RAD51, and they are essential for BRCA2 function in HR as shown by mouse knockout studies (Connor et al. 1997, Ludwig et al. 1997, Friedman et al. 1998, Patel et al. 1998, Jonkers et al. 2001). BRCA2 stimulates RAD51 assembly onto ssDNA, and the BRC repeats are critical for this, perhaps by acting cooperatively. It is still unclear whether each BRC repeat can have a distinct function or they can act in a redundant manner. Supporting the former possibility, BRC missense mutations disrupting the interaction with RAD51 have been identified in breast cancer patients (Pellegrini et al. 2002), and conservation between the repeats in a given species is relatively low. It was also shown that BRCA1-4 has a higher affinity to RAD51 monomers than BRC5-8; therefore, BRC repeats may not be functionally equivalent (Carreira & Kowalczykowski 2011). On the other hand, it was shown that an artificial fusion gene consisting of a single BRC repeat and RPA can display HR function (Saeki et al. 2006).

DBD
In the C-terminus of BRCA2, there is a DNA-binding domain (DBD) containing three oligonucleotide-binding (OB) folds, a Tower domain and a helix-turn-helix (HTH) motif. The DBD interacts not only with single-stranded DNA but also with DSS1 (Yang et al. 2002), which is a small 70 amino acid protein identified from the genomic region on chromosome 7q21.3 that was deleted in an inherited developmental syndrome – split hand/foot malformation. Recently, DSS1 has been reported to target BRCA2 to RPA (Zhao et al. 2015), and it functions in the replacement of RPA with RAD51 on resected ssDNA (see the ‘Mediator function of BRCA2 in loading of RAD51 onto ssDNA’ section below).

According to the breast cancer information core database, more than 25% of cancer-associated missense mutations map to the C-terminal region (residues 2500–2850) (Szabo et al. 2000), which includes the sequence through which BRCA2 binds to DSS1 (Jeyasekharan et al. 2013). Recent analysis of cancer-associated BRCA2 mutations has led to the identification of a nuclear export signal (NES) in the C-terminal region of BRCA2 (Jeyasekharan et al. 2013). The NES is masked by the interaction with DSS1. Interestingly, a common cancer-associated BRCA2 mutation, D2723H, impairs the binding of BRCA2 to DSS1, leading to its mislocalization to the cytoplasm and disruption of RAD51 loading onto damaged chromatin. Notably, this mutation is likely to decrease RAD51 foci formation even in the presence of normal BRCA2, suggesting that this mutation acts in a dominant-negative manner.

The three OB domains in BRCA2 are structurally very similar to the canonical OB fold, like the one in RPA (Murzin 1993), consisting of a highly curved β-sheet that closes on itself to form a β barrel. Both the OB2 and OB3 folds have the obvious groove that is characteristic of the ssDNA-binding sites of canonical OB folds (Yang et al. 2002). Using electron microscopy, Thorslund and coworkers unveiled that purified human BRCA2 selectively binds to single-stranded DNA in tailed duplexes and replication fork structures (Thorslund et al. 2010). The Tower domain is capable of binding duplex DNA; however, full-length BRCA2 is likely to interact primarily with ssDNA.

C-terminal RAD51 binding site
Esashi and coworkers demonstrated that RAD51 directly interacts with a region near the BRCA2 C-terminal end that has no homology with the BRC repeats. Phosphorylation of this region at Ser3291 by cyclin-dependent kinase (CDK) disrupts the C-terminal BRCA2–RAD51 interaction (Esashi et al. 2005). The level of phosphorylation at this residue is low during S phase when RAD51 activity is high and increases as the cell enters mitosis. DNA damage elicits a block of this phosphorylation, suggesting that this modification can modulate BRCA2 activity. Unlike BRC repeats, the C-terminal RAD51-binding domain selectively interacts with RAD51 oligomers and RAD51 nucleoprotein
filaments. This region protects RAD51 nucleoprotein filaments formed on ssDNA from dissociation by the BRC repeats. The FA-D1 patient cell line, EUFA423, expresses truncated BRCA2 lacking the C-terminal 192 amino acid residues, which means that the C-terminal RAD51-binding domain (residues 3265–3330) is lost in this patient. This cell line showed impairments in RAD51 focus formation and HR activity (Wang et al. 2004). In addition, an individual with HBOC has been reported to carry a deletion of the C-terminal 224 residues of BRCA2 (Håkansson et al. 1997). These observations underscore the importance of tumor suppression of the C-terminal RAD51-binding region that regulates RAD51 nucleoprotein filament formation (Esashi et al. 2007, Ayoub et al. 2009).

Mediator function of BRCA2 in loading of RAD51 onto ssDNA

RAD51 protein itself can bind to both ssDNA and dsDNA. In HR, because RAD51 must initially bind to resected ssDNA tails at the DSB, and the ssDNA is quickly coated with RPA, RAD51 requires a targeting factor that mediates its interaction with ssDNA. It has been shown that full-length purified human BRCA2 is able to enhance RAD51 presynaptic assembly on RPA-coated ssDNA, promoting RPA-RAD51 exchange. BRCA2 can stimulate RAD51 ssDNA binding in vitro, while inhibiting the ability of RAD51 to bind dsDNA (Carreira et al. 2009, Shivji et al. 2009). Mechanistically, BRCA2 stabilizes ATP-bound RAD51-ssDNA filaments by blocking ATP hydrolysis (Carreira et al. 2009, Jensen et al. 2010). Unlike yeast Rad52, which plays a dominant mediator role for yeast Rad51, BRCA2 does not bind RPA directly. How, then, does BRCA2 regulate RPA-RAD51 exchange?

A key factor turns out to be DSS1, the small and highly acidic protein that interacts with O81 of BRCA2 (Yang et al. 2002). Purified human DSS1 in the presence of BRCA2 stimulates RAD51 binding to RPA-covered ssDNA, compared with BRCA2 alone (Liu et al. 2010). In contrast, DSS1 alone does not activate RAD51 binding to RPA-ssDNA. Furthermore, Zhao and coworkers demonstrated that DSS1 targets BRCA2 to RPA, and DSS1 functions as a DNA mimic to promote the removal of RPA from ssDNA, thereby promoting exchange with RAD51 on ssDNA (Zhao et al. 2015).

It is known that there are other molecules involved in RAD51 regulation. These include RAD51AP1 (Wiese et al. 2007) and TONSL/MMS22L (Duro et al. 2010, O’Donnell et al. 2010). Further analysis will shed more light on the possible interplay between these proteins and HR mechanisms underlying disorders like FA or HBOC.

Additional regulators of HR and RAD51 function

XPG, which is affected in xeroderma pigmentosum complementation group G (XP-G), has been reported to form a complex with BRCA2 and DSS1. Trego and coworkers searched for novel XPG partners and unexpectedly found that XPG interacts with BRCA2, RAD51 and PALB2 (Trego et al. 2016). XPG forms foci in S phase, but not in G1 cells. Because XPG depletion caused a decreased presence of RAD51 and BRCA2 in the chromatin fraction, this protein is likely to contribute to HR.

Foci formation by proteins involved in HR, including BRCA1 and RAD51, is tightly regulated during the cell cycle, and they normally accumulate at the site of DNA damage in S and G2 phase, when the cell has sister chromatid DNA. The mechanism by which BRCA1 foci formation is antagonized in G1 phase by proteins that inhibit DNA end resection, such as 53BP1 and RIF1, has been revealed recently (Chapman et al. 2013, Escribano-Diaz et al. 2013, Zimmermann et al. 2013). In addition to this mechanism, it was recently reported that PALB2–BRCA2 cannot bind to BRCA1 specifically in G1 phase owing to the ubiquitination of PALB2. This modification is mediated by the KEAP1–CRL3 ubiquitin ligase, leading to the suppression of HR in G1 (Orthwein et al. 2015). Furthermore, this PALB2 ubiquitination is antagonized by a deubiquitinate USP11. Interestingly, KEAP1 mutations have been reported in breast cancers (Hartikainen et al. 2015).

Genome maintenance and tumor suppression by BRCA2

As discussed previously, monoallelic BRCA2 mutation causes HBOC, whereas biallelic mutations are characteristic of the FA-D1 subgroup, which displays a particularly severe form of FA, with very early onset of leukemia and solid tumors (Hirsch et al. 2004, Wagner et al. 2004). The malignancies observed in FA-D1 patients are not breast or ovarian cancer; however, this is not surprising because these patients are infants whose endocrine and reproductive systems are immature. BRCA1-deficient breast cancers are typically ‘basal-like’ and ‘triple negative’ for epidermal growth factor receptor 2 (HER2), progesterone receptor and estrogen receptor, and they are more recalcitrant to conventional therapy. On the other hand, breast cancer...
stemming from mutated BRCA2 is clinically categorized as similar to a common sporadic form of breast cancer (Roy et al. 2011). The reason why similar HR deficiencies lead to such distinct clinical entities is still poorly understood (Roy et al. 2011).

In line with the two-hit hypothesis proposed by Knudson (1971), it has been considered that the malignant cells in patients carrying monoallelic BRCA2 mutations obligatorily harbor LOH affecting the wild-type allele, leading to the loss of BRCA2 function. However, a recent study showed that out of 90 BRCA-deficient breast cancers, ten cases did not lose chromosomes that harbored normal copies of BRCA genes and did not show signatures that indicate loss of BRCA functions (Nik-Zainal et al. 2016). Furthermore, loss of mutant alleles can occur in BRCA-associated breast cancer (King et al. 2007). Thus, in addition to the loss of all BRCA function, BRCA haploinsufficiency may also promote carcinogenesis, and some of the cancers arising in the HBOC patients may not be HR deficient. Indeed, BRCA1+/− mutated cells are defective in response to replication stress (Pathania et al. 2014). As studies on mice showed (Ludwig et al. 1997, Jonkers et al. 2001), in carcinogenic steps, the cells tend to lose Tp53 (or an equivalent checkpoint gene) before loss of BRCA/HR function to avoid cell death and/or senescence.

Therapeutic implications of HR defects in HBOC

Defective HR is an important target for chemotherapy in HBOC patients (Konstantinopoulos et al. 2015). Platinum-based chemotherapy is a well-established and widely used modality for cancer treatment. As cisplatin and its derivative carboplatin induce intrastrand and interstrand crosslinks (Deans & West 2011), BRCA-deficient, hence ICL repair-deficient, HBOC cells are naturally sensitive to these drugs (De Picciotto et al. 2016). Indeed, BRCA-mutated HBOC patients appear to have a better short-term prognosis compared with non-BRCA patients, perhaps owing to the better response to chemotherapy (Konstantinopoulos et al. 2015), though this may not be the case for long-term survival (McLaughlin et al. 2013). Based on the discovery that PARP inhibition induces a dramatic cell killing in cells deficient in HR (Bryant et al. 2005, Farmer et al. 2005), an exciting opportunity to develop novel chemotherapeutic drugs has emerged. This is an instance where two distinct but important DNA repair activities are simultaneously inhibited, leading to cell death (synthetic lethality). An initial explanation that this lethality was due to impaired base excision repair, with an increased level of single-strand breaks that are converted to toxic DSBs by replication, is now challenged, and revised models have been proposed (Helmeday 2011, Konstantinopoulos et al. 2015).

Resistance to chemotherapy drugs invariably appears after the initial clinical response during prolonged treatment. A number of resistance mechanisms have been proposed. It has been suggested that secondary mutations in BRCA genes that restore the wild-type reading frame, leading to recovered HR activity, are the major mechanisms for the acquired resistance (Edwards et al. 2008, Sakai et al. 2008). This is analogous to the reversion mosaicism in hematopoietic cells sometimes observed in FA patients, which may mitigate progression of bone marrow failure (Soulier et al. 2005). Genome instability due to HR defects and selection may contribute to these phenomena. Another mechanism for acquired resistance in BRCA2-deficient tumors is the increased replication fork stability without restoring HR (Chaudhuri et al. 2016). This could be due to several mechanisms including PTIP deficiency that inhibits the access of MRE11 nuclease to stalled replication forks (Chaudhuri et al. 2016) or loss of the nucleosome remodeling factor CHD4 (Guillemette et al. 2015). In BRCA1-mutated tumors, normal levels of HR activities might be restored by the loss of 53BP1 or REV7/MAD2L2 (Bouwman et al. 2010, Boersma et al. 2015, Xu et al. 2015). In the absence of BRCA1, these genes function to prevent end resection of DSBs, blocking the subsequent HR reaction.

BRCA-deficient tumors may accumulate an enormous number of mutations due to HR defects and genome instability during the carcinogenic process. Thus, these cells may carry higher numbers of tumor-specific peptide antigens that are presented to tumor-infiltrating lymphocytes. This hypothesis has been tested in clinical samples, leading to the conclusion that BRCA1/2-mutated high-grade serous ovarian cancer may be more sensitive to recently developed immune checkpoint inhibitors, such as anti-PD-1 or anti-PD-L1 antibodies (Strickland et al. 2016).

How defects in HR and ICL repair affect hematopoietic stem cells or promote cancer development?

Deficiencies in DNA repair limit the renewal capacity of aging hematopoietic stem cells (Rossi et al. 2007), and FA patients have higher levels of DNA damage in
these cells, leading to the upregulation of p53 and cell death/senescence (Ceccaldi et al. 2012). The origin of the endogenous damage in FA is an important issue that needs to be resolved (Garaycoechea & Patel 2014) because this knowledge may allow us to develop a novel strategy for preventing bone marrow failure and cancer. Likewise, it is important to know by what mechanism the genome is destabilized in BRCA mutation carriers.

Endogenous aldehydes and lipid peroxidation products have been proposed as major sources of spontaneous DNA damage in FA (Garaycoechea et al. 2012). Using a mutant cell panel derived from the chicken DT40 cell line, it was shown that cells lacking Fancd2 or Brca2 are particularly sensitive to formaldehyde at concentrations similar to those in normal human serum (Ridpath et al. 2007). Patel and coworkers indicated in a series of papers that aldehyde detoxifying enzymes ALDH2 (which mainly catalyzes acetaldehyde) and ADH5 (which mainly catalyzes formaldehyde) play critical roles in FA model mice in the suppression of bone marrow failure and leukemogenesis (Langevin et al. 2011, Garaycoechea et al. 2012, Oberbeck et al. 2014, Pontel et al. 2015). These results clearly indicate that endogenous aldehydes can damage DNA in hematopoietic stem cells. As East Asians often carry an enzymatically defective ALDH2 variant allele (ALDH2*2), we examined ALDH2 genotypes in our cohort of Japanese FA patients (Hira et al. 2013). In line with the mouse studies, our results indicated that the ALDH2 variant allele accelerates the progression of bone marrow failure in these patients. Strikingly, we identified several FA children who had homozygous ALDH2 mutations. These patients displayed particularly grave symptoms, including an extremely early onset of myelodysplasia (Hira et al. 2013, Yabe et al. 2016). This combined FA–ALDH2 deficiency could be considered to be a distinct disease entity. It will be exciting to see whether ALDH2 can be a drug target to prevent bone marrow failure in FA patients. A compound that stimulates ALDH2 activity has already been developed (Perez-Miller et al. 2010). It will also be interesting to test how ALDH2 status can affect cancer development in HBOC among East Asian populations.

Another source of DNA damage can be DNA replication fork stalling, which likely contributes to genome instability in FA or HBOC. The nascent DNA strand at the blocked fork is protected by RAD51 filaments stabilized by the C-terminal domain of BRCA2 in a manner independent of HR (Schlacher et al. 2011). In the absence of BRCA2, BRCA1 or FANCD2, the stalled fork cannot be protected and is degraded by MRE11 nuclease, leading to the loss of genetic information or genome rearrangements (Schlacher et al. 2012). This mechanism might be important for genome stability and tumor suppression provided by BRCA1/2 or FA genes.

Genes involved in pre-mRNA splicing and in the biogenesis and export of messenger ribonucleoprotein (mRNP) also have an important role for genome stability (Paulsen et al. 2009). R-loops consisting of DNA–RNA hybrids and a displaced single-stranded DNA often arise when transcription is perturbed (e.g., upon collision of transcription bubbles and replication forks). Thus, R-loops may be a chief source of replication stress and cancer-associated genome instability. Bhatia and coworkers demonstrated the accumulation of R-loops in BRCA2-depleted cells (Bhatia et al. 2015). Furthermore, recent studies asked whether the FA pathway coordinates transcription–replication conflicts and is involved in R-loop resolution (García-Rubio et al. 2015, Schwab et al. 2015). Indeed, human and mouse cells deficient in FA gene function accumulate R-loops, indicating that the FA pathway does play a critical role in R-loop resolution. MMC-induced FANCD2 foci levels are reduced by the expression of RNaseH1, which digests RNA in RNA–DNA hybrids. These studies imply that the accumulation of R-loops might contribute to hematopoietic stem cell exhaustion in FA. It will also be interesting to know how R-loops trigger the activation of the FA pathway.

Of note, it has also been proposed that cytokines that are upregulated in FA, such as TNF-α or TGF-β, may directly harm hematopoietic stem cells or modulate DNA damage repair in the stem cell compartment (Du et al. 2014, Zhang et al. 2016). This line of investigation may inform the development of novel therapeutic strategies for FA.

Conclusions
As summarized in this review, there has been a lot of progress toward the mechanistic understanding of HR repair and genome stability in this decade. Furthermore, we have seen an exciting development of PARP inhibitors as novel and promising cancer therapeutics. Detailed knowledge about the pathogenesis of HBOC and FA has been obtained.

Despite this progress, obvious questions are still lingering in the field. It is a true enigma that HR deficiency leads to carcinogenesis in a tissue-specific manner, although such specificity is also often the case for the other
hereditary cancer syndromes. From a practical point of view, a large number of variants of unknown significance (VUS) generated from genetic testing of BRCA1 or BRCA2 pose a significant problem in the interpretation of the test results. In the long run, accumulated knowledge about segregation of the genotype and an individual’s cancer susceptibility within families may eventually clarify the significance of VUS. At the moment, careful evaluation of DNA repair capacity in lymphocytes from cases with VUS might be useful (Pathania et al. 2014, Vaclová et al. 2015). It would be particularly useful to construct a collection of isogenic knock-in cells with candidate variants using the CRISPR–CAS9 system (Paquet et al. 2016). Endogenous aldehydes may include at least several molecular species (Xie et al. 2016), and they may induce various types of DNA damage, such as monoadducts, interstrand crosslinks or DNA–protein crosslinks. Which of these actually contributes to FA pathology, and how endogenous aldehydes are generated in cells should be elucidated in the near future.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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