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REVIEW ARTICLE

The role of the Fanconi anemia network in the response to DNA replication stress

Kerstin Gari[1], and Angelos Constantinou[2]

[1]*DNA Damage Response Laboratory, Cancer Research UK, London Research Institute, Clare Hall Laboratories, South Mimms, UK, and* [2]*Department of Biochemistry, University of Lausanne, Lausanne, Switzerland*

Abstract

Fanconi anemia is a genetically heterogeneous disorder associated with chromosome instability and a highly elevated risk for developing cancer. The mutated genes encode proteins involved in the cellular response to DNA replication stress. Fanconi anemia proteins are extensively connected with DNA caretaker proteins, and appear to function as a hub for the coordination of DNA repair with DNA replication and cell cycle progression. At a molecular level, however, the raison d'être of Fanconi anemia proteins still remains largely elusive. The thirteen Fanconi anemia proteins identified to date have not been embraced into a single and defined biological process. To help put the Fanconi anemia puzzle into perspective, we begin this review with a summary of the strategies employed by prokaryotes and eukaryotes to tolerate obstacles to the progression of replication forks. We then summarize what we know about Fanconi anemia with an emphasis on biochemical aspects, and discuss how the Fanconi anemia network, a late acquisition in evolution, may function to permit the faithful and complete duplication of our very large vertebrate chromosomes.

Keywords: *Cancer; genomic instability; DNA repair; DNA recombination; DNA damage signaling*

Part I: tolerance of DNA replication stress

As they travel along DNA, replication machineries frequently encounter obstacles, such as proteins tightly bound to DNA, secondary DNA structures or DNA template lesions. Although prokaryotes and eukaryotes may share similar strategies to tolerate replication obstacles, there are significant differences in the management of replication stress in these two life forms. We will first briefly discuss how replication forks overcome obstacles in *Escherichia coli*, and then introduce the replication stress response in eukaryotes, with a special focus on the intrinsic differences between prokaryotes and eukaryotes, and the limitation of an extrapolation of data obtained from *E. coli*.

Replication and tolerance of replication stress in E. coli

In *E. coli*, replication starts bi-directionally from a single origin of replication, and ends in the diametrically opposite terminus region (Hill, 1992). The concerted actions of the replicative helicase DnaB, DnaG primase, and the DNA polymerase III holoenzyme, which comprises two DNA polymerase III core polymerases, two β sliding clamp processivity factors, and a single γ complex clamp loader, allow the coordinated replication of both the leading and the lagging strand of the DNA template.

The mechanisms of tolerance to replication blocks depend on the nature of the obstacles. We will discuss here three categories of replication obstacles: (1) obstacles that affect either the lagging or the leading strand, (2) gaps in one of the strands that lead to the formation of a double-strand break (DSB) when encountered by a replication fork, and (3) obstacles that block both strands of the DNA.

1. Tolerance of strand-specific lesions

A lesion on the lagging strand does not necessarily cause the replisome to stall, as the lagging strand polymerase can simply extend the next downstream primer, leaving leading strand synthesis unaffected (Figure 1A). Indeed,

Address for Correspondence: Angelos Constantinou, Department of Biochemistry, University of Lausanne, Ch. des Boveresses 155, 1066 Epalinges s/Lausanne, Switzerland. E-mail: angelos.constantinou@unil.ch

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a lagging strand lesion blocks only the synthesis of a single Okazaki fragment. The lagging strand polymerase remains associated with the replisome and can resume DNA replication at the next downstream primer. This bypasses the lesion, and leaves a single-stranded gap behind (Higuchi *et al.*, 2003; McInerney and O'Donnell, 2004).

How DNA synthesis resumes when lesions block the extension of the leading strand is still subject to debate. Some evidence suggests that after UV radiation, replication forks may undergo transient fork reversal (Courcelle *et al.*, 2003). During fork reversal, the nascent DNA strands are annealed and the replication fork is converted into a four-way junction. As a

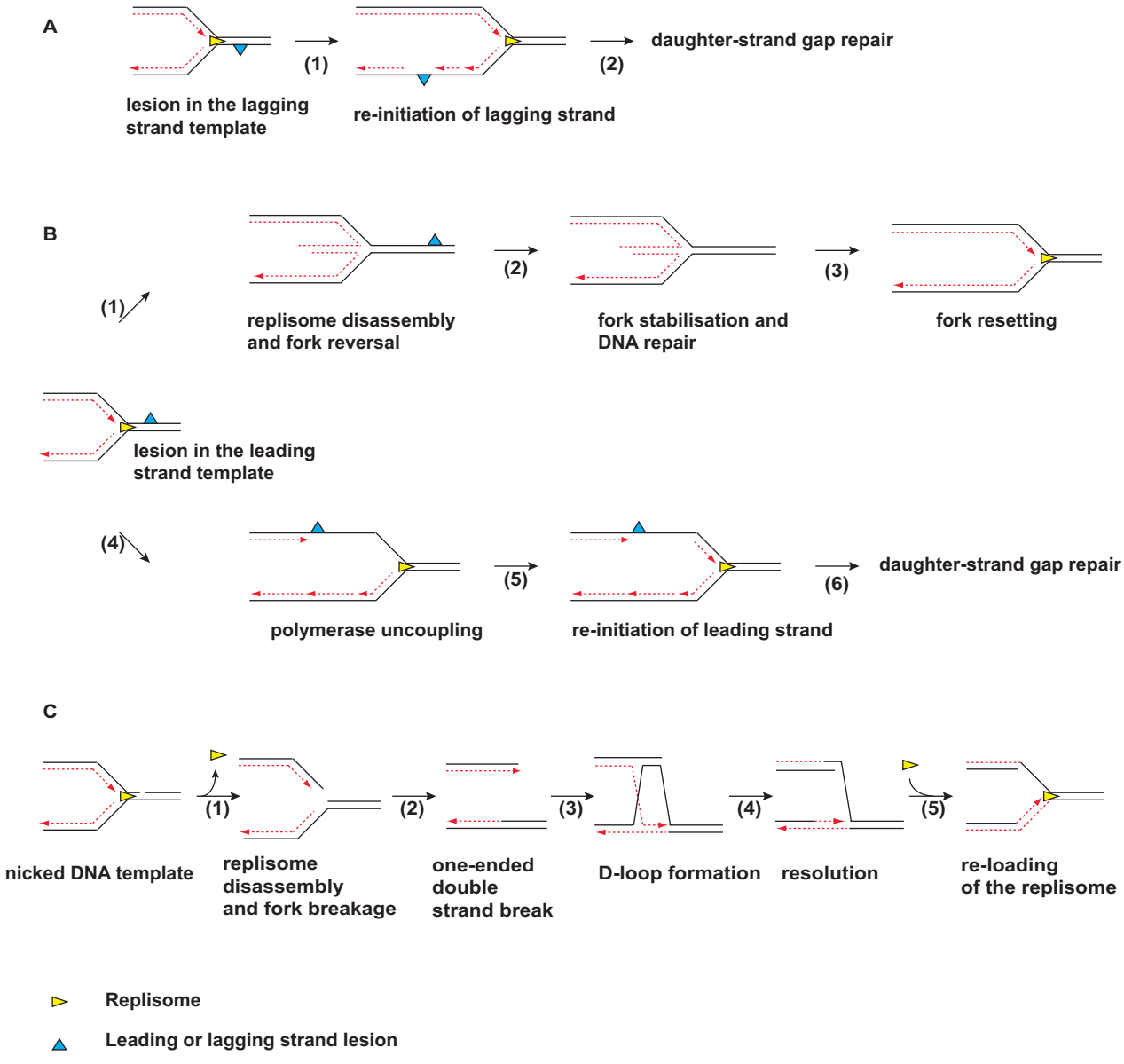

Figure 1. Replication fork collisions with lesions in the lagging or leading strand in *E. coli.* (A) When a replication fork collides with a lesion in the lagging strand template, the lagging strand polymerase remains associated with the replisome and resumes DNA replication at the next downstream primer (1). The single-stranded gap left behind has to be repaired afterwards (2). (B) Lesions on the leading strand template may cause transient replication fork reversal (1), allowing repair enzymes to gain access to the lesion (2). Replication resumes once the replication block has been removed (3). Alternatively, the encounter of a replication fork with a lesion in the leading strand template could cause polymerase uncoupling (4). Leading strand synthesis might be re-initiated downstream of the lesion (5), followed by daughter-strand gap repair (6). (C) When a replication fork runs into a single-strand interruption, the disassembly of the replisome is accompanied by replication fork breakage (1) and the formation of a free dsDNA end (2). RecBCD-mediated recombination creates a recombination intermediate with a D-loop (3). After Holliday junction resolution (4), the replisome is reloaded via PriA (5). Arrows indicate the 3′-end of each strand. Newly synthesized strands are depicted as dashed lines.

consequence, the branch point of the replication fork is moved away from the blocking lesion. Thus, replication fork reversal may facilitate the access of DNA repair enzymes to the lesion (Courcelle and Hanawalt, 2003). After DNA repair, a replication fork structure would be re-established and DNA replication could resume (Figure 1B).

Alternatively, bacteria could make use of three specialized DNA polymerases that can replicate directly past template lesions (Fuchs *et al.*, 2004; Goodman, 2002). These translesion DNA polymerases can be accurate (error-free) or mutagenic (error-prone). Whereas in theory a switch between high-fidelity polymerases and translesion DNA polymerases at the replication fork would allow direct bypass of the lesion, accumulating evidence suggests that translesion DNA synthesis occurs in a post-replicative manner. Indeed, leading and lagging strand synthesis can be uncoupled when the leading strand is blocked (Higuchi *et al.*, 2003; Pages and Fuchs, 2003), and leading strand synthesis can be re-initiated downstream of a leading strand block (Heller and Marians, 2006). Re-priming of the leading strand allows resumption of DNA synthesis and leads to the formation of single-stranded gaps that are left behind the replication fork and repaired in a post-replicative manner (Figure 1B).

These results seem to contradict the general opinion that leading strand synthesis is continuous. They are, however, in full agreement with two articles published almost 40 years ago. Okazaki *et al.* observed that virtually all nascent DNA at replication forks is present as small fragments, suggesting that both lagging and leading strands can be synthesized in a discontinuous manner (Okazaki *et al.*, 1968). At that time, it could also be shown that newly synthesized DNA strands in UV-irradiated cells are initially smaller than in control cells (Rupp and Howard-Flanders, 1968). This suggests that the new strands contain gaps, which are presumably situated opposite the UV photoproducts, and which can be filled in a post-replicative way by translesion DNA polymerases, or via homologous recombination (Rupp *et al.*, 1971). Whereas most of the *in vivo* data obtained since then are in line with a model of discontinuous leading strand synthesis, results obtained from experiments *in vitro* rather point to a continuous leading strand synthesis, a view that has prevailed over the last decades (Wang 2005).

Conclusions. Both lagging and leading strand DNA synthesis can be discontinuous in bacteria. Thus, DNA lesions in one of the template DNA strands are not impenetrable blocks to replication forks, since DNA synthesis can be re-primed downstream of the lesion. If one considers that *E. coli* is able to duplicate its 4.6 megabase genome from a single origin of replication within 40 min, a speed that corresponds roughly to the one measured *in vitro* (Mok and Marians, 1987), it becomes clear that there is hardly any time for repair events taking place at the fork. By leaving lesions behind, replication forks can proceed while the lesions are dealt with by post-replication repair (Langston and O'Donnell, 2006; Lehmann and Fuchs, 2006).

2. Recombination-dependent recovery of collapsed replication forks

Gaps or nicks in one of the DNA template strands constitute a special danger to replication forks (Figure 1C), as a DNA double-strand break is formed when replication forks run into ssDNA interruptions (Kuzminov, 1995; Michel *et al.*, 2007). After disassembly of the replisome, the recombinase RecA assembles on the broken molecule and catalyzes pairing and strand invasion with the homologous duplex DNA, forming a displacement (D) loop. The 3′-end of the broken DNA duplex is used to prime leading strand DNA synthesis with the donor duplex as a template. The re-establishment of a replication fork from a recombination intermediate depends on the PriA helicase, which promotes the re-assembly of a functional replisome at the D-loop (Liu and Marians, 1999; Sandler and Marians, 2000; Xu and Marians, 2003).

3. Recovery from obstacles that block both strands of the DNA duplex

Mutations which impair the replicative helicase DnaB or the accessory helicase Rep, whose supposed function is to clear the DNA from proteins ahead of ongoing replication forks (Heller and Marians, 2007; Matson *et al.*, 1994), provoke the arrest of replication forks.

Inactivation of DnaB or Rep causes the accumulation of DNA double-strand breaks in double-strand break repair-deficient *recBC* mutant cells (Myers and Stahl, 1994; Michel *et al.*, 1997). When additionally RuvAB, the complex essential for branch migration of Holliday junctions during homologous recombination (West, 1997), is inactivated, the formation of double-strand breaks is suppressed (Seigneur *et al.*, 1998). Taken together, these results suggest that the RuvAB complex is required for the formation of double-strand breaks upon replication arrest in cells with impaired DnaB or Rep function. It has been proposed that RuvAB can generate a DNA double-strand end by promoting the annealing of the two nascent DNA strands, thereby forming a four-way junction (Figure 2). After replication fork reversal, several alternative pathways have been proposed to occur (Lambert *et al.*, 2007). First, regressed forks could be processed by branch migration in the opposite direction. Second, regressed forks could be viewed as DNA double-strand breaks and be processed by the RecBCD complex to initiate homologous recombination via RecA

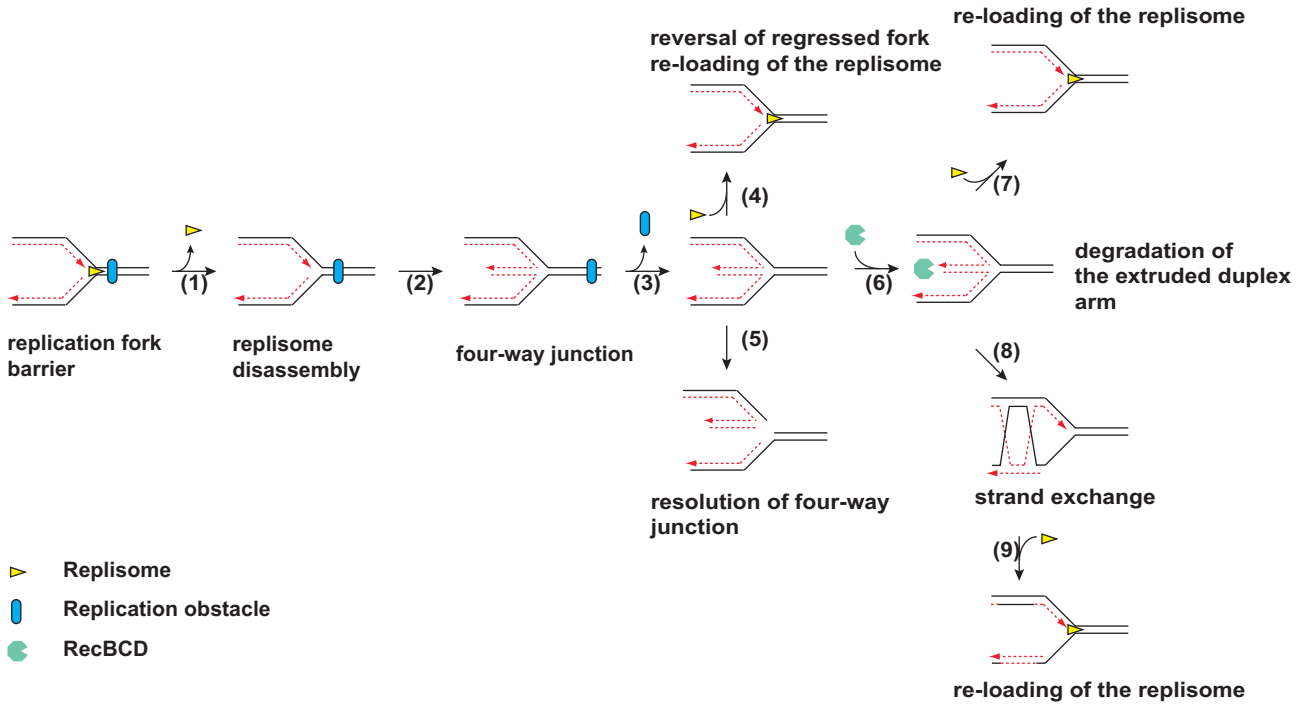

Figure 2. Replication fork restart in *E. coli*. A replication fork is arrested at a replication fork barrier. After disassembly of the replisome (1), and replication fork regression (2), the replication fork barrier is removed (3). The regressed fork can be processed either by branch migration in the opposite direction and PriA-mediated replisome re-loading (4) or RuvABC-mediated Holliday junction cleavage (5). Alternatively, RecBCD can process the reversed fork (6) in two ways. It can degrade the regressed fork and create a 3′-arm replication fork-like structure on which the replisome is loaded via PriA (7), or it can process the regressed fork in a way that RecA-dependent recombination can occur (8). After RuvABC-mediated cleavage, the replisome is loaded via PriA (9).

(Flores *et al.*, 2001). Alternatively, RecBCD (exoV)-dependent degradation of the regressed fork could regenerate a replication fork-like structure (Michel *et al.*, 2004). Third, regressed forks could be cleaved by RuvABC (Seigneur *et al.*, 1998). All pathways rely on additional proteins to remove the obstacle, and on the PriA-directed reloading of the replisome in order to restart DNA replication (Sandler and Marians, 2000).

More recently, replication fork reversal in bacteria has been observed when replication forks are arrested by defects in the replisome components (Flores *et al.*, 2001; Grompone *et al.*, 2002; 2004; Le Masson *et al.*, 2008), suggesting that reversed forks can be induced by different replication defects.

Conclusions. Since *E. coli* possesses only a single origin of replication, replication forks have to be rescued at any price in order to successfully replicate DNA. The repair of collapsed replication forks by recombination enzymes has been clearly demonstrated in bacteria (Kogoma, 1997; Michel *et al.*, 2004). Recombination-dependent replication depends on the activity of the PriA protein, which can recruit the DnaB replicative helicase to RecA-mediated recombination intermediates (Liu and Marians, 1999; Sandler and Marians, 2000; Xu and Marians, 2003).

Replication in eukaryotes

Although the fundamental processes of DNA replication described above for *E. coli* rely on the same principles in eukaryotes, and orthologs for most of the *E. coli* proteins have been found in yeast and higher eukaryotes, substantial differences in the management of replication stress exist.

Firstly, prokaryotes possess a single origin of replication, while higher eukaryotes have approximately 50,000 replication origins, spaced on average every 30–150 kb, although theoretically each replication fork has time to replicate up to 1.5 Mb of DNA during S phase (Blow and Gillespie, 2008). This implies that a stalled replication fork in eukaryotes does not necessarily prevent completion of DNA replication, as in principle an adjacent replication fork can replicate up to the site of the arrested fork. In contrast, when a replication fork collapses in *E. coli*, the only way to complete DNA replication is to restart DNA synthesis from the broken replication intermediate.

Secondly, the re-assembly of a processive replisome at recombination intermediates has never been demonstrated in mammalian cells, and no functional equivalent of PriA has been identified in eukaryotes.

Eukaryotes instead possess a sophisticated replication fork surveillance system to maintain immobilized replication fork structures in a stable and active state, until the block is removed and replication can resume (Lopes *et al.*, 2001; Tercero and Diffley, 2001; Cobb *et al.*, 2003; Lucca *et al.*, 2004; Harper and Elledge, 2007).

1. Origin plasticity

In eukaryotes, DNA replication is initiated at multiple origins that are distributed throughout the genome. In late mitosis and early G1, the origin recognition complex (ORC) and the Cdc6 and Cdt1 proteins mediate the loading of the MCM2-7 replicative helicase complex at origins (Coleman *et al.*, 1996; Nishitani *et al.*, 2000; Bell, 2002), forming a pre-replicative complex that is licensed for replication (Diffley, 2004; Blow and Dutta, 2005). Licensed origins remain "dormant" until activation by the S phase kinases Cdk2/CyclinE and Cdc7/Dbf4, which promote the assembly of the replication machinery (Bell and Dutta, 2002).

Most eukaryotic cells lack an identifiable origin consensus sequence. The current view is that specific features of chromatin structure and function define the position of replication origins and the temporal program of origin activation throughout S phase (Gilbert, 2002; 2004).

Accumulating evidence suggests that the number of potential origins in the genome exceeds the number of origins used during normal S phase (Figure 3). MCM2-7

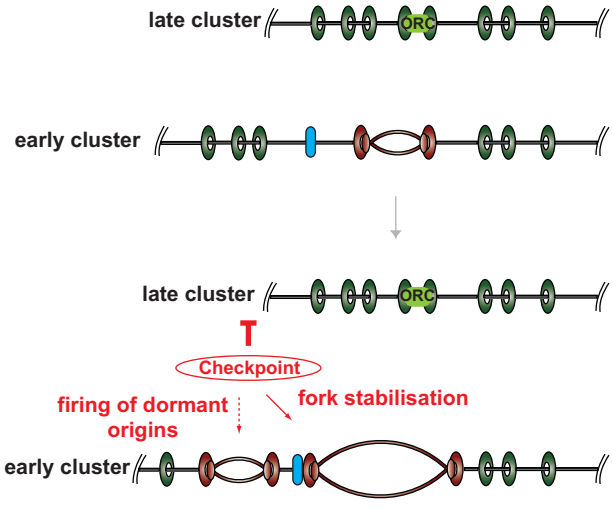

Figure 3. Dormant origins ensure completion of DNA replication. A single ORC complex loads several MCM2-7 double hexamers (dark green), which constitute potential replication origins. Clusters of replication origins that are programmed to fire early and late in S phase are represented. When a replication fork stalls, a nearby dormant origin fires within the active replication cluster, thereby ensuring completion of DNA replication. The checkpoint response preferentially inhibits the firing of late replication clusters, and promotes stabilization and repair of blocked replication forks in early replication clusters.

complexes bound to chromatin outnumber ORC complexes by a factor of 10 to 100 (Lei *et al.*, 1996; Donovan *et al.*, 1997; Edwards *et al.*, 2002), and can spread away from loading sites (Ritzi *et al.*, 1998; Edwards *et al.*, 2002). As the ORC complex becomes dispensable for replication once the MCM proteins are clamped around DNA (Hua and Newport, 1998; Rowles *et al.*, 1996; Edwards *et al.*, 2002), it is thought that the excess of MCM2-7 complexes constitutes a pool of candidate origins that can be activated if required to ensure the completion of DNA replication (Lucas *et al.*, 2000; Edwards *et al.*, 2002; Hyrien *et al.*, 2003; Blow and Dutta, 2005).

It has been recently demonstrated that replication origins that remain "silent" in normal conditions are activated and become essential for cell survival after treatment with replication-blocking agents (Woodward *et al.*, 2006; Ge *et al.*, 2007; Ibarra *et al.*, 2008). This notion of origin plasticity is in line with work published 30 years ago, in which it was proposed that cells use only a small fraction of origins under normal conditions, but that they are able to increase the number of initiation sites when necessary (Taylor, 1977). These results might have fallen into oblivion because they seem to contradict the observation that damage-induced checkpoint activation in S phase inhibits the firing of late origins of replication. This is, however, only paradoxical at first glance. In mammalian cells, origins of replication fire in clusters (Figure 3). During unperturbed DNA replication, a number of origins within a replication cluster stay dormant, and are passively replicated from neighboring origins. In response to replication stress, the checkpoint response in S phase inhibits only the firing of late replication clusters, while preserving the cell's ability to fire new replication origins within clusters that contain stalled replication forks (Gilbert, 2007). Since origin choice is a stochastic event, the probability that a particular dormant origin is activated increases when nearby replication forks are stalled.

Recently, a computer model of DNA replication has been developed (Blow and Ge, 2009). The model is in agreement with experimental data and shows that the stochastic nature of origin firing suffices to explain how DNA replication can be completed when some replication forks are blocked irreversibly. Consistent with this, it has been shown recently that a replication fork, which moves towards one defined DSB in budding yeast, is rescued by an adjacent replication fork arising from a normally silent origin (Doksani *et al.*, 2009). In this study, a single DSB was found not to be sufficient to activate the S phase checkpoint, suggesting that the rescue of a terminated fork by dormant origin firing in yeast operates when the number of DNA lesions is below the threshold for checkpoint activation. In Xenopus egg extracts, however, Plk1 was found to be necessary for the firing of supplemental replication origins when the

replication checkpoint is activated (Trenz *et al.*, 2008). Plk1 binds to the chromatin-bound MCM complex following phosphorylation of MCM2 at serine 92 by ATM/ATR (Trenz *et al.*, 2008). Plk1-bound MCM complexes are checkpoint-blind and can be activated under replication stress conditions. Thus, it is possible that in metazoans, active mechanisms operate within active replication clusters to facilitate the firing of new origins in response to replication stress.

Conclusions. The firing of dormant origins in eukaryotes is a simple, robust and essential mechanism for the completion of DNA replication in cells exposed to replication-blocking lesions (Woodward *et al.*, 2006; Ge *et al.*, 2007; Ibarra *et al.*, 2008). Without redundant origins, higher eukaryotic cells succumb to the blockage of active replication forks, presumably because the restart of DNA synthesis from collapsed replication forks is not possible, or not efficient enough to terminate DNA replication. If two converging replication forks stall simultaneously, the firing of a dormant origin between them would allow completion of DNA replication without the intervention of complex recombination mechanisms.

2. Checkpoint surveillance of replication forks

Strictly speaking, cell cycle checkpoints refer to mechanisms by which the cell actively arrests its progression through the cell cycle until it can ensure that an earlier process has been completed. The DNA damage response in S phase plays roles beyond cell cycle arrest. Activation of the DNA damage response in S phase will interrupt progression through S phase, stabilize stalled replication intermediates, and promote repair of damaged replication intermediates (Lopes *et al.*, 2001; Tercero and Diffley, 2001; Cobb *et al.*, 2003; Lucca *et al.*, 2004; Harper and Elledge, 2007).

At the top of the DNA replication checkpoint are the phosphoinositode-3-kinase-related kinases ATM (ataxia telangiectasia mutated) and ATR (ATM related), which sense DNA damage and activate a complex signaling network through regulated phosphorylation of more than 700 proteins (Matsuoka *et al.*, 2007). Whereas ATM is activated by either DNA double-strand breaks or changes in the chromatin structure (Bakkenist and Kastan, 2003; Shiloh, 2003; Kozlov *et al.*, 2006), ATR most strongly responds to agents which inhibit DNA replication, such as UV irradiation and hydroxyurea (Osborn *et al.*, 2002; Kumagai and Dunphy, 2006). The common structure within the variety of possible DNA intermediates at stalled replication forks, which is recognized by ATR and its partner protein ATRIP, is RPA-coated ssDNA (Zou and Elledge, 2003; Shechter *et al.*, 2004). Although the ATM and ATR pathways seem to be two parallel pathways at first sight, increasing evidence suggests that they are strongly interconnected. Recently, it has been demonstrated that UV and hydroxyurea not only activate ATR, but also ATM, and furthermore that ATM activation is ATR-dependent (Stiff *et al.*, 2006). Vice versa, ATR is activated by double-strand breaks in an ATM-dependent way (Cuadrado *et al.*, 2006; Jazayeri *et al.*, 2006).

Tolerance of replication stress in eukaryotes

As in *E. coli*, the consequences of replication blocks depend on the nature of the obstacle. Here we will discuss how eukaryotes replicate DNA under conditions of nucleotide starvation (1), in the presence of natural pause sites (2), of DNA lesions on one of the template strands (3), and of DNA interstrand crosslinks (4).

1. Replication under conditions of nucleotide starvation

Hydroxyurea (HU) inhibits DNA polymerases by depleting the nucleotide pool without interfering with DNA helicases. This leads to the formation of ssDNA ahead of the stalled fork, and robust checkpoint activation (Figure 4A). It has been shown in budding yeast that replication forks remain stable for several hours during HU treatment, and that replication resumes after removal of the block (Lopes *et al.*, 2001; Sogo *et al.*, 2002). When checkpoint function is impaired, however, replication forks are unstable, aberrant DNA structures accumulate, and replication does not resume (Lopes *et al.*, 2001; Sogo *et al.*, 2002). Similar observations have been made in fission yeast (Noguchi *et al.*, 2003; 2004). Interestingly, using electron microscopy, it has been shown in budding yeast that 10% of HU-treated checkpoint-deficient cells accumulate DNA structures which correspond to regressed replication forks, in contrast to only 1% of checkpoint-proficient cells (Sogo *et al.*, 2002). One possible explanation is that the formation of a regressed fork in HU-treated cells is a pathological event, which is normally prevented by the DNA replication checkpoint. Alternatively, the half-life of regressed forks may be prolonged in checkpoint defective cells. Under normal conditions, replication fork reversal may be a very transient phenomenon, which can hardly be detected by electron microscopy.

In response to nucleotide shortage, the firing of dormant origins in mammalian cells contributes significantly to the completion of DNA replication. It has been shown that deoxynucleotide limitation in Chinese hamster cells results in an increase in the density of active origins in the genome (Anglana *et al.*, 2003). The slowing of DNA chain elongation is compensated by an increase in the number of active replication forks. Furthermore, a reduction of MCM proteins in *C. elegans* and in human cells, which has no consequences in the absence of rep-

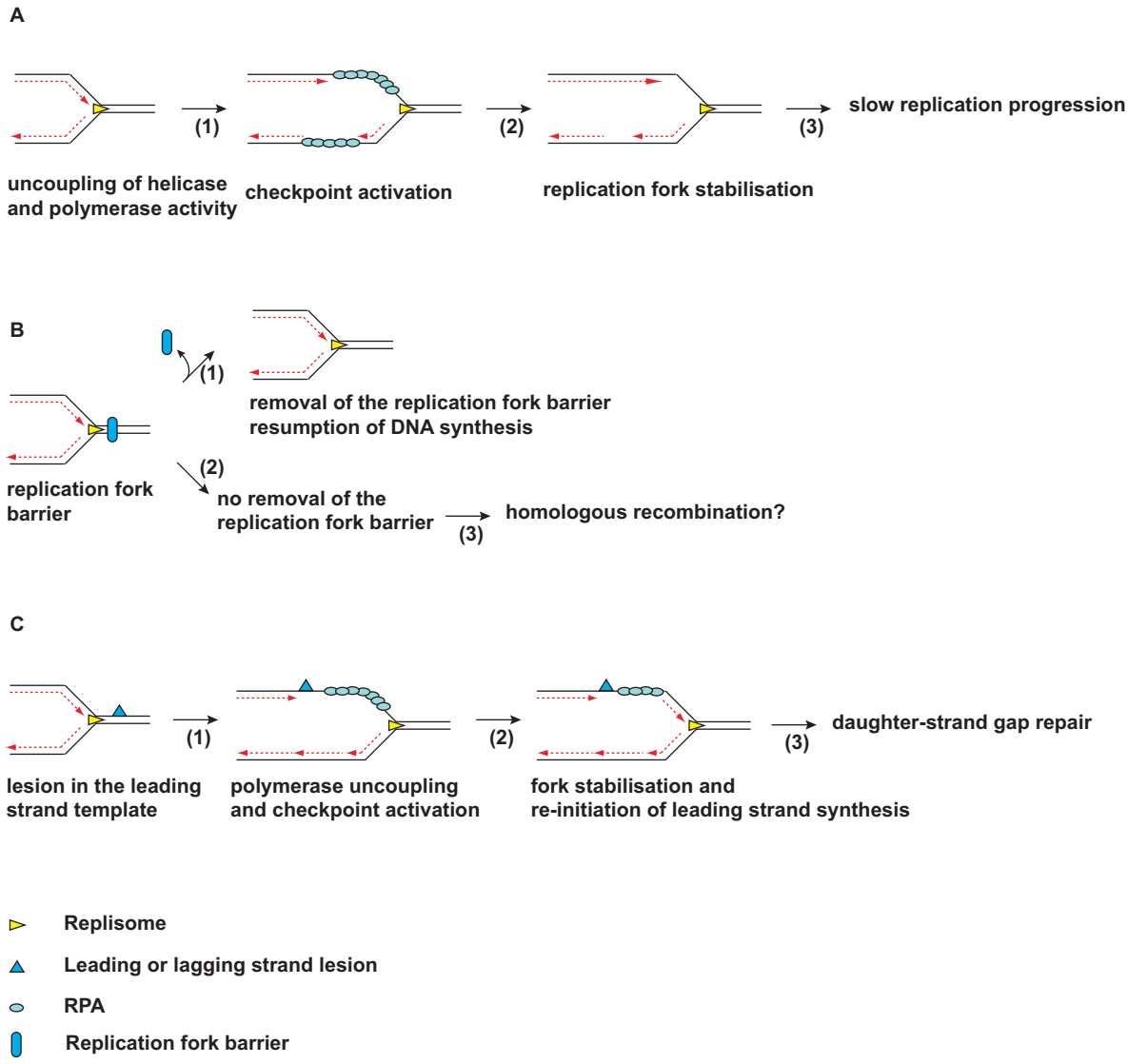

Figure 4. Replication fork stalling in eukaryotes. (A) Depletion of nucleotides causes uncoupling of the replicative helicase from the DNA polymerases (1). The resulting single-stranded DNA is rapidly coated by RPA and activates the DNA replication checkpoint, which leads to replication fork stabilization (2). The stabilized forks can progress slowly (3). (B) Replication fork barriers lead to replication fork stalling. When they are removed by helicases, forks usually resume DNA synthesis after a moment of pausing (1). In the case that they cannot be removed (2), homologous recombination seems to come into play (3). (C) A lesion in one of the template strands (here the leading strand is showcased) leads to the uncoupling of leading and lagging strand DNA polymerase activities and a robust checkpoint activation due to single-stranded DNA stretches coated by RPA (1). Forks are stabilized, and DNA synthesis is re-primed downstream of the lesion (2). Gaps opposite the lesions are filled in by post-replication repair (3).

lication stress, causes a dramatic hypersensitivity to HU (Woodward *et al.*, 2006; Ge *et al.*, 2007).

Conclusions. Studies of the cellular response to hydroxyurea illustrate the essential roles of replication origin redundancy and of replication fork surveillance systems in the tolerance of replication stress.

2. Replication fork barriers and fragile sites

Forks also pause during unperturbed S phase at regions that are intrinsically difficult to replicate (Figure 4B). These sites contain proteins tightly bound to DNA, and can be programmed or accidental. Most of our knowledge so far comes from studies in yeast.

Weak replication fork barriers induce only a transient pausing of replication forks. In yeast, protein complexes bound to centromeres have been reported to result in the pausing of replication forks, independently of the direction of fork progression (Greenfeder and Newlon, 1992). In contrast, pausing at tRNA genes occurs only when the direction of fork progression is opposed to the direction of tRNA transcription (Deshpande and Newlon, 1996). Paused forks need not be deleterious, as long as the replicative machinery remains associated.

Strong replication fork barriers block further progression of a replication fork. The best studied example is the polar rDNA locus of budding yeast, which has been reported to restrict DNA replication to the direction of DNA transcription (Brewer and Fangman, 1988), probably to avoid collisions between replication forks and the transcription machinery. Interestingly, a recombination hot-spot (HOT1) was found to overlap with this replication fork barrier (Keil and Roeder, 1984; Voelkel-Meiman *et al.*, 1987). Although this connection is intriguing, and replication fork collapse at the barrier was originally proposed to be the cause of recombination, the exact link between replication fork stalling and recombination remains obscure, and factors other than the act of pausing seem to play a role (Labib and Hodgson, 2007).

Forks also pause at accidental barriers, such as pre-replicative complexes at origins of replication, protein complexes at telomeres, or other non-histone protein–DNA complexes (Makovets *et al.*, 2004; Miller *et al.*, 2006; Wang *et al.*, 2001). In contrast, the RNA polymerase II machinery does not seem to cause replication fork stalling (Ivessa *et al.*, 2003), unless it is blocked itself in regions of GC-rich DNA (Wellinger *et al.*, 2006).

Under normal conditions, replication fork pausing or stalling at replication fork barriers does not seem to elicit a checkpoint response (Calzada *et al.*, 2005; Lambert *et al.*, 2005), perhaps because of the lack of exposed ssDNA (Lucchini and Sogo, 1994; Gruber *et al.*, 2000). Hence, the lack of replication fork stabilization, and, thus, a higher risk of fork collapse, might be one explanation for the increased level of recombination. On the other hand, there is compelling evidence that pausing per se does not cause replication forks to fall apart (Calzada *et al.*, 2005; Pacek *et al.*, 2006). Instead, stalled replication forks have been shown to remain stable for extended periods and to simply resume synthesis without recombination (Prado and Aguilera, 2005). Interesting insights come from studies in yeast, which show that pausing of replication forks, at least at programmed pause sites, is an active process (Calzada *et al.*, 2005; Dalgaard and Klar, 2000; Katou *et al.*, 2003), and more than just a collision with an obstacle. Whether this is also the case with accidental replication fork barriers, however, has not yet been established.

Certain regions in the genome of eukaryotes are particularly vulnerable and experience replication stress even in the absence of physical obstacles blocking fork progression. Replication fork slowing due to DNA polymerase impairment, for example, results in chromosomal breakage and translocations at specific sites in eukaryotic chromosomes. These otherwise stable regions are referred to as "fragile sites" (Richards, 2001). It has been proposed that breakage at fragile sites results from the slowing of replication and the presence of certain sequence elements, such as inverted repeats (Admire *et al.*, 2006; Lemoine *et al.*, 2005). Impairing polymerase function presumably increases the amount of ssDNA exposed and favours the formation of secondary structures. Recently, a tRNA region has been associated with preferential chromosomal breakage, suggesting that replication fork stalling at tRNA replication fork barriers might initiate the formation of fragile sites (Admire *et al.*, 2006). ATR plays a key role in the maintenance of fragile site stability (Casper *et al.*, 2002), supporting the notion that chromosome gaps and breaks at fragile sites are caused by unreplicated single-stranded DNA (Glover *et al.*, 2005).

3. Bypass of DNA lesions on one of the template strands

As in *E. coli*, increasing evidence suggests that replication forks in eukaryotes can re-prime both lagging and leading strand synthesis downstream of a lesion (Figure 4C). After UV irradiation, gaps in the nascent strands have been detected in yeast and mammalian cells (Lehmann, 1972; Meneghini, 1976; Lopes *et al.*, 2006). Using a combination of electron microscopy and two-dimensional gel electrophoresis, Lopes *et al.* (2006) provide direct evidence that in yeast cells leading and lagging strand DNA synthesis can be uncoupled at irreparable UV lesions and that small single-stranded gaps accumulate along the replicated DNA, suggesting that re-priming events downstream of a lesion can occur on both template strands. Further studies with cells mutated in either translesion DNA synthesis or homologous recombination have indicated that gaps left opposite the damage are first attempted to be filled by translesion polymerases, and only if this fails, by homologous recombination (Lopes *et al.*, 2006).

Ten translesion DNA polymerases capable of synthesizing DNA past template lesions have been identified in mammalian cells (Bebenek and Kunkel, 2004; Friedberg *et al.*, 2005). Monoubiquitination of PCNA in response to DNA damage is a key step in the activation of translesion DNA synthesis to bypass DNA lesions (Stelter and Ulrich, 2003). The monoubiquitinated form of PCNA has been shown to exhibit increased affinity for polη, polι and REV1 (Kannouche *et al.*, 2004; Watanabe *et al.*, 2004; Bienko *et al.*, 2005; Guo *et al.*, 2006; Plosky *et al.*, 2006). In principle, translesion DNA synthesis can occur either directly at the stalled fork or in a post-replicative manner. Consistent with the latter possibility, monoubiquitinated PCNA has been shown to persist even after DNA lesions have been removed (Niimi *et al.*, 2008). Using a DNA fiber labeling methodology to visualize cellular replicons, and sediment gradient velocity to analyze newly synthesized DNA, the role of monoubiquitinated PCNA in post-replicative gap filling has been further demonstrated (Edmunds *et al.*, 2008). In addition, however, these authors observed that the non-catalytic function

of REV1 was necessary to support the progression of replication forks through damaged DNA, independently of PCNA ubiquitination (Edmunds *et al.*, 2008). This suggests that in addition to postreplicative repair, REV1 may also assist replication forks directly.

One popular model for bypassing UV-induced lesions involves replication fork reversal, and implies progression of the lagging strand beyond the leading strand. Once the replication fork has regressed, and the newly synthesized DNA strands are annealed, the lagging strand can serve as a template to extend the leading strand past the lesion. This "template switching" model, proposed already in 1976 (Higgins *et al.*, 1976), however, is still not supported by compelling structural evidence. Lopes *et al.* (2006) detected only four reversed forks in 2100 forks examined in UV treated eukaryotic cells.

Conclusions. As in bacteria, lesions in one of the template DNA strands do not preclude DNA synthesis, which can be re-primed downstream of the lesion. Gaps are left behind and can be filled in by post-replication repair.

4. Replication-coupled repair of interstrand DNA crosslinks

Interstrand DNA crosslinks (ICL) present probably the most challenging lesion to overcome during DNA replication, as they covalently link both template strands. As ICLs are highly toxic, especially to dividing cells, ICL-inducers, such as mitomycin C, cisplatin, and nitrogen mustard, are widely used in cancer treatment (McHugh *et al.*, 2001).

Bacteria and yeasts repair ICLs using a combination of nucleotide excision repair and homologous recombination repair pathways (Dronkert and Kanaar, 2001; Lehoczky *et al.*, 2007). The situation in mammalian cells is less clear. A remarkable difference between mammals and yeast is that only cells defective for *XPF* or *ERCC1* are hypersensitive to interstrand DNA crosslinking agents, whereas other nucleotide excision repair-deficient cells are not, or only mildly, sensitive (Hoy *et al.*, 1985; Damia *et al.*, 1996).

Several lines of evidence suggest that the repair of ICLs in mammalian cells occurs mainly during DNA replication, features DSBs as repair intermediates (Akkari *et al.*, 2000; De Silva *et al.*, 2000; Rothfuss and Grompe, 2004), and involves the homologous recombination machinery (Takata *et al.*, 2001; Thompson and Schild, 2001; Godthelp *et al.*, 2002b). Cells exposed to ICLs depend on the Mus81-Eme1 endonuclease for DSB formation (Haber and Heyer, 2001; Hanada *et al.*, 2006), and on ERCC1-XPF for the resolution of crosslink-induced DSBs (Niedernhofer *et al.*, 2004). The ssDNA gaps that result from the unhooking of the crosslink are filled in by translesion DNA polymerases, which are able to bypass the remaining monoadduct (Zheng *et al.*, 2003; Wu *et al.*, 2004; Wittschieben *et al.*, 2006). Finally, homologous recombination plays a crucial role in the restoration of the damaged DNA (Cipak *et al.*, 2006; Zhang *et al.*, 2007). Several other proteins have also been implicated in the repair of interstrand DNA crosslinks, notably mismatch repair proteins (Zhang *et al.*, 2002; 2007; Wu *et al.*, 2005), and the Fanconi anemia proteins, which will be discussed below.

Most models for ICL repair in eukaryotic cells are based on our knowledge of the genes that are necessary for ICL tolerance, on the biochemical properties of some of the proteins encoded by those genes, and on a certain amount of speculation. Recently, Raschle and colleagues have set up an *in vitro* system, using *Xenopus* egg extracts, to study more directly replication-coupled repair of a plasmid containing a site-specific ICL (Raschle *et al.*, 2008). Unlike most models for ICL repair, which infer that a single replication fork collides with the lesion, collapses, and restarts via homologous recombination, Raschle and colleagues propose that ICL repair during replication occurs when two forks converge at the ICL. According to their study, the production of two intact and continuous duplex molecules from a damaged plasmid appears to occur in a stepwise manner (Figure 5). First, two replication forks converge at the ICL. The leading strands of both replication forks initially pause 20–40 nucleotides from the ICL. After 20 to 45 minutes, one of the two leading strands advances to within one nucleotide of the ICL. In the meantime, lagging strand products are being degraded. After 30 more minutes, the ICL is uncoupled and the growing leading strand is extended beyond the lesion in a DNA polymerase ζ–dependent manner. Fully repaired daughter duplexes are eventually produced, probably via the concerted action of nucleotide excision repair and homologous recombination.

Conclusions. Interstrand DNA crosslinks are particularly threatening to replicating cells, since they completely block replication fork progression. ICL repair involves the interplay of many different repair pathways, notably homologous recombination, nucleotide excision repair, mismatch repair and the Fanconi anemia network.

Role of homologous recombination in S phase

The DNA replication checkpoint acts as a first protective barrier to stabilize stalled replication forks (Dimitrova and Gilbert, 2000; Lopes *et al.*, 2001; Cobb *et al.*, 2003), and replication can resume once the block has been removed. But even in the case when a stalled replication fork collapses, restart of DNA replication via homologous recombination might not be necessary to complete DNA replication, as long as there is an adjacent fork which manages to replicate up to the site of replication arrest.

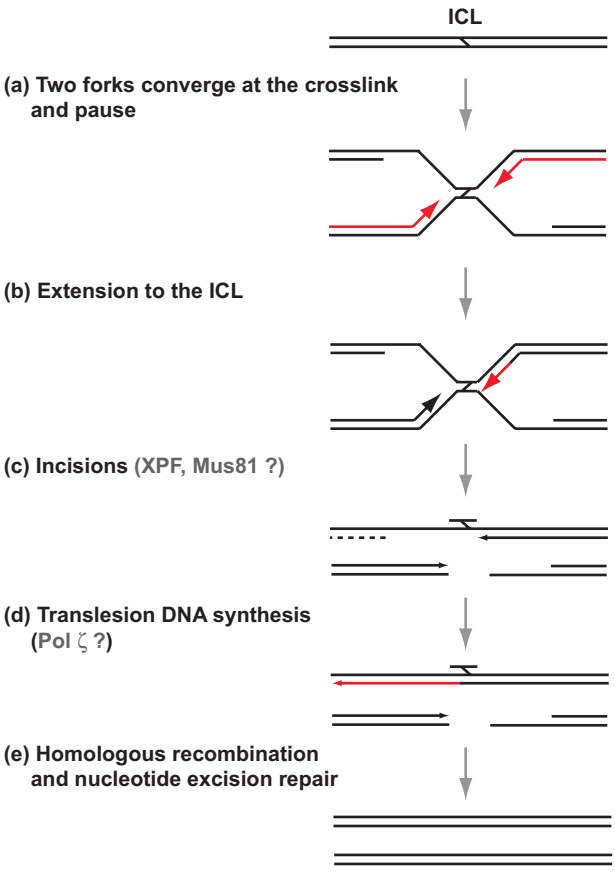

Figure 5. Replication-coupled repair of DNA interstrand crosslinks. (a) Two adjacent replication forks converge at an interstrand DNA crosslink (ICL). Both replication forks initially stall 20–40 nucleotides away from the lesion. (b) The leading strand of one of the replication forks is extended to within 1 nt of the ICL. (c) The crosslink is unhooked via dual incisions, and the two sister duplexes are uncoupled. (d) Translesion DNA polymerases (possibly REV1 and polymerase ζ) then extend the leading strand past the lesion. (e) Repair via the combined action of homologous recombination and nucleotide excision repair eventually produces two continuous and fully replicated duplex molecules. This model, as originally proposed by Raschle and colleagues (2008), is based on the analysis of ICL repair in *Xenopus* egg extracts.

In yeast, the checkpoint response to replication stress restrains the activity of recombination proteins (Boddy *et al.*, 2003; Kai *et al.*, 2005; Meister *et al.*, 2005; Alabert *et al.*, 2009). The replication checkpoint suppresses the resection of DNA ends in an Mrc1-dependent manner, thereby preventing the repair of DSBs by homologous recombination (Alabert *et al.*, 2009). Recombination, however, is necessary to complete DNA replication, as un-replicated DNA regions accumulate in *rad51*Δ cells treated with MMS (Alabert *et al.*, 2009). In the presence of an active checkpoint response, which inhibits the resection of DNA ends, recombination proteins most likely assemble on pre-existing single-stranded DNA intermediates at stalled replication forks (Alabert *et al.*, 2009). Taken together, these results suggest that homologous recombination at replication forks is highly controlled, but becomes inevitable where no adjacent forks can complete DNA replication, or when two neighboring replication forks are blocked. Accordingly, it has been shown in fission yeast that recombination proteins are recruited to replication forks stalled at the strong replication fork barrier RTS1 (replication termination sequence), and that recombination is required for cell survival (Lambert *et al.*, 2005). The fact that gross chromosomal rearrangements occur, however, suggests that recombination in this situation, though indispensable for cell survival, is at the expense of genome stability (Pellicioli and Foiani, 2005).

The collapse of a replication fork is often thought to result in the formation of a one-ended double-strand break, which is repaired via strand invasion into a homologous template and re-establishment of a replication fork, as would be the case in *E. coli* (Figure 1C). In *S. cerevisiae*, however, the two ends of a double-strand break are detected when a site-specific single-strand break is replicated (Cortes-Ledesma and Aguilera, 2006). This suggests that two replication forks converge at the nick and generate a two-ended double-strand break. Alternatively, the nick could be bypassed provided that it is located on the lagging strand template and the two sister chromatids are held together by cohesins. The repair of these replication-coupled double-strand breaks occurs then by sister chromatid exchange (Cortes-Ledesma and Aguilera, 2006).

Although direct evidence for replication restart from recombination intermediates is missing in higher eukaryotes, it has been shown that under certain circumstances, a highly processive copying mechanism similar to *E. coli* recombination-initiated replication, termed "break-induced replication" (BIR) can be engaged at the MAT locus in yeast (Malkova *et al.*, 1996). After invasion of the 3′-end from a one-ended double-strand break into a homologous duplex, a replication fork is established, and both leading and lagging strand DNA synthesis is initiated, being able to copy hundreds of kilobases (Holmes and Haber, 1999). Although originally identified as an error-prone RAD51-independent pathway (Kraus *et al.*, 2001), more recently a RAD51-dependent branch of break-induced replication has been demonstrated (Davis and Symington, 2004; Malkova *et al.*, 2005). Recent results suggest that BIR occurs by several rounds of strand invasion, DNA synthesis and dissociation (Smith *et al.*, 2007). These frequent dissociation and re-invasion events may limit BIR in favour of gene conversion by second-end capture, thereby avoiding chromosomal rearrangements caused by promiscuous recombination between dispersed repeated sequences (Smith *et al.*, 2007).

It has not been rigorously established whether or not a similar pathway exists in higher eukaryotes. It is possible that recombination-coupled DNA replication is suppressed in mammalian cells. In eukaryotic cells, a number of helicases control homologous recombination and antagonize strand invasion via distinct mechanisms, to promote gene conversion, inhibit crossovers, and prevent recombination-induced gross chromosomal rearrangements in mitotic cells. RecQ helicases (notably BLM and Sgs1), Srs2, RTEL1, and Mph1 all antagonize RAD51-mediated displacement loops (Krejci *et al.*, 2003; Veaute *et al.*, 2003; Bugreev *et al.*, 2007; Hu *et al.*, 2007; Barber *et al.*, 2008; Prakash *et al.*, 2009).

Consistent with the notion that DNA recombination functions essentially after DNA replication, cytological observations have shown that in S phase, RAD51 foci do not co-localize with active replication factories, but associate with post-replicative chromatin (Tashiro *et al.*, 2000). Furthermore, using an N-end rule degron-RAD51, Su and colleagues have demonstrated that chicken DT40 cells can progress until the end of S phase without RAD51 (Su *et al.*, 2008). RAD51 is essential, however, in G2, to resolve aberrant DNA structures that have accumulated during S phase.

Conclusions. In eukaryotes, replication forks are probably not, or only in exceptional cases, restarted by homologous recombination. This, by no means, contradicts the fact that homologous recombination is indispensable for completing the replication process (Murray *et al.*, 1994; Merrill and Holm, 1998). It merely suggests that homologous recombination in S phase is a post-replication repair pathway that deals with DNA lesions which remain after DNA synthesis.

Part II: Fanconi anemia

In higher eukaryotes, the proteins associated with Fanconi anemia (FA) play an important role in the maintenance of genomic integrity and in the cellular tolerance of replication stress. The first description of Fanconi anemia patients was reported in 1927 by the Swiss paediatrician Guido Fanconi (Fanconi, 1927). The clinical manifestations of Fanconi anemia include a variety of congenital abnormalities, severe bone marrow failure and cancers (Fanconi, 1967). It is a recessive disorder with multiple genetic complementation groups. To date, 12 autosomal Fanconi anemia genes (*FANCA*, *FANCC*, *FANCD1=BRCA2*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ=BRIP1*, *FANCL*, *FANCM*, *FANCN =PALB2*), and one Fanconi anemia gene located on the X chromosome (*FANCB*) have been identified (Table 1). The frequency of heterozygous carriers is estimated at 1:300 in the general population, and the disease strikes about 1 in 360,000 newborns (Friedberg *et al.*, 2006). Information on Fanconi anemia mutations is available at the URL http://www.rockefeller.edu/fanconi/mutate/.

Clinical features of Fanconi anemia

At birth, Fanconi anemia patients can exhibit a broad range of physical defects (Dokal, 2000; Tischkowitz and Hodgson, 2003). The most common skeletal abnormalities are hypoplasia of the thumb and radius. Vertebral anomalies, hip dislocations or scoliosis may also occur. Areas of the skin are often hypo- or hyper-pigmentated (café-au-lait spots). Other features include developmental delay, genital abnormalities, microphtalmia and microcephaly. One third of Fanconi anemia patients have renal and urinary tract malformations. Mental retardation, conductive deafness, gastrointestinal malformations and cardiac defects are less common.

Haematological abnormalities arise in the first decade of life. The blood count is usually normal at birth. Between age 5 and 10, pancytopenia, the severe depression of all haematopoietic lineages, arises as a consequence of bone marrow aplasia and the myelodisplastic syndrome. By the age of 29, 37% of affected individuals have developed acute myeloid leukaemia (AML) (Alter, 2003).

Patients with Fanconi anemia who reach adulthood have a high risk of developing squamous cell carcinomas in rapidly proliferating epithelia of the oral cavity, the oesophagus, the gastrointestinal tract, the anus, the uterus cervix and the vulva. Hepatic tumors in Fanconi anemia patients may be a secondary effect related to the treatment with androgens, which are used to stimulate the production of red blood cells (Tischkowitz and Hodgson, 2003). A compilation study of cancer cases described between 1927 and 2001 reveals that if the competing risks of aplastic anemia, leukaemia and liver tumors were removed, 76% of Fanconi anemia patients would develop solid tumors by the age of 45 (Alter, 2003).

Fanconi anemia is a chromosomal instability syndrome

Guido Fanconi initially proposed that Fanconi anemia was caused by a chromosome translocation (Fanconi, 1964). His hypothesis directed attention to chromosomes, which turned out to be unstable in cells derived from Fanconi anemia patients (Schroeder *et al.*, 1964; Schmid *et al.*, 1965; Swift and Hirschhorn, 1966). These cells are characterized by broken chromosomes and the formation of radial chromosomes due to the pairing of homologous or non-homologous metaphase chromosomes. Abnormalities in chromosome

Table 1. Fanconi anemia complementation groups. Chromosome location is from OMIM, predicted molecular weight and number of amino acids (aa) are from UniProtKB/Swiss-Prot. Protein motifs: BRCA2 is endowed with eight RAD51 binding BRC repeats, three oligonucleotide/oligosaccharide-binding (OB) folds similar to the ssDNA binding OB folds of RPA and a helix-turn-helix (HTH) motif that binds dsDNA. The ARM repeat is a right-handed super helix of a-helices involved in protein–protein interactions. The tetratricopeptide (TPR) repeat is a degenerated motif present in tandem arrays of 3–16 motifs which fold the protein into a right-handed superhelical scaffold for protein–protein interactions. The WD40 motif usually assumes a b-propeller fold which functions as a site for protein–protein interaction. The structure of the plant homeodomain (PHD) finger is similar to the structure of the RING finger motif that can act as E3 ubiquitin ligase. ERCC4: excision repair cross complementation group 4 nuclease domain.

Group	Gene	Location	Identification	Protein	Motifs
A	*FANCA*	16q24.3	(Foe *et al.*, 1996) (Apostolou *et al.*, 1996)	163 kDa 1455 aa	
B	*FANCB*	Xp22.3	(Meetei *et al.*, 2003a)	98 kDa 859 aa	
C	*FANCC*	9q22.3	(Strathdee *et al.*, 1992)	63 kDa 558 aa	
D1	*BRCA2*	13q12.3	(Howlett *et al.*, 2002)	384 kDa 3418 aa	BRC repeats (Bork *et al.*, 1996) (OB) folds DNA binding and HTH (Yang et al., 2002)
D2	*FANCD2*	3p25.3	(Timmers *et al.*, 2001)	166 kDa 1471 aa	ARM repeat (Smogorzewska *et al.*, 2007)
E	*FANCE*	6p22-p21	(de Winter *et al.*, 2000a)	59 kDa 536 aa	non-canonical helical repeat (Nookala *et al.*, 2007)
F	*FANCF*	11p15	(de Winter *et al.*, 2000b)	42 kDa 374 aa	non-canonical helical repeat (Kowal *et al.*, 2007)
G	*FANCG(XRCC9)*	9p13	(de Winter *et al.*, 1998)	69 kDa 622 aa	Tetratricopeptide repeats (Blom *et al.*, 2004)
I	*FANCI*	15q25-q26	(Smogorzewska *et al.*, 2007) (Sims *et al.*, 2007) (Dorsman *et al.*, 2007)	149 kDa 1328 aa	ARM repeat, Lipocalin fold (Smogorzewska *et al.*, 2007)
J	*BRIP1 (BACH1)*	17q22	(Levran *et al.*, 2005) (Levitus *et al.*, 2005) (Litman *et al.*, 2005)	141 kDa 1249 aa	DEAH helicase (Cantor *et al.*, 2001)
L	*FANCL (PHF9)*	2p16.1	(Meetei *et al.*, 2003)	43 kDa 375 aa	PHD finger, WD40 repeats (Meetei *et al.*, 2003)
M	*FANCM (HsHef)*	14q21.3	(Meetei *et al.*, 2005)	232 kDa 2048 aa	DEAH helicase, ERCC4 (Meetei *et al.*, 2005; Mosedale *et al.*, 2005)
N	*PALB2*	16p12.1	(Reid *et al.*, 2007) (Xia *et al.*, 2007)	131 kDa 1186 aa	WD40 repeats (Xia *et al.*, 2006)

structures occur both spontaneously, and after induction of DNA damage with agents that covalently link the two anti-parallel strands of the DNA double helix, such as mitomycin C, diepoxybutane, nitrogen mustard, and photoactivated psoralens (Auerbach and Wolman, 1976). Survival of Fanconi anemia cells in the presence of crosslinking agents is also severely reduced, a feature that is used to diagnose Fanconi anemia (Auerbach, 1993).

The occurrence of cancer in a chromosome instability syndrome is certainly not surprising, but also developmental abnormalities and bone marrow failure can be readily explained on the basis of genome instability. Broken or unrepaired DNA is expected to trigger senescence or apoptosis leading to increased cell death, which may cause pattern alternation during development and organ failure later in life. Selection against cell death-inducing signals may allow clonal expansion of genetically unstable cells.

The disabling of Fanconi anemia proteins has been associated with aberrant activation of the NF-kB and MAPK signaling pathways (Briot *et al.*, 2008), resulting in over-secretion of the cytokine TNF-α, which is known to impinge on self-renewal of haematopoietic stem cells (Bryder *et al.*, 2001; Dybedal *et al.*, 2001). Increased NF-kB signaling in Fanconi anemia cells may result from chronic activation of the DNA damage response kinase ATM (Kennedy *et al.*, 2007), which relays the DNA damage signal to the NF-kB pathway (Wu ZH *et al.*, 2006b). Thus, the high incidence of double-strand DNA breaks may readily explain bone marrow failure in Fanconi anemia.

Fanconi anemia proteins

In addition to the 13 FA complementation group proteins, two further proteins termed Fanconi anemia-associated proteins (FAAP) have been identified. Here,

the FA proteins will be classified as distinct functional entities: (1) the Fanconi anemia ubiquitin ligase complex, commonly known as the FA core complex (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FAAP100); (2) FANCM and FAAP24; (3) FANCD2 and FANC; (4) BRCA2 and PALB2; and (5) BRIP1.

Although FANCM was originally identified as part of the FA core complex (Meetei *et al.*, 2005), we choose here not to classify FANCM as a classical FA core complex subunit, as it is known now that FANCM is not essential for the assembly of the FA core complex, and that it can also function independently of the FA core complex (as discussed below).

1. The Fanconi anemia core complex

FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FAAP100 form a ubiquitin ligase complex (Meetei *et al.*, 2003a; 2003b), which is detected throughout the cell cycle (Alpi *et al.*, 2007) and involved in the monoubiquitination of FANCD2 and FANCI (Garcia-Higuera *et al.*, 2001; Smogorzewska *et al.*, 2007). Disabling mutations in any of the genes encoding subunits of the FA complex destabilize the whole complex (Garcia-Higuera *et al.*, 2001).

Assembly of the FA core complex. A number of protein–protein interaction studies have provided a rough picture of the molecular architecture of the FA core complex, which forms through the sequential assembly of sub-complexes (Figure 6). FANCC and FANCE interact directly and depend on each other for stability and nuclear localization (Medhurst *et al.*, 2001; Pace *et al.*, 2002; Taniguchi and D'Andrea, 2002; Leveille *et al.*, 2006). The partner of FANCA is FANCG, and the stability of one depends on the physical integrity of the other (Garcia-Higuera *et al.*, 1999; Kruyt *et al.*, 1999; Waisfisz *et al.*, 1999; Reuter *et al.*, 2000). FANCB interacts directly with FANCL and FAAP100 to form a stable sub-complex (Medhurst *et al.*, 2006; Ling *et al.*, 2007). FANCA binds the FANCB-FANCL heterodimer (Medhurst *et al.*, 2006). This interaction directs the assembly of a FANCA-FANCG-FANCB-FANCL sub-complex in the nucleus, and is necessary for the nuclear retention of FANCA (Medhurst *et al.*, 2006). The FANCA-FANCG-FANCB-FANCL sub-complex is stable in FA group C, group E and group F cells, suggesting that FANCA-FANCG-FANCB-FANCL and FANCC-FANCE-FANCF form independent entities (Medhurst *et al.*, 2006). FANCF is a flexible adaptor protein allowing assembly of the Fanconi anemia core complex: its amino-terminus interacts directly with the FANCC-FANCE heterodimer, whereas its carboxyl-terminus binds FANCG (Leveille *et al.*, 2004).

FANCL contains a plant homeodomain (PHD) and functions as an E3 ligase for monoubiquitination of the Fanconi anemia proteins FANCD2 and FANCI (Meetei

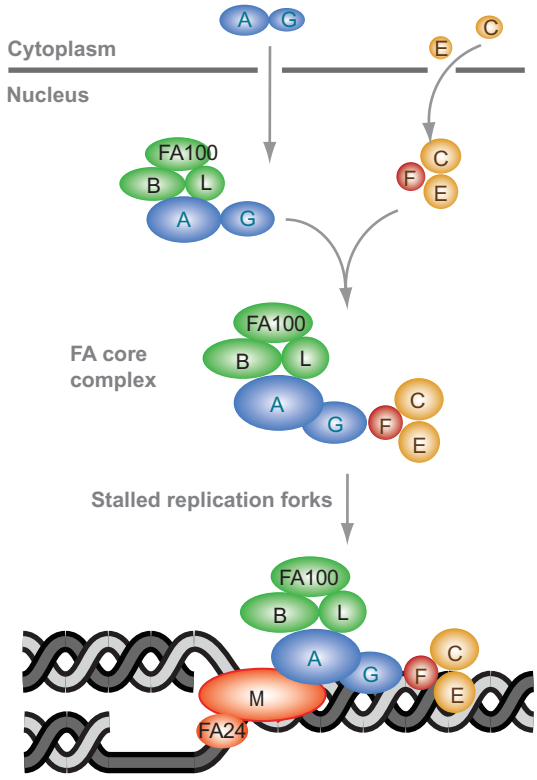

Figure 6. Assembly of the Fanconi anemia core complex. The FA core complex is an assembly of sub-complexes. The FANCA-FANCG and FANCE-FANCC heterodimers translocate in the nucleus. FANCA and FANCG then associate with FAAP100, FANCB and FANCL, whereas FANCC-FANCE bind FANCF. FANCF is a flexible adaptor protein allowing the assembly of the FA core complex (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FAAP100). FANCM and FAAP24 are required for the recruitment of the FA core complex to chromatin, presumably to damaged replication intermediates.

et al., 2003a; Gurtan *et al.*, 2006; Smogorzewska *et al.*, 2007; Alpi *et al.*, 2008), which will be discussed below. The other recognizable motifs in the FA ubiquitin ligase complex are protein–protein interaction modules. FANCL contains WD40 repeats involved in the assembly of the FA core complex (Gurtan *et al.*, 2006), and FANCG comprises tetratricopeptide repeats known to form superhelical scaffolds for protein–protein interactions (Blom *et al.*, 2004). Crystals of the carboxyl-termini of FANCE and FANCF have revealed almost entirely α-helical proteins with an elongated solenoidal structure (Kowal *et al.*, 2007; Nookala *et al.*, 2007). Secondary structure predictions suggest that FANCA, FANCB, FANCC and FANCG may adopt a similar fold. Fanconi anemia helical repeats show a strong preference for hydrophobic residues, mainly leucine, at eight specific positions (Nookala *et al.*, 2007). This non-canonical helical repeat may represent a variant in a family of helical repeat motifs which mediate protein–protein interactions, such as TPR, ARM and

HEAT repeats. The hydrophobic properties of FA core complex proteins are potential sources of artifacts in protein–protein interaction studies and cause solubility problems during expression and purification of recombinant proteins.

Phosphorylation of FA core complex proteins. Several FA core complex proteins are phosphorylated. After DNA damage, FANCA is phosphorylated at serine 1449 by ATR. This posttranslational modification, which is not observed during an unchallenged S phase, is necessary for cellular resistance to mitomycin C (Collins *et al.*, 2008). FANCG is phosphorylated at serine 7 (Qiao *et al.*, 2004), a modification associated with the formation of a complex containing FANCG, FANCD2 and the recombination repair proteins BRCA2 and XRCC3 (Wilson *et al.*, 2008). During mitosis, FANCG is phosphorylated at serine 383, and at serine 387 by CDC2 (Mi *et al.*, 2004). The integrity of these phosphorylation sites is necessary for the complementation of FANCG-deficient cells (Mi *et al.*, 2004). Finally, FANCE is phosphorylated by Chk1 at threonine 346 and serine 374 (Wang *et al.*, 2007). Whereas this phosphorylation is required for resistance to mitomycin C, it is apparently not necessary for FANCD2 monoubiquitination and normal cell cycle progression (Wang *et al.*, 2007).

Role of the FA core complex. Whereas for a long time the only function of the FA core complex was thought to be the monoubiquitination of FANCD2, compelling evidence suggests now that the FA complex has additional roles beyond FANCD2 monoubiquitination (Matsushita *et al.*, 2005). Whereas a recombinant FANCD2 protein artificially fused to ubiquitin can correct Fancd2 mutant cells for DNA damage sensitivity, it cannot complement cells with disabled FA core complex proteins, suggesting that the FA core complex is directly required for cellular tolerance to interstrand DNA crosslinking agents (Matsushita *et al.*, 2005).

Conclusions. FA core complex proteins are highly regulated by several phosphorylation steps. The majority of these phosphorylation events, however, are neither required for the formation of the FA complex, nor for the monoubiquitination of FANCD2. The FA core complex is likely to perform multiple functions to promote tolerance of replication stress. We know that it promotes the monoubiquitination of FANCD2 and FANCI, but other putative functions of the FA core complex remain to be discovered.

2. FANCM and FAAP24

During the synthesis phase of the cell cycle, the FA core complex associates with chromatin and the nuclear matrix in a DNA damage-induced manner (Qiao *et al.*, 2001; Mi and Kupfer, 2004). The stable association of the FA complex with chromatin in S phase depends on FANCM and FAAP24 (Mosedale *et al.*, 2005; Kim *et al.*, 2008). FANCM was discovered by virtue of its association with the FA ubiquitin ligase complex (Meetei *et al.*, 2005). The protein is not essential for the assembly of the complex, although a reduction in the abundance of the FA complex has been noted in DT40 *Fancm*$^{-/-}$ cells (Mosedale *et al.*, 2005).

During mitosis, FANCM is phosphorylated by Plk1, and targeted for degradation by the β-TRCP subunit of the SCF E3-ubiquitin ligase (Kee *et al.*, 2009). Regulated degradation of FANCM in mitosis is necessary to release the FA core complex from chromatin. Interestingly, expression of a non-degradable recombinant FANCM protein induces chromosomal aberrations and sensitivity to MMC, suggesting that the timely degradation of FANCM is an important step in FA-mediated repair events (Kee *et al.*, 2009).

FANCM is a branch point DNA translocase. Unlike the FA ubiquitin ligase complex, FANCM associates with chromatin throughout the cell cycle (Kim *et al.*, 2008). It contains an amino-terminal SF2 helicase domain and a carboxyl-terminal ERCC4-like nuclease domain (Meetei *et al.*, 2005; Mosedale *et al.*, 2005). In contrast to the FA core complex proteins, FANCM is highly conserved, and orthologs are found in a variety of species (Figure 7A): Pyrococcus furiosus Hef (Nishino *et al.*, 2005), Saccharomyces cerevisiae Mph1 (Schurer *et al.*, 2004) and Schizosaccharomyces pombe Fml1 (Sun *et al.*, 2008).

A crystallographic analysis of the Hef helicase domain from *P. furiosus* has revealed that it is composed of two classical RecA-like folds, which contain the conserved helicase motifs necessary for ATP hydrolysis and translocation, and an intervening Hef-specific domain, structurally similar to the "thumb" domain of polymerases, and critical for the recognition and the processing of branched DNA structures (Nishino *et al.*, 2005). It is noteworthy that other branch-recognizing helicases, such as *E. coli* RecG and RecQ, also contain specific domains important for their branch-specific helicase activity (Singleton *et al.*, 2001; Bennett and Keck, 2004; Nishino *et al.*, 2005). Unlike in Hef, however, these domains are located before (RecG) and after (RecQ) the helicase core.

Interestingly, in their study Nishino and colleagues also identified a putative Hef homolog in humans (the hypothetical protein KIAA1596, later known as FANCM), and proposed that it was a candidate for the processing of branched DNA molecules (Nishino *et al.*, 2005), in analogy to Hef which can specifically recognize and process fork-structured DNA and Holliday junctions (Komori *et al.*, 2004). Indeed, human FANCM and *S. pombe* Fml1 exhibit high and specific affinity for branched DNA structures (Figure 7B), and translocate the branch point of model replication forks and

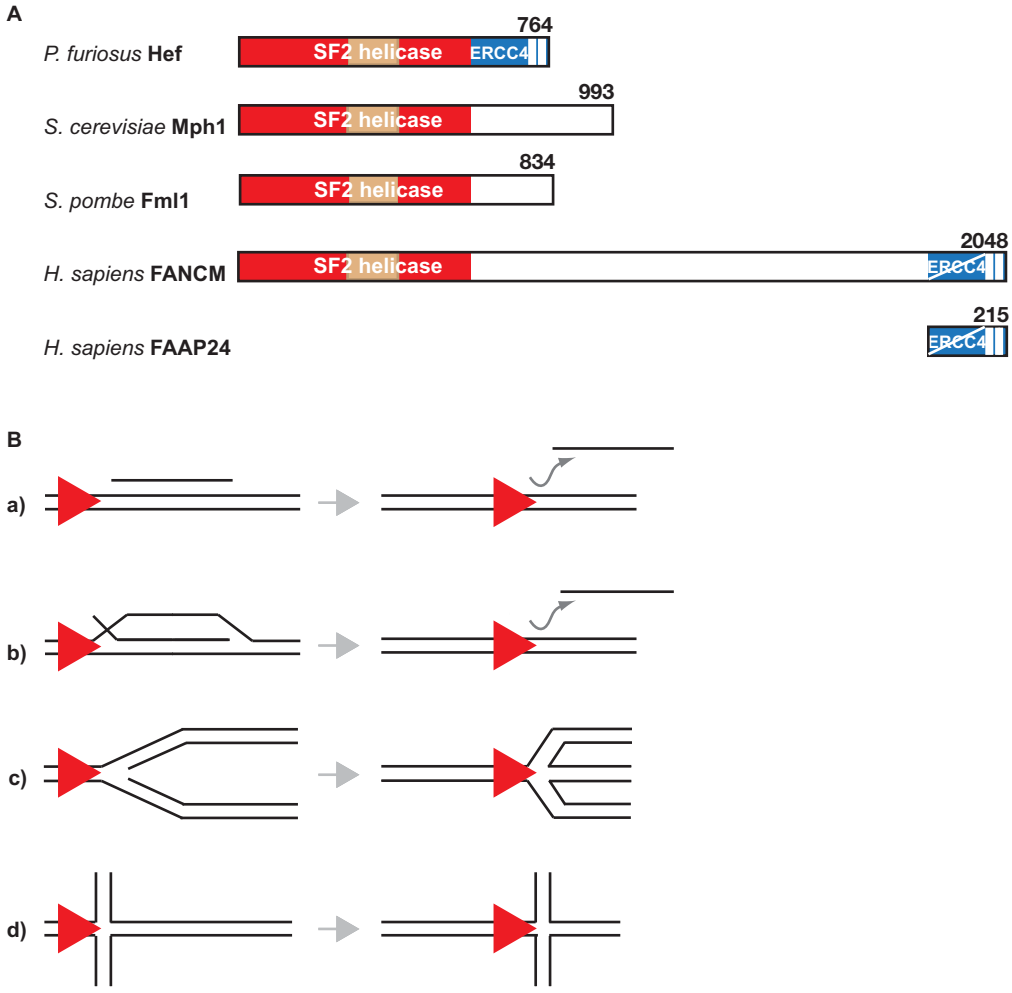

Figure 7. FANCM-like proteins and DNA substrates. (A) FANCM and orthologs (Hef, Mph1 and Fml1) share an amino-terminal SF2 helicase domain (red), which is interrupted by an intervening sequence (light brown) essential for recognition of branched DNA structures. Hef and FANCM also contain a C-terminal ERCC4 nuclease motif (blue) and helix-hairpin-helix DNA-binding motifs (white boxes). In FANCM, the ERCC4 motif is degenerated, but required for the homotypic interaction with FAAP24. (B) FANCM can displace the third strand of a triple helix (a). FANCM and orthologs can dismantle displacement (D) loops (b), promote replication fork reversal (c), and translocate four-way junctions (d).

Holliday junctions in an ATPase-dependent manner (Meetei *et al.*, 2005; Gari *et al.*, 2008a; 2008b; Sun *et al.*, 2008; Xue *et al.*, 2008). FANCM and Fml1 poorly unwind the two strands of duplex DNA (Gari *et al.*, 2008b; Sun *et al.*, 2008), but have the ability to translocate along double-stranded DNA (Meetei *et al.*, 2005; Gari *et al.*, 2008a; 2008b; Sun *et al.*, 2008; Xue *et al.*, 2008) and to promote replication fork reversal *in vitro*, that is the conversion of a replication fork into a four-way junction (Gari *et al.*, 2008a; Sun *et al.*, 2008). Furthermore, FANCM, Fml1 and Mph1 were found to dismantle displacement (D)-loops in vitro (Gari *et al.*, 2008a; Sun *et al.*, 2008, Prakash, 2009).

These biochemical studies suggest that FANCM and orthologs are likely to fulfil their important roles in DNA damage tolerance by acting directly on DNA replication and DNA repair intermediates.

FANCM promotes DNA damage signaling and DNA repair. Displacement loops arise during double-strand break repair via homologous recombination, when one strand of the broken DNA molecule invades a homologous duplex, which serves as a template for repair. Several studies suggest that dissociation of displacement loops by FANCM-like proteins may promote gene conversion and prevent crossover formation. In *S. pombe*, the proportion of crossovers produced during repair of a broken plasmid is increased in *fml1* Δ mutants (Sun *et al.*, 2008). In *S. cerevisiae*, Mph1 suppresses crossovers during recombinational repair of chromosomal DSBs (Prakash *et al.*, 2009). In DT40 cells, a higher frequency of sister chromatid-exchanges is observed in the absence of chicken FANCM (Mosedale et al., 2005). D-loop displacement by FANCM-like proteins (Figure 8) may limit crossover products by driving the recombinational repair

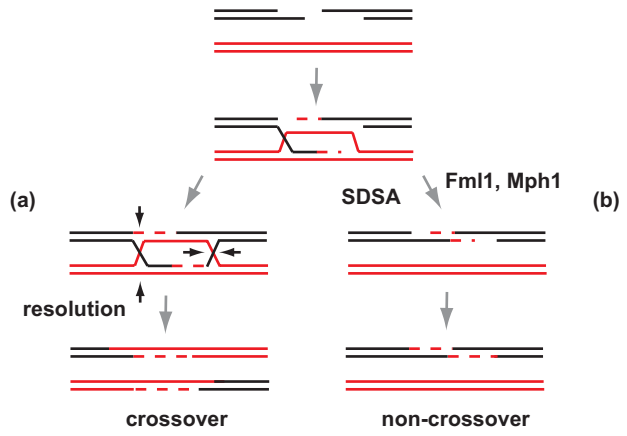

Figure 8. Mechanisms for gene conversion with or without crossovers. After D-loop formation, extension from the 3′-end of the invading strand is templated by the donor duplex. Two sub-pathways of homologous recombination with different genetic outcomes can then occur. (a) Two Holliday junctions are formed and resolved in opposite orientations to yield crossover products. (b) Synthesis-dependent strand-annealing (SDSA): The D-loop is dismantled before Holliday junction formation and the extended DNA strands can now anneal. Fml1 and Mph1 can catalyze D-loop disruption. Gap filling and ligation complete the repair process.

process into the synthesis-dependent strand-annealing sub-pathway of homologous recombination (Sun *et al.*, 2008; Prakash *et al.*, 2009).

Surprisingly, while limiting the formation of crossovers during mitotic DSB repair, Fml1 seems to promote homologous recombination when a replication fork is stopped at the replication termination sequence RTS1 (Sun *et al.*, 2008). Fork reversal by Fml1 may promote RAD51-mediated gene conversion, as it generates a double-stranded DNA end, which can be processed into a 3′ single-stranded DNA tail amenable to the assembly of a RAD51 nucleoprotein filament (Sun *et al.*, 2008), as described in Figure 2.

Interestingly, FANCM is also required for DNA damage signaling *in vivo*, as it has been shown to promote efficient activation of the ATR-mediated checkpoint-signaling pathway (Collis *et al.*, 2008). Facilitation of checkpoint signaling by FANCM depends on its branch-point translocase activity, consistent with the notion that fork remodeling by FANCM reinforces the assembly of a DNA damage-signaling complex at stalled forks (Collis *et al.*, 2008).

FAAP24. Members of the ERCC4/XPF family of endonucleases are usually functional as heterodimers, as observed with XPF-ERCC1 (Sijbers *et al.*, 1996) and Mus81-Eme1 (Boddy *et al.*, 2001; Chen *et al.*, 2001; Ciccia *et al.*, 2003; Gaillard *et al.*, 2003). The two partner proteins interact with each other by homotypic interactions between their ERCC4 nuclease domains (Boddy *et al.*, 2001; McCutchen-Maloney *et al.*, 1999). It is noteworthy that only the nuclease domains of XPF and Mus81 are active, whereas the nuclease domains in the corresponding partner proteins ERCC1 and Eme1 are degenerated (Boddy *et al.*, 2001; Enzlin and Scharer, 2002).

When FANCM was discovered to be a new member of this family of proteins, databases were searched for an ERCC4 motif containing protein that could interact with the ERCC4 motif of FANCM. This approach led to the discovery of FAAP24 (Ciccia *et al.*, 2007). FAAP24 (FA-associated protein of 24 kDa) associates with the FA core complex, and is required for normal levels of FANCD2 monoubiquitination (Ciccia *et al.*, 2007). FAAP24 knockdown cells are sensitive to interstrand DNA crosslinking agents. In contrast to Mus81-Eme1 and XPF-ERCC1, in FANCM-FAAP24 both ERCC4 nuclease domains appear to be degenerated (Ciccia *et al.*, 2007; Meetei *et al.*, 2005), which explains why no nuclease activity has yet been associated with the FANCM-FAAP24 heterodimer. FAAP24 exhibits affinity for single-stranded DNA, and binds to branched DNA molecules with single-stranded arms, such as flaps and splayed arms (Ciccia *et al.*, 2007).

In vitro, FAAP24 is dispensable for the biochemical activities of FANCM, such as a recognition of branched DNA and branch-point translocation (Gari *et al.*, 2008b). *In vivo*, however, FAAP24 appears to be required for the stable association of FANCM with chromatin (Kim *et al.*, 2008). Like FANCM, FAAP24 is necessary for ATR-mediated checkpoint signaling (Collis *et al.*, 2008), possibly by playing an architectural role necessary for the assembly and stability of the ATR/Chk1 signaling complex at stalled replication forks.

Dual role for FANCM-FAAP24. FANCM and FAAP24 interact with HCLK2 (Collis *et al.*, 2008), a regulator of the stability of PI3K-related kinases (Takai *et al.*, 2007). FANCD2 foci formation and monoubiquitination depend on the presence of HCLK2 (Collis *et al.*, 2007). The FANCM-FAAP24-HCLK2 DNA damage signaling complex, however, forms independently of the FA ubiquitin ligase complex (Collis *et al.*, 2008). Thus, it appears that the FANCM-FAAP24 heterodimer has two independent functions: firstly, it promotes the recruitment of the FA core complex to chromatin, and secondly, it facilitates ATR/Chk1 checkpoint signaling. An additional level of complexity can be anticipated from the observation that FANCM and FAAP24 do not always interact in the cell, suggesting that FANCM and FAAP24 may also play independent roles (Collis *et al.*, 2008).

The translocase activity of FANCM is dispensable for the chromatin association of the FA ubiquitin ligase complex and for normal FANCD2 monoubiquitination (Xue *et al.*, 2008), but necessary for cellular tolerance to interstrand DNA crosslinking agents and checkpoint signaling (Collis *et al.*, 2008; Xue *et al.*, 2008).

Conclusions. The important role of FANCM-like proteins in the remodeling of branched DNA intermediates is conserved throughout evolution and distinct from the role of FANCM in the FANCD2 monoubiquitination pathway, which has been acquired later in evolution.

3. FANCD2 and FANCI

The discovery of FANCD2 (Timmers *et al.*, 2001) and its monoubiquitination at lysine 561 by the FA ubiquitin ligase complex (Garcia-Higuera *et al.*, 2001) was taken up with enthusiasm in the FA field, as it provided a first grasp as to the function of the FA pathway. Monoubiquitinated FANCD2 preferentially associates with chromatin (Wang *et al.*, 2004), a step which is also dependent on BRCA1 and the phosphorylated form of histone H2A, γH2AX (Bogliolo *et al.*, 2007). Furthermore, FANCD2 accumulates within DNA repair foci containing BRCA1, RAD51 and BRCA2 (Garcia-Higuera *et al.*, 2001; Hussain *et al.*, 2004), the latter controlling the activity of RAD51 during DNA double-strand break repair by homologous recombination (Yang *et al.*, 2005; Esashi *et al.*, 2005; 2007).

Interestingly, the more recently identified FANCI has turned out to be a paralog of FANCD2 with sequence homologies and similar properties (Sims *et al.*, 2007; Smogorzewska *et al.*, 2007). Both proteins are monoubiquitinated by the FA core complex in response to DNA replication stress, and re-localize to chromatin and the same nuclear foci. They have also been found to co-immunoprecipitate, and their stability and monoubiquitination are mutually interdependent (Sims *et al.*, 2007; Smogorzewska *et al.*, 2007), suggesting that they work together in a complex.

Monoubiquitination of FANCD2. Monoubiquitination is a reversible regulatory modification that can control protein function (Hicke, 2001). The conjugation of ubiquitin moieties to proteins (Figure 9A) necessitates the concerted action of three types of enzymes (Hicke, 2001; Pickart, 2001). An E1 enzyme activates ubiquitin through ATP hydrolysis, and promotes formation of a high-energy thiolester bond between ubiquitin and the active site cysteine of an E2-conjugating (UBC) enzyme. An E3 enzyme eventually mediates the conjugation of the ubiquitin moiety to the substrate. More than 30 E2 enzymes have been identified and hundreds of putative E3 enzymes confer substrate specificity via different mechanisms of ubiquitin transfer.

Recent studies have provided fresh insights into the E1-E2-E3 cascade, which mediates the monoubiquitination of FANCD2. A yeast two-hybrid screen with FANCL as a bait led to the discovery of UBE2T, the E2 enzyme in the FANCD2 monoubiquitination pathway (Machida *et al.*, 2006; Alpi *et al.*, 2007; 2008). FANCL, the E3 ligase subunit of the FA complex (Alpi *et al.*, 2008; Meetei *et al.*, 2003a), recruits UBE2T via its plant homeodomain (PHD)/RING finger variant motif, whereas its WD40 repeats bind and stabilize the FA complex (Gurtan *et al.*, 2006; Machida *et al.*, 2006). FANCE binds directly to FANCD2, suggesting that it plays an important role in the interaction of the FA ubiquitin ligase complex with its substrate (Pace *et al.*, 2002; Gordon and Buchwald, 2003; Gordon *et al.*, 2005). It is still unclear how the E2 enzyme UBE2T, the FA ubiquitin ligase complex, and the substrate FANCD2 are brought together in living cells, as they are recruited independently to chromatin (Alpi *et al.*, 2007).

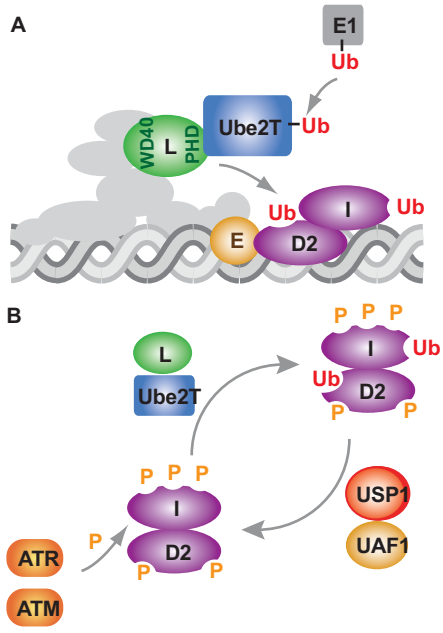

Figure 9. Regulated monoubiquitination of FANCD2. (A) Monoubiquitination of FANCD2 necessitates an E1 enzyme, which promotes the formation of a thiolester bond between ubiquitin and Ube2T (the E2 enzyme), and the FA ubiquitin ligase complex, which mediates the conjugation of the monoubiquitin moiety to FANCD2 and FANCI. FANCL, the E3 ligase, interacts with the FA core complex via its WD40 repeats and with Ube2T via its PHD motif. FANCE in the FA core complex interacts directly with FANCD2. FANCI restricts monoubiquitination of chicken FANCD2 to lysine 563. (B) The FANCD2 monoubiquitination and deubiquitination cycle: Phosphorylation of FANCI is a molecular switch for the monoubiquitination of FANCD2. The phosphorylation of FANCD2 by ATR is necessary for its DNA damage-dependent monoubiquitination. FANCD2 is deubiquitinated by USP1, a cysteine protease that is strongly stimulated by its partner protein UAF1.

The monoubiquitination of chicken FANCD2 at lysine 563 has been reconstituted *in vitro* (Alpi *et al.*, 2008). The reaction minimally requires E1, UBE2T (E2), FANCL (E3), ubiquitin, FANCD2 and FANCI. Alpi and colleagues noted that the WD40 repeats of FANCL form an RWD-like domain (RING finger proteins/WD repeat proteins/yeast DEAD-like helicases), which stimulates the activity of UBE2T. Remarkably, FANCI was found indispensable for reconstitution of site-specific ubiquitination

of chicken FANCD2 at lysine 563 (Alpi *et al.*, 2008). How FANCI restricts ubiquitination of FANCD2 to lysine 563, however, is not yet known. FANCD2 and FANCI interact, but the two proteins do not form a stable and stoichiometric heterodimer (Ishiai *et al.*, 2008; Smogorzewska *et al.*, 2007).

So far, the functional role for FANCI monoubiquitination has remained elusive. FANCI was not ubiquitinated in the reconstituted *in vitro* system, and FANCI monoubiquitination is dispensable for FANCD2 monoubiquitination and for tolerance to DNA lesions *in vivo* (Alpi *et al.*, 2008; Ishiai *et al.*, 2008). The crucial post-translational modification of FANCI is phosphorylation at multiple Ser/Thr-Gln motifs, which provides a molecular switch for turning on the FANCD2 monoubiquitination pathway (Ishiai *et al.*, 2008).

ATR is required for efficient FANCD2 monoubiquitination (Andreassen *et al.*, 2004). In response to ICLs, FANCD2 is phosphorylated in an ATR-dependent manner (Pichierri and Rosselli, 2004). Phosphorylation takes place on threonine 691 and serine 717, and is required for the damaged-induced monoubiquitination of FANCD2 (Ho *et al.*, 2006). ATM also phosphorylates threonine 691 during normal S phase progression, and thereby contributes to the monoubiquitination of FANCD2 (Ho *et al.*, 2006).

Deubiquitination of FANCD2. The deubiquitination of FANCD2 is as important as its monoubiquitination (Figure 9B). FANCD2 is deubiquitinated by USP1 (Nijman *et al.*, 2005), a cysteine protease that also hydrolyzes monoubiquitin from PCNA (Huang *et al.*, 2006). USP1 localizes to chromatin and associates with FANCD2 (Nijman *et al.*, 2005). Its stability and enzymatic activity are strongly stimulated by UAF1 (Cohn *et al.*, 2007). UAF1 contains WD40 repeats predicted to form a complete propeller structure that is crucial for the formation of an active USP1/UAF1 heterodimer (Cohn *et al.*, 2007). Persistence of FANCD2 monoubiquitination caused by inactivation of the USP1 gene sensitizes DT40 cells to interstrand DNA crosslinking agents (Oestergaard *et al.*, 2007). Consistent with this, bone marrow cells from USP1$^{-/-}$ mice are hypersensitive to mitomycin C (Kim *et al.*, 2009). The phenotypic features of USP1$^{-/-}$ mice are similar to that of FA mice (Kim *et al.*, 2009). As expected, the depletion of USP1 results in increased levels of chromatin-bound monoubiquitinated FANCD2; surprisingly, however, FANCD2 does not form nuclear foci in the absence of USP1 (Kim *et al.*, 2009). In summary, monoubiquitination of FANCD2 promotes chromatin association of FANCD2, but deubiquitination of FANCD2 is necessary for foci formation and cellular tolerance to DNA crosslinks.

USP1 is capable of auto-cleavage at a ubiquitin-like diglycine motif (Huang *et al.*, 2006), thereby maintaining its appropriate level in the cell (Oestergaard *et al.*, 2007). This reaction, however, is not essential for the regulation of USP1 activity. *In vitro*, the cleavage products of USP1 are held together by UAF1 in a complex that remains active (Cohn *et al.*, 2007), and the non-self-cleavable USP1 mutant complements hypersensitivity to UV and cisplatin in DT40 cells (Oestergaard *et al.*, 2007). USP1 levels are cell cycle-regulated by transcriptional control and by proteasomal degradation (Nijman *et al.*, 2005). USP1 levels peak in S phase and decline when cells exit S phase. Transcription of *USP1* is also rapidly suppressed following UV irradiation (50 J m^{-2}), allowing accumulation of monoubiquitinitated FANCD2 in response to high doses of DNA photoproducts (Cohn *et al.*, 2007).

Conclusions. Although the actual role of FANCD2 monoubiquitination remains to be deciphered, it is known to constitute a pivotal step in the cellular response to replication stress. It is for example conceivable that the monoubiquinated form of FANCD2 constitutes a specific binding interface for the assembly of yet unknown proteins (Matsushita *et al.*, 2005).

One important question to be addressed is the nature of the DNA structure that triggers the binding of FANCD2 to chromatin. FANCD2 is monoubiquitinated and forms foci very rapidly following ionizing radiation (Rothfuss and Grompe, 2004). Thus, after IR, FANCD2 appears to be primarily activated by DSBs or resected DSBs, rather than stalled replication forks. FANCD2 also accumulates rapidly at localized UV damage sites in the nucleus of replicating cells, in an H2AX- and BRCA1-dependent manner (Bogliolo *et al.*, 2007). Whatever the triggering DNA structure may be, however, it is likely that other proteins, not yet identified, mediate the recruitment of FANCD2 to chromatin, since FANCD2 alone exhibits only weak affinity for DNA *in vitro* (Park *et al.*, 2005).

USP1 plays an important role in the recycling of monoubiquitinated FANCD2. Deubiquitination of FANCD2 may also be important to release FANCD2 from DNA damage signaling and repair complexes, allowing subsequent repair steps to take place in order to complete the repair process (Oestergaard *et al.*, 2007).

4. BRCA2 and PALB2

In contrast to the other complementation groups, FA-D1 (Howlett *et al.*, 2002), FA-J (Levitus *et al.*, 2005; Levran *et al.*, 2005; Litman *et al.*, 2005), and FA-N (Rahman *et al.*, 2007; Reid *et al.*, 2007; Xia *et al.*, 2007) cells display normal levels of FANCD2 monoubiquitination, suggesting that the corresponding gene products work either downstream in the FANCD2 monoubiquitination pathway or in a separate branch of the Fanconi anemia network.

The discovery of biallelic inactivation of *BRCA2* in FA group D1 was a major breakthrough in the Fanconi anemia field, as one of the FA proteins could be linked to the

well-characterized homologous recombination repair pathway (Howlett *et al.*, 2002). In addition, the fact that *BRCA2*, a renowned gene linked to familial breast and ovarian cancers, is part of the FA network has propelled Fanconi anemia to the forefront of cancer research. The connection of Fanconi anemia and homologous recombination was further enforced when FA group N cells were found to bear mutations in the *PALB2* gene (Rahman *et al.*, 2007; Reid *et al.*, 2007; Xia *et al.*, 2007). PALB2 promotes the association of BRCA1 with BRCA2 (Zhang *et al.*, 2009). The C-terminal WD40 repeats of PALB2 bind directly to the amino-terminus of BRCA2 (Xia *et al.*, 2006), and the N-terminal coiled-coil motif of PALB2 associates with the coiled-coil motif of BRCA1 (Zhang *et al.*, 2009). PALB2 is necessary for the stable association of BRCA2 with chromatin (Xia *et al.*, 2006), whereas BRCA1 targets PALB2 and BRCA2 to DNA damage sites, enabling DNA repair by homologous recombination (Zhang *et al.*, 2009).

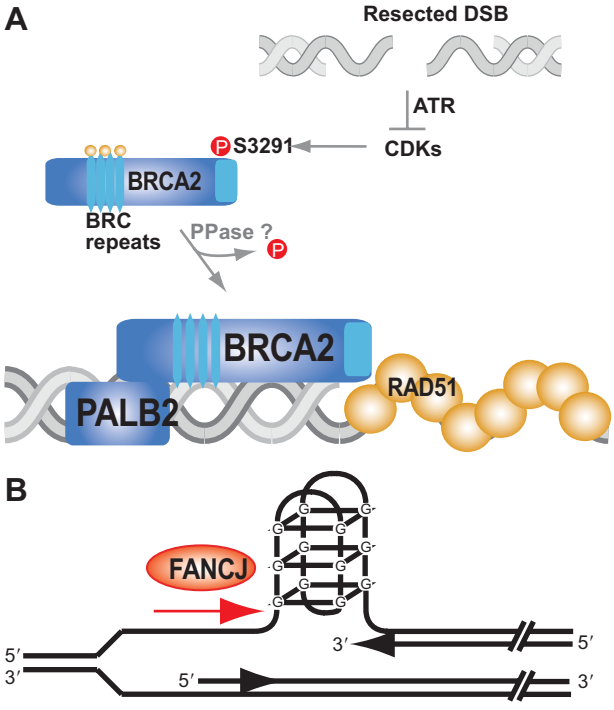

Figure 10. Roles of BRCA2, PALB2 and BRIP1. (A) The activity of RAD51 is regulated by BRCA2 (FANCD1). PALB2 (FANCN) promotes the stable association of BRCA2 with chromatin. Monomeric RAD51 interacts with BRC motifs in the central region of BRCA2, whereas the C-terminus of BRCA2 interacts with and stabilizes RAD51 nucleoprotein filaments. Phosphorylation of BRCA2 at serine 3291 by CDK blocks the interaction of multimeric RAD51 with the C-terminus of BRCA2. Activation of the DNA damage response by ATM and ATR leads to a down-regulation of CDK activity, and removal of the inhibitory phosphate on BRCA2. BRCA2 can then catalyze RAD51 nucleoprotein filament formation at resected DNA double-strand breaks. (B) FANCJ unwinds G-quadruplex structures, which may arise in guanine-rich regions during transient denaturation of DNA by the replication machinery.

BRCA2 regulates the activity of RAD51. In its active form, RAD51 forms a polymer wrapped around single-stranded DNA (Figure 10A). This RAD51 nucleoprotein filament catalyzes homology search and strand exchange between broken DNA and an intact homologous template during DNA double-strand break repair by homologous recombination (West, 2003). The activity of RAD51 is regulated by direct interactions with a series of BRC motifs located in the central region of BRCA2 and with the carboxyl-terminus of BRCA2 (Wong *et al.*, 1997; Davies and Pellegrini, 2007; Esashi *et al.*, 2007).

The crystallographic structure of a RAD51–BRC4 complex reveals that the BRC4 polypeptide is in continuous contact with RAD51 and mimics the structure of the RAD51 domain involved in the interaction between RAD51 monomers (Pellegrini *et al.*, 2002). Hence, the BRC repeats in BRCA2 prevent RAD51 oligomerization, as they compete for the binding interface involved in RAD51 self-association (Pellegrini *et al.*, 2002).

Although attractive, this description of the BRC–RAD51 interaction is probably oversimplified. Indeed, BRC repeats are multifaceted. A BRCA2 fragment containing all eight BRC repeats has been shown to stimulate strand exchange *in vitro* (Shivji *et al.*, 2006), and under specific conditions, BRC repeats can associate with RAD51 nucleoprotein filaments (Galkin *et al.*, 2005). Furthermore, a sequence of 36 residues in the C-terminus of BRCA2 (BRCA2Exon27) interacts specifically with RAD51 nucleoprotein filaments, and protects these filaments from disruption by the BRC repeats (Davies and Pellegrini, 2007; Esashi *et al.*, 2007). Interaction between RAD51 and BRCA2Exon27 is inhibited by CDK-mediated phosphorylation of BRCA2 at serine 3291 (Esashi *et al.*, 2005; Yata and Esashi, 2009), consistent with a tight regulation of the RAD51/BRCA2 interaction during cell cycle. Following cellular exposure to DNA damaging agents, phosphorylation of Ser3291 is decreased, which allows RAD51 filament formation (Esashi *et al.*, 2005).

Since BRCA2 is directly involved in the enzymology of homologous recombination, it is not surprising that both FA group D1 and FA group N cells are deficient in RAD51 foci formation in response to interstrand DNA crosslinking agents (Godthelp *et al.*, 2002a; Xia *et al.*, 2007). The co-localization of FANCD2 with RAD51 and BRCA1 in damaged induced repair foci (Garcia-Higuera *et al.*, 2001), and the fact that FANCG and FANCD2 directly interact with BRCA2 (Hussain *et al.*, 2003; 2004; Wilson *et al.*, 2008), suggest a further link between the Fanconi anemia network and homologous recombination.

BRCA2- and PALB2-deficient cells differ from other FA subtypes. Major phenotypic and clinical differences exist between FA group D1 and N, and the other FA groups. Firstly, impaired RAD51 foci formation distinguishes FA-D1 and FA-N cells from all other FA complementation

groups. None of the other FA proteins are necessary for the stable accumulation of RAD51 in damage-induced repair foci (Godthelp *et al.*, 2005). Secondly, repair of enzymatically-induced DNA double-strand breaks by homologous recombination is not, or only mildly, defective in human and chicken DT40 FA mutant cells (Hinz *et al.*, 2006). This is in contrast with the marked homologous recombination defect observed with bona fide homologous recombination mutants, including BRCA2 and PALB2 mutants.

In the clinic, BRCA2 and PALB2 patients present syndromic associations that are different from those exhibited by typical Fanconi anemia patients (Hirsch *et al.*, 2004; Patel, 2007; Reid *et al.*, 2007). BRCA2 and PALB2 patients suffer from early onset leukaemia and solid tumors of the brain (medulloblastoma) and kidney (Wilms tumor), which are rarely observed among Fanconi anemia patients from other complementation groups where pancytopenia initially predominates.

Conclusions. The Fanconi anemia network includes BRCA2 and PALB2, two key regulators of homologous recombination. BRCA2 can sequester RAD51, transport RAD51 to DNA repair sites, stimulate RAD51 filament formation or disassemble RAD51 filaments, and, thus, provides a structural platform required for the fine regulation of RAD51.

BRCA2 and PALB2 most likely constitute a functional group that is connected with, but distinct from, the other Fanconi anemia proteins. Consistent with this, the knockdown of BRCA2 in *FANCD2* mutant cells further sensitizes these cells to mitomycin C (Ohashi *et al.*, 2005), suggesting that FANCD2 and BRCA2 have independent functions.

5. BRIP1

BRCA1 mutations have not been associated with Fanconi anemia, but the BRCA1-interacting protein BRIP1 (also known as BRCA1-associated C-terminal helicase BACH1) has turned out to be the defective protein in FA-J cells (Levitus *et al.*, 2005; Levran *et al.*, 2005; Litman *et al.*, 2005). FANCJ is a 5′–3′ DNA helicase (Cantor *et al.*, 2004), which minimally requires 15 single-stranded nucleotides for DNA unwinding, and binds preferentially to fork-like structures (Gupta *et al.*, 2005).

The *Caenorhabditis elegans* homolog of FANCJ is DOG-1 (Youds *et al.*, 2008). DOG-1 was named after the mutator phenotype that results from *dog-1* disruption: deletion of guanine-rich DNA (Cheung *et al.*, 2002). Guanine-rich DNA can form G-quadruplexes (Wang *et al.*, 1991; Maizels, 2006; Phan *et al.*, 2006), a DNA structure with planar arrays of four guanines (G-quartets) stabilized by Hoogsteen type bonds (Figure 10B). More than 3000,000 potential G4-DNA sites have been identified in the human genome (Huppert and Balasubramanian, 2005). These structures, which may arise during transient denaturation of DNA by the replication and transcription machineries (Maizels, 2008), are predicted to block the progression of DNA replication forks (Figure 10B). Unbiased analyses of the left arm of chromosome V revealed that fragile sites in *dog-1* animals are confined to G4 DNA sites ($G_{3-5}N_{1-7}G_{3-5}N_{1-7}G_{3-5}N_{1-7}$) (Kruisselbrink *et al.*, 2008). With the observation that human FANCJ can unwind G-quadruplex DNA *in vitro* (Wu *et al.*, 2008), there is now compelling evidence for FANCJ playing a role in the removal of replication-blocking DNA structures. Genetic instability at G4-DNA sites causes small deletions, but can also produce gross chromosomal rearrangements in DOG-1/FANCJ deficient cells (Zhao *et al.*, 2008).

In addition to its ability to unwind G4-DNA, FANCJ can undo Hoogsteen base pairs, inhibit RAD51-mediated strand exchange, and displace streptavidin bound to biotinylated oligonucleotides (Sommers *et al.*, 2009). This suggests that the action of FANCJ is not restricted to G4-DNA, but might rather contribute to the removal of a broad spectrum of obstacles ahead of replication forks.

Whereas the connection of FANCJ to BRCA1 is interesting, a recent study has shown that this interaction is dispensable for the function of FANCJ in the cellular response to interstrand DNA crosslinks (Peng *et al.*, 2007). The contribution of FANCJ in crosslink repair, which remains to be defined at a molecular level, depends on its interaction with MutL alpha (Peng *et al.*, 2007). This new connection between FANCJ and the mismatch repair machinery is potentially important, as mismatch repair proteins have been implicated in the recognition and in the removal of interstrand DNA crosslinks (Duckett *et al.*, 1996; Yamada *et al.*, 1997; Zhang *et al.*, 2002; Barber *et al.*, 2005; Zheng *et al.*, 2006).

Conclusions. FANCJ is a structural helicase whose role in the unwinding of G-quadruplex DNA is well understood. Increasing evidence suggests now that it might play a more general role in the removal of replication fork-blocking structures.

The Fanconi anemia network in the replication stress response

Fanconi anemia cells are not exclusively hypersensitive to interstrand DNA crosslinks. FA cells are also hypersensitive to physiological levels of formaldehyde, which induce protein–DNA crosslinks (Ridpath *et al.*, 2007). Another study in hamster cells shows that the FA complex subunit FANCG is also critical for tolerance to mono-adducts generated by 6-thioguanine, ethylnitrosourea or methyl methanesulfonate (Tebbs *et al.*, 2005).

FANCD2 forms foci in response to a variety of replication stress conditions. Intrastrand photoproducts

generated by UV light activate the FANCD2 monoubiquitination pathway, as can agents that perturb replication without actually inducing chemical alterations in the DNA, such as hydroxyurea or aphidicolin (Garcia-Higuera *et al.*, 2001; Pichierri *et al.*, 2004a; Wang *et al.*, 2004). FANCD2 foci also form rapidly after ionizing radiation (Taniguchi *et al.*, 2002b; Rothfuss and Grompe, 2004). It should be noted that even during a non-challenged S phase, FANCD2 is monoubiquitinated and localized to chromatin (Taniguchi *et al.*, 2002a).

Fanconi anemia proteins are known to contribute to the stability of common fragile sites, that is regions in the DNA that are intrinsically difficult to replicate (Howlett *et al.*, 2005). In the *Xenopus* egg extract system, depletion of FANCA or FANCD2 provokes an accumulation of DSBs during DNA replication (Sobeck *et al.*, 2006). Furthermore, FANCD2 forms foci in response to endogenous signals of re-replication induced by the depletion of geminin (Zhu and Dutta, 2006).

Taken together, these observations suggest that Fanconi anemia proteins play a crucial role in preserving genomic integrity during DNA replication, without being confined to interstrand DNA crosslinks. The notoriously pronounced sensitivity of FA cells to ICL-inducing agents might simply reflect the severity of the lesion and the complexity of the repair response involved.

Several lines of evidence suggest that Fanconi anemia proteins function at the interface of DNA damage signaling, homologous recombination and translesion DNA synthesis (Thompson *et al.*, 2005; Mirchandani and D'Andrea, 2006; Wang, 2007), allowing cells to organize an appropriate response to the replication problem encountered. Fanconi anemia proteins promote optimal repair of damaged replication intermediates to limit the severity of mutagenesis and prevent gross chromosomal rearrangements (Hinz *et al.*, 2006).

We will discuss in the following chapters how the Fanconi anemia network contributes to the maintenance of genomic integrity under a variety of replication stress conditions, most probably by coordinating (1) the checkpoint response, (2) homologous recombination and (3) translesion DNA synthesis together with other caretaker proteins, such as (4) the MRN-complex and (5) BLM.

1. Fanconi anemia proteins and the checkpoint response

We have discussed how FANCM facilitates ATR-mediated checkpoint signaling. Other FA proteins also play an important role in damage-induced checkpoint signaling.

In response to re-replication, FANCA and FANCD2 are required for the activation of the G2/M checkpoint (Zhu and Dutta, 2006). Defects in re-replication-induced checkpoint activation lead to cell death in FANCA or FANCD2 knockdown cells (Zhu and Dutta, 2006).

In response to ionizing radiation, FANCD2 is phosphorylated by ATM on serine 222, serine 1401, serine 1404 and serine 1418 (Taniguchi *et al.*, 2002b). Phosphorylation of FANCD2 at Ser222 is required for the checkpoint response to IR in S phase, but not for FANCD2 monoubiquitination, FANCD2 foci formation and cellular tolerance to mitomycin C (Taniguchi *et al.*, 2002b). This suggests that the ATM-FANCD2 S phase checkpoint pathway is functionally separate from the activation of FANCD2 by the FA core complex. After ionizing radiation, FANCC was also found to be necessary for the sustained maintenance of the G2/M checkpoint (Freie *et al.*, 2004). Interestingly, whereas radiation sensitivity is not a hallmark of FA cells, clinical radiosensitivity in FA patients has been reported in the literature (Marcou *et al.*, 2001; Alter, 2002).

In response to treatment with mitomycin C, FA cells exhibit only partial ATR-mediated inhibition of DNA synthesis, suggesting that FA proteins are necessary for the full activation of checkpoint signaling in response to crosslinks (Pichierri and Rosselli, 2004). Consistent with this, FANCC is necessary for the inhibition of late replication origins after DNA crosslinking (Phelps *et al.*, 2007).

2. Fanconi anemia proteins and homologous recombination

With the notable exception of FANCD1 (BRCA2) and FANCN (PALB2), FA proteins do not seem to play an essential role in the repair of DNA double-strand breaks by homologous recombination (Mosedale *et al.*, 2005; Hinz *et al.*, 2006; Sun *et al.*, 2008; Prakash *et al.*, 2009). It is conceivable, however, that FA proteins play a role in the regulation or the fine-tuning of homologous recombination specifically at stalled replication forks.

A possible scenario is that FA proteins promote tolerance to DNA lesions during DNA replication by assisting the homologous recombination machinery specifically in the context of stalled replication forks, as observed with the FANCM ortholog Fml1 (Sun *et al.*, 2008). Furthermore, the FA core complex proteins and FANCM may contribute to the regulation of homologous recombination sub-pathways. In accordance with this idea, it has been shown that FANCM orthologs promote gene conversion at stalled replication forks, while preventing crossover formation during DSB repair (Prakash *et al.*, 2009; Sun *et al.*, 2008; and Figure 8).

3. Fanconi anemia proteins and translesion DNA synthesis

It has been known for a long time that Fanconi anemia cells are hypomutable, suggesting that FA proteins facilitate translesion DNA synthesis. In normal cells, the

majority of spontaneous genetic alterations at the *hprt* locus are point mutations, whereas deletions are the predominating mutations in FA cell lines (Papadopoulo *et al.*, 1990; Laquerbe *et al.*, 1999). Similarly, an increase in the ratio of spontaneous deletions versus base substitutions is observed within the *hprt* gene in *fancg* mutant hamster cells (Hinz *et al.*, 2007). In DT40 chicken cells, the knockout of *Fancc* causes a defect in mutational repair at the immunoglobulin V gene locus (Niedzwiedz *et al.*, 2004). Furthermore, Fanconi anemia proteins are necessary for normal mutagenic tolerance to photoactivated psoralens (Papadopoulo *et al.*, 1990; Guillouf *et al.*, 1993). In a *SupF* mutagenesis assay, FA group A and G cells also exhibit a reduction in spontaneous and UVC-induced mutations (Mirchandani *et al.*, 2008).

In DT40 chicken cells, an epistatic relationship has been established between FANCC, REV1 and the DNA polymerase ζ subunit REV3 after treatment with cisplatin (Niedzwiedz *et al.*, 2004). Moreover, FANCD2 and REV1 co-localize following hydroxyurea-induced replication fork stalling in Hela cells (Niedzwiedz *et al.*, 2004). Mirchandani and colleagues report that FANCA and FANCG, but not FANCD2, are necessary for the accumulation of REV1 in spontaneous and damaged-induced repair foci (Mirchandani *et al.*, 2008). These authors further suggest that the BRCT domain in REV1 is necessary for FANCA/FANCG-mediated REV1 foci formation.

Conclusions. Without doubt, FA proteins contribute to error-prone repair, notably through REV1 and DNA polymerase ζ, but whether FA proteins play a direct or indirect role in translesion DNA synthesis has not yet been established. At a biochemical level, no direct interaction between translesion DNA polymerases and Fanconi anemia proteins has been observed so far. Another important question to be addressed is whether FA proteins assist REV1 at stalled replication forks, or during post-replicational gap repair mediated by monoubiquitinated PCNA.

4. Fanconi anemia proteins and the MRE11/RAD50/NBS1-complex

Fanconi anemia proteins are connected with several caretaker proteins implicated in chromosomal instability syndromes, such as the MRE11/RAD50/NBS1 complex (MRN). MRE11 exhibits exo- and endo-nuclease activities, as well as strand dissociation and strand-annealing activities (Trujillo *et al.*, 1998; Paull and Gellert, 1998, 1999; Trujillo and Sung, 2001). RAD50 is related to proteins of the structural maintenance of chromosome (SMC) family (Aravind *et al.*, 1999). The MRN complex associates early with DSBs and contributes to checkpoint activation and DSB repair (Petrini and Stracker, 2003). NBS1 is phosphorylated by ATM in response to ionizing radiation (Lim *et al.*, 2000) and by ATR in response to lesions that interfere with the progression of replication forks (Pichierri and Rosselli, 2004).

ATM-mediated phosphorylation of FANCD2 on serine 222 depends on NBS1 and MRE11 (Nakanishi *et al.*, 2002), and ATR-mediated phosphorylation of FANCD2 depends on NBS1 (Pichierri and Rosselli, 2004). In response to cellular treatment with mitomycin C, FANCD2 co-localizes with NBS1, and FANCC is necessary for the formation of MRN nuclear foci (Nakanishi *et al.*, 2002; Pichierri *et al.*, 2004a).

5. Fanconi anemia proteins and BLM

Disabling mutations in *BLM* cause Bloom's syndrome, a disorder with a hyper-recombination phenotype, which is of particular interest with regards to our understanding of tumorigenesis, as it predisposes patients to the full range of cancers observed in the general population (German, 1995).

BLM is a 3′–5′ DNA helicase of the RecQ family (Cheok *et al.*, 2005; Bachrati and Hickson, 2008), which stabilizes damaged replication forks, while limiting recombination events (Bachrati and Hickson, 2003; Rassool *et al.*, 2003; Wu and Hickson, 2003; Wu, 2007).

In vitro, BLM can process a variety of DNA substrates, notably branched DNA structures, such as Holliday junctions (Karow *et al.*, 2000; Wu, 2007). In association with Topo IIIα, BLM can catalyze the dissolution of double Holliday junctions, via branch migration and DNA decatenation (Wu and Hickson, 2003; Wu L *et al.*, 2006). Since there are structural similarities between recombination intermediates with double Holliday junctions and small unreplicated DNA duplexes flanked by two converging replication forks (Branzei *et al.*, 2006), the ability of BLM/Topo IIIα to dissolve Holliday junctions *in vitro* may reflect a role for BLM in the disentanglement of intertwined replicated DNA molecules. This notion is supported by the recent observation that BLM prevents chromosome missegregation (Chan *et al.*, 2007). The authors found that during mitosis BLM is localized to ultra fine anaphase bridges, which connect daughter nuclei. These structures are thought to consist of uncondensed DNA caused by incomplete DNA replication and DNA repair processes. Interestingly, the formation of these ultra fine anaphase bridges is highly increased in BLM-deficient cells, suggesting that BLM suppresses their formation (Chan *et al.*, 2007).

Several connections between BLM and FA proteins have been observed. BLM and Topo IIIα were immunopurified from Hela cells together with the FA core complex (Meetei *et al.*, 2003b). BLM and Ub-FANCD2 co-immunoprecipitate and co-localize in nuclear foci following treatment with hydroxyurea or DNA crosslinkers (Pichierri *et al.*, 2004b). Moreover, BLM foci formation and phosphorylation in response to

DNA crosslinkers depend on the integrity of the FA core complex (Pichierri *et al.*, 2004b). Consistent with this, Hirano and colleagues reported that chicken FANCC and FANCD2 were necessary for mitomycin C-induced formation of GFP-BLM foci (Hirano *et al.*, 2005).

Conclusions. BLM and FA proteins cooperate in the management of crosslinks during DNA replication, perhaps in the resolution of replicated molecules entangled by DNA interstrand crosslinks.

Part III: Conclusions and outlook

We can anticipate that additional associations between the FA network and other proteins involved in the maintenance of genome stability will be reported in the near future. The remarkable number of discoveries that have been made in recent years suggest that Fanconi anemia proteins contribute to an integrated network which provides the cell with the capacity to coordinate the action of molecular machines involved in the detection of DNA lesions, in DNA repair, in DNA replication and in the regulation of cell cycle progression and cell survival. Fanconi anemia proteins are implicated in the general response to replication stress, rather than functioning specifically in the repair of interstrand DNA crosslinks.

Hypothesis for the role of Fanconi anemia proteins in S phase

In the G1 or G2 phases of the cell cycle, base excision repair, nucleotide excision repair, non-homologous end-joining and/or homology-directed repair pathways may suffice to ensure the maintenance of genomic integrity. In S phase, FA proteins may represent an additional layer in the DNA damage response that is necessary to connect ongoing DNA repair activities with incoming replication forks, and to coordinate the restoration of sister chromatids once two replication forks have converged at a site of damage (Figure 11).

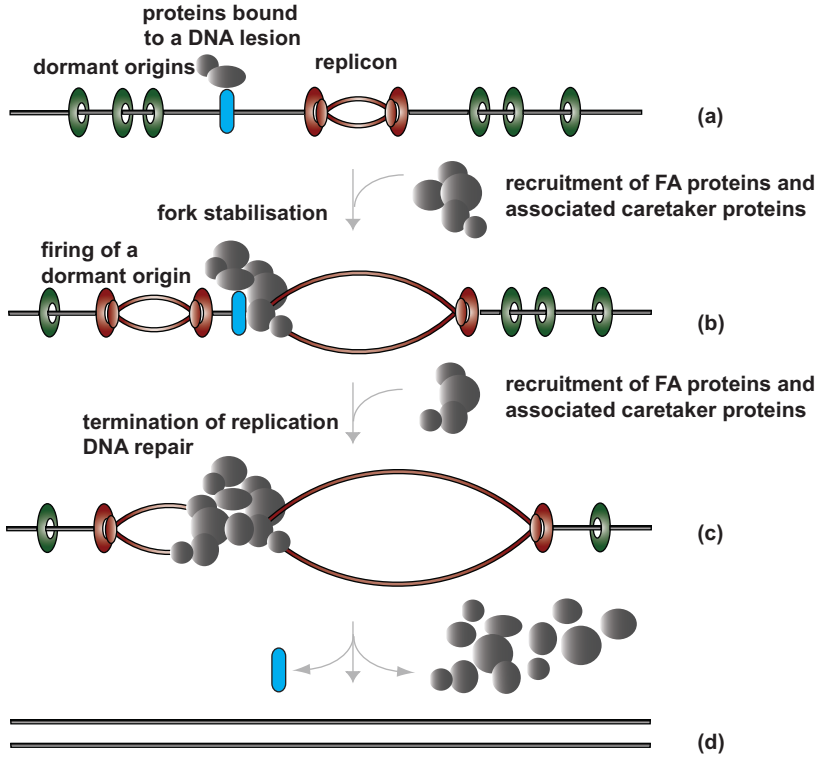

Figure 11. Model for the role of FA proteins in S phase. (a) Schematic representation of an active replication cluster. An active replicon is depicted in red. Pairs of MCM complexes representing dormant origins of replication are shown in green. The left-going replication fork progresses towards a DNA lesion (blue box) that has been recognized by DNA damage binding proteins (in grey). Since we do not know exactly the temporal order of assembly and the location of Fanconi anemia and Fanconi anemia-associated proteins in S phase, all DNA caretaker proteins are depicted in grey. For instance, the lesion could be a DSB bound by the MRN complex and FANCD2. (b) Once the replication fork stalls at the lesion, additional FA and FA-associated proteins are recruited to the damaged replication fork. FANCM could target the branch point of the stalled fork and recruit the FA core complex. When a replication fork is blocked, DNA repair and DNA remodeling activities alter locally the structure of chromatin, and a normally dormant origin can fire and complete DNA replication. (c) Adjacent replication forks have now converged at the lesion, and additional caretaker proteins are being recruited to complete the repair process in a post-replicative manner. BLM, for instance, interacts with the FA core complex and may be necessary to resolve untangled DNA duplexes. (d) Two fully replicated and intact sister chromatids are eventually produced. A color version of the figure is available online. (See colour version of this figure online at www.informahealthcare.com/bmg)

We still do not know how, when, and where exactly FA proteins are recruited to chromatin during S phase. These questions are not resolved yet because most localization studies are based on the observation of nuclear foci revealed by immunofluorescence staining. A widely spread misconception is to equate replication foci with replication forks, and to confuse replication factories, which contain PCNA and RPA, with post-replication repair factories, which also contain PCNA and RPA.

One possible scenario is that FANCM can bind to stalled replication forks, thereby promoting the stable association of the FA core complex with chromatin. An attractive alternative model is that Fanconi anemia proteins can sense and signal DNA lesions and/or DNA repair intermediates in S phase, even before catastrophic clashes occur with ongoing replication forks. DNA lesions are not just free chemical alterations in the DNA awaiting collisions with incoming replications forks, but they are usually bound by a variety of proteins that recognize specific types of lesions (Friedberg *et al.*, 2006). Thus, replication forks presumably do not collide with physical alterations in the DNA, but with protein complexes assembled around DNA lesions.

Experimental evidence that replication forks do not simply run into lesions and collapse comes from a recent study in budding yeast in which Doksani and colleagues investigated the fate of a replication fork starting from a defined origin of replication and progressing towards a defined DNA double-strand break (Doksani *et al.*, 2009). They found Mre11, Sae2 and Tel1/ATM to counteract the progression of the replication fork towards the DSB, and to prevent the degeneration of replication forks that are terminally arrested at the DSB (Doksani *et al.*, 2009). In this experimental setup, DNA caretaker proteins bound at the lesion controlled DNA chain elongation and the integrity of the replication intermediate, and DNA replication was rescued by the activation of dormant origins of replication (Doksani *et al.*, 2009).

Along these lines, Fanconi anemia proteins could be recruited to chromatin by protein complexes bound to DNA damage, and function as mediators or molecular matchmakers that coordinate ongoing DNA repair and DNA replication (Figure 11). The completion of DNA replication in the vicinity of a replication-blocking lesion may be achieved via adjacent replication forks coming from the opposite direction. The assembly of DNA repair complexes on chromatin may induce topological transitions in chromatin, which would indirectly facilitate the firing of nearby dormant origins. In this respect, DNA repair complexes may determine both termination and initiation sites of DNA replication in S phase.

We now need to understand how FA proteins are recruited to chromatin, which proteins depend on FA proteins to act at damage sites, how the FA network communicates with the replication machinery, in which order molecular machines come into play in S phase, and how this network modulates the balance between cell death and pro-survival signals. Research on Fanconi anemia represents a fantastic opportunity to understand how the cell integrates a variety of signals and biochemical activities to achieve homeostasis in S phase.

Hypothesis for the role of Fanconi anemia proteins in crosslink repair

The fact that Fanconi anemia cells are particularly sensitive to interstrand DNA crosslinking agents suggests that they play a central role in ICL tolerance. Their actual function, however, has remained elusive to date.

It will be important to understand how the fork remodeling activity of FANCM contributes to ICL tolerance. Studies in yeast demonstrate that fork regression, i.e. the conversion of a replication fork into a four-way junction, is a rare or transient event, which is observed more frequently in checkpoint-deficient cells than in wild-type cells (Sogo *et al.*, 2002; Lopes *et al.*, 2006). On the other hand, replication structures have only been studied in cells exposed to hydroxyurea and UV light. Considering the fact that HU-stalled replication forks are stabilized by the replication checkpoint, and that UV-lesions can be bypassed, replication fork reversal in these situations seems neither desired nor required. When an interstrand DNA crosslink has to be removed to complete replication, however, replication fork remodeling may be indispensable to facilitate the assembly of DNA repair proteins.

Recently, Raschle and colleagues have developed a cell-free system using *Xenopus* extracts to study replication-coupled repair of interstrand DNA crosslinks (Raschle *et al.*, 2008). In their experimental setup, the collision of a replication fork with an ICL triggers a checkpoint response and the monoubiquitination of FANCD2. Surprisingly, however, replication forks initially pause 20–40 nucleotides away from the ICL (Figure 5), which can most simply be explained by the enormous size of the replisome and the steric constrictions associated with. It would be very interesting to use this system and address whether the fork remodeling activity of FANCM (Gari *et al.*, 2008a) is required for replisome displacement and subsequent ICL repair.

Another open question in ICL repair is what triggers the activation of an ATR-dependent checkpoint response, since polymerase and helicase uncoupling is not possible at an ICL and, therefore, no extended regions of single-stranded DNA should be present. One possibility is that ATR is recruited to the lagging strand template, which is partially single-stranded (Raschle *et al.*, 2008). Alternatively, the assembly of an ATR signaling complex and the activation of the FANCD2 monoubiquitination pathway could depend on the formation of a substrate

generated by the branch point translocase activity of FANCM (Collis *et al.*, 2008).

Above, we have discussed evidence for a role of FA proteins in translesion synthesis and recombination repair. In addition, FANCA has been found to co-localize with the structure-specific endonuclease XPF after psoralen crosslinking (Sridharan *et al.*, 2003). Furthermore, ERCC1 appears to be necessary for FANCD2 monoubiquitination and foci formation, following DSB formation induced by hydroxyurea or mitomycin C (McCabe *et al.*, 2008). This suggests that DNA processing by ERCC1/XPF after DSB formation is necessary for activation of the FANCD2 monoubiquitination pathway, and provides further connections between FA proteins and DNA repair factors involved in crosslink repair.

Taken together, during crosslink repair, FA proteins may orchestrate the coordinated action of nucleases, translesion DNA polymerases and the homologous recombination machinery to restore fully replicated and repaired daughter duplexes.

Clinical perspectives

A better understanding of the FA network is likely to have a significant impact on human health. Whereas genomic instability can promote the acquisition of new biological properties conferring growth advantages (Hanahan and Weinberg, 2000), tumor cells experience constitutive replication stress and rely on functional DNA damage tolerance pathways to proliferate and resist chemotherapy (Sarasin and Kauffmann, 2008).

In the majority of human cancers, activation of oncogenes leads to replication fork collapse and double-strand break formation. This replication stress triggers the activation of a DNA damage response and the induction of apoptosis or senescence, until mutations, for instance of the p53 gene, allow escape from cell death pathways and development of full-blown cancer (Bartkova *et al.*, 2005; Gorgoulis *et al.*, 2005; Bartkova *et al.*, 2006; Di Micco *et al.*, 2006). Constitutive replication stress is therefore an essential feature that distinguishes cancer cells from normal cells (Halazonetis *et al.*, 2008). Thus, cancer cells may be selectively sensitive to small molecule inhibitors that target pathways involved in tolerance to DNA lesions in S phase. PARP inhibitors, for instance, selectively sensitize *BRCA2*-deficient tumor cells (Bryant *et al.*, 2005).

A number of DNA damage tolerance pathways coexist and provide redundant strategies for the survival of cells with damaged DNA (Friedberg *et al.*, 2006). We still know little about functional hierarchies between DNA caretaker proteins, and about how these proteins influence each other. A better understanding of DNA maintenance and DNA damage tolerance systems may eventually allow the predict of the response of cancer cells with diverse genetic backgrounds to radio- and chemotherapy, and to develop rational therapeutic interventions based on the identification and the selective targeting of the DNA damage tolerance systems that are crucial for the proliferation of specific types of tumors (Kennedy and D'Andrea, 2006; Kennedy *et al.*, 2007).

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