Abstract Chromosomal aberrations are often associated with incomplete genome duplication, for instance at common fragile sites, or as a consequence of chemical alterations in the DNA template that block replication forks. Studies of the cancer-prone disease Fanconi anaemia (FA) have provided important insights into the resolution of replication problems. The repair of interstrand DNA crosslinks induced by chemotherapy drugs is coupled with DNA replication and controlled by FA proteins. We discuss here the recent discovery of new FA-associated proteins and the development of new tractable repair systems that have dramatically improved our understanding of crosslink repair. We focus also on how FA proteins protect against replication failure in the context of fragile sites and on the identification of reactive metabolites that account for the development of Fanconi anaemia symptoms.

Fanconi anaemia

Fanconi anaemia (FA) was first recognised as a clinical entity by Guido Fanconi in 1927 (Fanconi 1927). The disease manifests with a variety of congenital abnormalities at birth and by a severe depression of all bone marrow-derived haematopoietic cell lineages (pancytopenia) during childhood (Tischkowitz and Hodgson 2003). Patients are at high risk of developing acute myeloid leukaemia and, in adulthood, squamous cell carcinomas of the gastrointestinal and of the female reproductive tracts (Alter et al. 2003). FA cells feature a high frequency of broken and radial chromosomes (Schroeder et al. 1964) and are highly sensitive to interstrand DNA crosslinks (ICLs) (Auerbach and Wolman 1976). Covalent bonds linking the two anti-parallel strands of DNA are extremely toxic, as they prevent DNA unwinding required for DNA replication and transcription. Hence, bi-functional adducts such as nitrogen mustards, mitomycin C or cisplatin are widely used in antitumor therapies (Deans and West 2011).

Fourteen FA genes have been identified to date, plus a 15th one, RAD51C, inactivated in one patient with multiple congenital anomalies (Vaz et al. 2010). The patient, however, had no symptoms of bone marrow failure or malignancies when diagnosed, so bi-allelic inactivation of RAD51C has been provisionally associated with a FA-like disorder. FA genes encode proteins implicated in an ubiquitin signalling pathway, in S phase checkpoint activation, in translesion DNA synthesis and in DNA double-strand break (DSB) repair by homologous recombination (Kee and D’Andrea 2010; Moldovan and D’Andrea 2009; Wang 2007).

The FA pathway

A basic molecular view of the FA pathway is depicted in Fig. 1. FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, the E3 ligase enzyme FANCL and an associated protein of 100 kDa (FAAP100) form a nuclear, ubiquitin ligase FA core complex (Garcia-Higuera et al. 2001; Ling et al. 2007; Meetei et al. 2003a). The FA core complex associates with chromatin and the nuclear matrix during S phase, in a DNA damage-induced and FANCM-dependent manner (Kim et al. 2008; Mi and Kupfer 2004; Qiao et al. 2001). FANCM is a DNA translocase that can bind and
move the branch point of three- and four-strand DNA structures akin to replication/repair intermediates (Gari et al. 2008a, b; Meetei et al. 2005; Xue et al. 2008). The DNA translocase forms a conserved DNA remodelling holoenzyme with the histone fold heterodimer MHF (Yan et al. 2010) and is assisted by the associated factor FAAP24 to regulate several independent aspects of the DNA damage response (Ciccia et al. 2007): (1) FANCM/FAAP24 interacts with the checkpoint protein HCLK2 and facilitates DNA damage signalling mediated by ATR (Collis et al. 2008); (2) FANCM associates with the Bloom’s complex (BLM-TopoIIIα-RMI1-RMI2) via direct contacts with the RMI1 and TopoIIIα subunits (Deans and West 2009; Meetei et al. 2003b); (3) FANCM/FAAP24 recruits the FA core complex to chromatin (Kim et al., through a direct FANCM–FANCF interaction (Deans and West 2009).

Genotoxic stress in S phase activates the FA core complex to monoubiquitinate FANCD2 and FANCI, two key interacting paralogs in the FA pathway (Dorsman et al. 2007; Garcia-Higuera et al. 2001; Sims et al. 2007; Smogorzewska et al. 2007). This signalling event has been proposed to stabilise the ubiquitinated FANCI–FANCD2 complex in chromatin, which in turn allows recruitment of DNA repair factors such as the nuclease FAN1 at damaged sites (Kratz et al. 2010; Liu et al. 2010; MacKay et al. 2010; Shereda et al. 2010; Smogorzewska et al. 2010; Stoepker et al. 2011; Yoshikiyo et al. 2010).

A number of posttranslational modifications regulate FA proteins: Phosphorylation of FANCI by ATR is a prerequisite for FANCD2 monoubiquitination (Ishiai et al. 2008). In mammalian cells, the phosphorylation of FANCD2 by ATR/ATM (T691 and S717) and by Chk1 (S331) is required for potent FANCD2 monoubiquitination and for resistance to DNA cross-linking agents (Ho et al. 2006; Zhi et al. 2009). Several phosphorylation steps also regulate FA core complex proteins. Phosphorylation of FANCA (S1449) by ATR, of FANCG (S7), and of FANCE (T346 and S374) by Chk1 all appear necessary for cellular tolerance to mitomycin C (Collins et al. 2008; Qiao et al. 2004; Wang et al. 2007).

An additional layer of complexity has emerged with the discovery that RAD18 contributes to the regulation of FANCD2 monoubiquitination (Geng et al. 2010; Palle and Vaziri 2011; Park et al. 2010a; Song et al. 2010b; Williams et al. 2011). RAD18 is an E3-ligase that monoubiquitinates proliferating cell nuclear antigen (PCNA) in response to replication fork stalling by bulky DNA damage (Prakash et al. 2005). PCNA functions as a scaffold for the recruitment of TLS polymerases that bypass DNA lesions. In response to bulky DNA adducts, the monoubiquitination of PCNA by RAD18 and the subsequent recruitment of TLS polymerases are necessary for efficient FANCD2 monoubiquitination (Song et al. 2010b). In cells exposed to mitomycin C or camptothecin, however, the monoubiquitination of FANCD2 is independent of PCNA, yet depends on RAD18 (Palle and Vaziri 2011; Williams et al. 2011). How exactly RAD18’s ligase activity participates in the FA pathway remains elusive.
Deubiquitination of FANCD2 is under the control of USP1 (Kim et al. 2009; Nijman et al. 2005), in association with UAF1 (Cohn et al. 2007). USP1 binds FANCI directly via its SUMO-like domain SLD2, which associates with the SUMO-like domain-interacting motif (SIM) on FANCI (Yang et al. 2011). Removal of the ubiquitin moiety on FANCD2 and FANCI is necessary for completion of the DNA repair process and ICL tolerance (Kim et al. 2009).

FANCI/BRIP1 interacts with BRCA1, unwinds DNA structures that block replication forks and facilitates checkpoint signalling (Bridge et al. 2005; Cantor et al. 2001; Gupta et al. 2005; Hiom 2010; Levitus et al. 2005; Litman et al. 2005). FANCI/BRIP1 also forms a complex with BLM, and the two helicases can unwind DNA substrates synergistically (Suhasini et al. 2011). Some symptoms in FA-J patients may be linked to a deficiency in BLM protein, as BLM is unstable in the absence of FANCJ (Suhasini et al. 2011). BRCA2/FANCD1 and PALB2/N are key regulators of homologous recombination. BRCA2, the product of the breast cancer susceptibility gene, provides a structural platform for the fine regulation of the strand exchange protein RAD51 (for a recent review, see (Holloman 2011). Additional insights into structural, functional and phenotypic features of Fanconi anaemia are developed below.

**FANCD2–FANCI and associated nucleases**

**Structure of FANCD2–FANCI**

A new view of the FA pathway has been provided by the recent crystal structure of the mouse FANCD2–FANCI complex (Joo et al. 2011). Each protein is made of four solenoid segments and two interspersed helical domains that fold into a saxophone-like structure (cartooned in Fig. 1). FANCD2 and FANCI interact in an antiparallel manner along their saxophone body, forming a narrow and shallow platform (Joo et al. 2011). Electron density maps and FANCI-DNA crystals have revealed that the FANCD2/ FANCI surface includes two sets of double- (dsDNA) and single- strand DNA (ssDNA) binding sites that could accommodate a replication intermediate comprising two converging forks at a crosslink (Fig. 1). This is consistent with earlier reports on the DNA-binding properties of FANCD2 and FANCI (Longerich et al. 2009; Park et al. 2005; Roques et al. 2009; Yuan et al. 2009). It is noteworthy that the purified FANCI–FANCD2 complex exhibits more affinity for branched DNA structures than either FANCD2 or FANCI proteins alone (Yuan et al. 2009). Three phosphorylation sites in FANCI as well as the FANCI and FANCD2 ubiquitination sites locate at the FANCD2–FANCI interface and are thought to stabilise the complex (Joo et al. 2011). Predictions suggest that the lysine–ubiquitin isopeptide bonds would lie within solvent free accessible tunnels from which the ubiquitin structural domain could emerge, on either side of the FANCD2–FANCI complex (Joo et al. 2011). This molecular view of FANCD2–FANCI shows how the complex may bind and protect stalled replication forks and control the recruitment of structure specific DNA endonucleases for coordinated unhooking of ICLs.

**FAN1**

The conjugation of FANCD2 with an ubiquitin moiety is not only a signal for the localisation and the retention of FANCD2 on chromatin but also for the recruitment of DNA repair nucleases. One such nuclease is FAN1, discovered independently by five groups that have reported congruent observations (Kratz et al. 2010; Liu et al. 2010; MacKay et al. 2010; Shereda et al. 2010; Smogorzewska et al. 2010); FAN1 stands for FANCD2-associated nuclease. The protein is necessary for chromosome stability and for cellular tolerance to cisplatin and mitomycin C. In cells treated with ICL-inducing agents, FAN1 forms nuclear foci that colocalise with FANCD2 and binds to the monoubiquitinated form of FANCD2. It contains an amino-terminal ubiquitin-binding motif, a RAD18-like CCHC zinc finger known as UBZ domain and a carboxy terminal VRR-nuc domain (virus-type replication-repair nuclease domain). The UBZ domain is both necessary and sufficient for interaction with FANCD2 and for targeting of FAN1 to DNA repair sites (Kratz et al. 2010; Liu et al. 2010; MacKay et al. 2010; Shereda et al. 2010). FAN1 exhibits 5′ flap endonuclease and 5′–3′ exonuclease activities and participates in DNA repair mechanisms dependent on homologous recombination (Kratz et al. 2010; Liu et al. 2010; MacKay et al. 2010; Smogorzewska et al. 2010). Together, these studies provide conclusive evidence of a strong link between Ub-FANCD2 and a structure specific endo/exo nuclease in ICL repair. Some nuances were brought in a follow-up study where the *Fan1* locus was disrupted by gene targeting in chicken DT40 cells (Yoshikiyo et al. 2010). First, FA proteins and FAN1 appear to have also non-epistatic functions because DT40 cells lacking both FANCC and FAN1 are more sensitive to cisplatin than either single mutant. Second, unlike FA-deficient cells that exhibit proliferation defects, the growth kinetic of *Fani* cells is similar to wild-type DT40 cells (Yoshikiyo et al. 2010). This may suggest that FAN1 does not function with FA proteins in the resolution of endogenous replication obstacles. Studies using defined repair systems (discussed below) will be necessary to understand precisely how FAN1 acts during repair of DNA damage induced by crosslinking agents.
SLX4

A second connexion with nucleases in the FA network emerged from the discovery of bi-allelic SLX4 mutations in six Fanconi anaemia patients (Kim et al. 2011; Stoepker et al. 2011). Based on this, SLX4 has been renamed Fanconi anaemia complementation group P (FANCP). With FANCM, FANCP is the second FA protein to be conserved in yeast. Inactivation of SLX4 sensitises cells to ICLs—FANCM, FANCP is the second FA protein to be conserved in the anaemia complementation group P (FANCP). With SLX4 mutations in Fanconi anaemia patients (Kim et al. 2011; Stoepker et al. 2011). Consistently, sub-cellular fractionation studies revealed that, unlike SLX1 and MUS81-EME1, the chromatin association of XPF-ERCC1 was specifically impaired in a FA-P cell line (EUFA1354) (Stoepker et al. 2011). This was confirmed by immunofluorescent studies showing that the formation of ERCC1 nuclear foci was impaired in EUFA1354 cells (Stoepker et al. 2011). These observations show that the association of SLX4 with XPF-ERCC1 is critical for the DNA damage response mediated by FA proteins.

Unlike FAN1, SLX4 is essential for the proliferation of chicken DT40 cells, suggesting that it plays an important role in the repair of DNA lesions that arise spontaneously (Yamamoto et al. 2011). Chicken SLX4 is connected to the Fanconi anaemia network by its UBZ domain, which is necessary for tolerance to ICL-inducing agents. SLX4 forms ICLs-induced foci in DT40 cells and associates with FANCD2 in pull-down experiments. The recruitment of SLX4 at DNA repair sites depends on the integrity of its UBZ domain and on the Fanconi anaemia core complex (Yamamoto et al. 2011). The observation that the function of SLX4 in ICL repair depends on the FA ubiquitination pathway in chicken DT40 cells awaits confirmation from studies based on human cellular systems. Additional work is also necessary to determine whether SLX4 UBZ domain binds Ub-FANCD2 directly. Finally, Fancc−/Slx4−/UBZD double mutants are more sensitive to ICLs inducing agents than either single mutant, suggesting that SLX4 and the FA core complex have also non-epistatic roles in ICL repair (Yamamoto et al. 2011).

FA proteins promote DNA damage signalling

Replication obstacles and stalled forks are signalled by ATR, which detects RPA-covered single-stranded DNA that accumulate as a result of uncoupling of MCM helicases and DNA polymerases (Byun et al. 2005; Zou and Elledge 2003). The replication checkpoint is triggered efficiently by the recognition of primed single-stranded DNA with a free 5′ end (MacDougall et al. 2007). Single-stranded DNA coated by the single-strand DNA-binding protein RPA is bound directly by ATRIP (ATR-interacting protein), which enables the association of the ATRIP–ATR complex at stalled forks (Zou and Elledge 2003). The 5′ junction between ssDNA and dsDNA is recognised by RAD17-RFC, which loads the 9–1–1 checkpoint clamp necessary for efficient ATR activation (Ellison and Stillman 2003; Majka et al. 2006; Zou et al. 2003). FA proteins stimulate DNA damage signalling in multiple ways.

Role of FANCM

Collis et al. (2008) provided initial evidence that FANCM functions in ATR-mediated checkpoint signalling, indepen-
dently of the Fanconi anaemia core complex. FANCM and FAAP24 associate with the checkpoint protein HCLK2. Depletion of FANCM in mammalian cells results in cellular features shared with ATR-defective cells, including spontaneous increase in DNA damage markers, increased frequency of cells with supernumary centrosomes and inefficient damage-induced Chk1 activation. Importantly, the integrity of the ATPase motif of FANCM is necessary for its role in checkpoint signalling (Collis et al. 2008; Huang et al. 2010). Subsequent studies confirmed that FANCM participates in Chk1 activation and limits replication-associated DNA damage (Luke-Glaser et al. 2010; Schwab et al. 2010). FANCM also promotes the retention of chicken TopBP1 in chromatin (Schwab et al. 2010) and the restart of stalled replication forks (Luke-Glaser et al. 2010; Schwab et al. 2010). Finally, Chk1 and FANCM protect each other from proteosomal degradation during DNA replication stress (Luke-Glaser et al. 2010). Together, these studies have unveiled a tight link between FANCM and S-phase checkpoint signalling.

Role of FANCI/BRIP1

FANCI/BRIP1 provides another link between Fanconi anaemia proteins and checkpoint signalling. The BRIP1 helicase interacts with TopBP1 (Gong et al. 2010). This interaction involves S-phase-specific phosphorylation of BRIP1 at Thr 1133 and the TopBP1 BRCT repeats 7 and 8, which undergo important conformational change upon binding with BRIP1 (Leung et al. 2011). In cells exposed to hydroxyurea, the helicase activity of BRIP1 and its interaction with TopBP1 are both required for the accumulation of RPA in chromatin, which in turn promotes the assembly of the ATR signalling complex and the phosphorylation of Chk1 (Gong et al. 2010). Hence, FANCI/BRIP1 is likely to facilitate ATR activation by unwinding DNA at stalled forks.

Signalling from a DNA crosslink

As crosslinks prevent the separation of DNA strands, and RPA covered single-stranded DNA is a critical determinant of ATR activation, it raises the question of how DNA damage signalling is implemented at ICL-stalled forks. Two reports suggest that Fanconi anaemia proteins sense ICLs directly and promote an ICL-specific mode of ATR activation (Ben-Yehoyada et al. 2009; Huang et al. 2010). Specifically after exposure to ICL-inducing agents, FANCM and FAAP24 proteins are necessary for the accumulation and the phosphorylation of RPA32 at damaged sites (Huang et al. 2010). Interestingly, the formation of RPA foci induced by ICLs occurs in the absence of microscopically detectable single-stranded DNA foci. In vitro, FAAP24 binds ICL-damaged DNA preferentially and promotes the recruitment of RPA (Huang et al. 2010). In a cell-free extract from *Xenopus* eggs, plasmid DNA containing a single ICL can induce ATR signalling in the absence of DNA replication (Ben-Yehoyada et al. 2009). ICL-induced ATR activation depends on xFANCD2 and xFANCL (Ben-Yehoyada et al. 2009) in contrast to human cells that do not seem to rely on FA core complex proteins for ICL-signalling (Huang et al. 2010). Using episomal-based chromatin IP from HEK293 cells to detect proteins bound to a plasmid bearing a defined interstrand DNA crosslink, Shen et al. (2009) found that the FA core complex proteins and FANCD2 were enriched on the damaged plasmid compared with the unmodified substrate (Shen et al. 2009). The recruitment of FA proteins to the ICL was not dependent on the plasmid undergoing DNA replication (Shen et al. 2009), consistent with the observations that FA proteins bind crosslinked DNA and promote ATR signalling of ICLs independently of DNA replication (Ben-Yehoyada et al. 2009; Huang et al. 2010).

In conclusion, FA proteins seem to bind DNA crosslinks before replication forks reach the lesion and promote an ICL-specific mode of ATR activation that is mechanistically distinct from signal activation induced by uncoupling of MCM helicase and DNA polymerase activities.

Indirect role of FA proteins

More recently, a genome-wide screen for genes necessary to maintain cell cycle arrest after exposure to ionizing radiation revealed that homologous recombination and Fanconi anaemia proteins play a critical role in S-phase checkpoint activation (Cotta-Ramusino et al. 2011). Consistently, the homologous recombination protein RAD51C has been implicated in Chk2 activation and cell cycle arrest in response to DNA damage (Badie et al. 2009). The involvement of homologous recombination (HR) proteins in checkpoint signalling suggests that cells may sense ongoing DNA repair (Cotta-Ramusino et al. 2011). Consequently, the role of Fanconi anaemia protein in DNA damage signalling may also result from their function in channelling DSBs into repair by homologous recombination (Adamo et al. 2010; Pace et al. 2010). Consistent with this, inhibition of the non-homologous end-joining machinery restores most of the IR-induced signalling defects in cells lacking FANCM, FANCL and FANCI (Cotta-Ramusino et al. 2011).

FA proteins promote replication-coupled ICL repair

The removal of interstrand DNA crosslinks mobilises an important part of the cellular DNA repair tool kit, including
helicas, nucleases, translesion DNA polymerases, DNA recombinases, and a battery of posttranslational modifications and scaffold proteins, which orchestrate the sequential intervention of DNA processing enzymes. The development of experimentally tractable systems, using DNA plasmids damaged with a single ICL at a defined position, has provided powerful means to tackle ICL repair in *Xenopus* egg extracts and in mammalian cells. ICLs can be repaired in G0/G1 cells by the combined action of the nucleotide excision repair machinery that unhooks and excises the crosslink and translesion DNA polymerases that ensure DNA repair synthesis (Ben-Yehoyada et al. 2009; Sarkar et al. 2006; Shen et al. 2009). In S/G2, the repair of ICLs is coupled with DNA replication and depends on Fanconi anaemia proteins and homologous recombination (Fig. 2). The Fanconi anaemia pathway has long been thought to promote homologous recombination, but this has been difficult to demonstrate with the widely used GFP reporter mammalian system developed in the laboratory of Maria Jasin, which measures the repair of a double-strand DNA break introduced by I-SceI endonuclease (Moynahan et al. 2001). The Jasin’s laboratory has now modified the reporter system to demonstrate that the Fanconi anaemia pathway facilitates replication-coupled homologous recombination in mammalian cells (Nakanishi et al. 2011). The authors used a triplex-forming oligonucleotide (TFO) conjugate to introduce a psoralen interstrand DNA crosslink at a specific site. After crosslink formation, the TFO was removed by reduction of a disulphide bond that links the oligonucleotide and the psoralen moiety. To measure repair with or without replication, they simply used plasmids containing or not the OriP EBV replication origin. Whereas DNA replication had little impact on recombination repair induced by a DSB, ICL-induced homologous recombination was greatly stimulated when coupled to replication and strongly dependent on the integrity of the FA pathway (Nakanishi et al. 2011).

**Replication-coupled ICL-repair in Xenopus egg extracts**

Major advances in our understanding of the mechanism of replication-coupled ICL repair have been made in the laboratory of Johannes Walter, using plasmids containing either nitrogen-mustard like or cisplatin ICL and *Xenopus* egg extracts (Raschle et al. 2008). Plasmids bearing a defined ICL are replicated by sequential incubation into high-speed supernatant of egg cytoplasm followed by highly concentrated nucleoplasm egg extracts. This procedure promotes synchronous and efficient replication. As the plasmid is small, two replication forks rapidly converge and stall at the ICL after random initiation of bidirectional replication. Fork stalling at the ICL induces the activation of the ATR signalling pathway and the monoubiquitination of FANCD2, suggesting that the system supports the DNA damage response. A detailed examination of plasmid-ICL replication in this system revealed that the leading strand of both forks first pause 20–40 nucleotides from the ICL (Fig. 2). A recent study from the same laboratory has demonstrated that the replicative DNA helicase complex is a 3′–5′ ssDNA translocase that unwinds DNA by steric exclusion (Fu et al. 2011). During replication-coupled repair of crosslinked plasmids, the observed pausing site 20–40 nt from the ICL corresponds to the footprint of the stalled replicative DNA helicase, composed of the hexameric ATPase MCM2-7, CDC45 and GINS.

![Fig. 2](image-url)
disappearance of MCM7 from the ICL site correlates with the leading strand nearing towards the ICL, suggesting that displacement of the replicative helicase facilitates the resumption of DNA synthesis towards the ICL (Fu et al. 2011).

After DNA replication, nucleolytic digestions occur on one of the parental DNA strands to “unhook” the crosslink (Fig. 2). As a result, one sister chromatid is broken, while the other sister duplex is restored, first by insertion of a nucleotide opposite the adduct and then by DNA repair synthesis dependent on Pol ζ (Raschle et al. 2008).

The “unhooking” of the crosslink and the insertion of a nucleotide opposite the unhooked crosslink are mediated by ubiquitylated FANCD2 and FANCI (Knipscheer et al. 2009). Both events are abolished when the FANCD2–FANCI complex is removed from the extract or when depleted extracts are complemented with recombinant FANCD2 bearing a lysine to arginine substitution in its monoubiquitination site. It is likely that Ub-FANCD2/Ub-FANCI recruits structure-specific nucleases to coordinate incisions in one of the parental DNA strands. A recent study has shown that human SNM1A can digest ICL-containing DNA from a single 5′ nick (Wang et al. 2011).

The data suggest that, in principle, an initial incision catalysed by XPF-ERCC1 would be sufficient to prime SNM1A exonucleolytic digestion of DNA past the ICL, yielding a gapped DNA intermediate with a covalently linked mononucleotide.

In DT40 cells, the Fanconi anaemia pathway interacts genetically with REV1 (Niedzwiedz et al. 2004), a dCMP transferase containing an ubiquitin-binding motif (Lehmann et al. 2007). Hence, ub-FANCD2/Ub-FANCI may also recruit REV1 to insert a nucleotide across the unhooked crosslink, followed by DNA polymerase ζ for DNA repair synthesis and restoration of one sister duplex (Raschle et al. 2008).

A recent replication-coupled repair study has used low speed supernatants from Xenopus eggs, which supports a single replication initiation event on plasmid DNA after chromatization and assembly into pseudo nuclei (Le Breton et al. 2011). Whether both Raschle et al. (2008) and Le Breton et al. (2011) show that concerted incisions occur in one of the parental DNA strands, incisions are observed in the later study whether one or both forks have reached the ICL. Consistent with this, ICL-plasmids containing the origin of replication from Epstein–Barr virus are repaired when introduced in mammalian cells (Nakanishi et al. 2011). Since EBNA1 initiates unidirectional DNA replication at OriP, this shows that crosslink repair can be accomplished from a single stalled fork.

In the final stage of ICL repair, the restored duplex is used as a template for repair of the broken sister by homologous recombination. Remarkably, recombination-dependent ICL repair can be recapitulated in the Xenopus egg extract system: Repair of the broken sister duplex is RAD51 dependent and leads to the formation of a recombination intermediate containing a hemicatenane (Long et al. 2011). As expected, formation of the recombination intermediates requires the presence of FANCD2–FANCI. Chromatin immunoprecipitation analyses showed that RAD51 and FANCD2–FANCI accumulate near the ICL independently of each other. Interestingly, RAD51 is targeted to ICL-stalled fork before the formation of a double-strand DNA break. This is consistent with a role for RAD51 in the protection of nascent DNA strands from degradation at stalled forks (Hashimoto et al. 2010). A similar role has been attributed to BRCA2, which prevents MRE11-mediated degradation of nascent DNA (Schlacher et al. 2011). Fork protection by BRCA2 depends on a RAD51-binding site at the carboxyl terminus of BRCA2, which stabilises RAD51 nucleoprotein filaments. It is therefore possible that BRCA2/FANCD1 targets RAD51 to replication forks stalled at ICLs.

Pathway choice

HR and non-homologous end joining (NHEJ) are competing DSBs repair pathways (Kass and Jasin 2010). Recruitment of DNA-PKcs to DSB sites is observed already two seconds after micro-irradiation (Uematsu et al. 2007). Ku70/Ku80 and DNA-PKcs are very abundant, largely outnumber HR factors, and would easily win in a DNA end binding competition based on the law of mass action (Meek et al. 2008). To prevent this, the repair of DSBs is highly regulated in S phase. Adamo et al. (2010) and Pace et al. (2010) have shown that elimination of NHEJ components in FA-deficient cells suffice to suppress hypersensitivity to DNA crosslinks and accumulation of chromosomal aberration and to correct homologous recombination defects. This suggests that the main function of Fanconi anaemia proteins is to prevent the toxic engagement of the non-homologous end joining machinery, thereby allowing recombination dependent crosslink repair (Adamo et al. 2010; Pace et al. 2010). There is, however, one difference between the two studies: Pace et al. (2010) find that only the knockout of Ku70 eliminates the cisplatin sensitivity of DT40 Fancc−/− cells; suppression of DNA-PKcs or Ligase IV provides no advantages. In contrast, Adamo et al. (2010) observe that deletion of lig-4 in Caenorhabditis elegans fcd-2 mutant strains restores meiotic and ICL repair defects and that the knockdown or drug inhibition of DNA-PKcs suppresses phenotypic features of transformed human cells lacking FA core complex or FANCD2 proteins. Whereas the reason for this difference is not clear yet, the consensus is that an important function of FA proteins is to shield replication-coupled repair intermediates from the promiscuous inter-
Foreword on supplementary replication origins and the rescue of stalled forks

During licensing of DNA replication origins, MCM2-7 complexes are loaded onto chromatin in large excess (Edwards et al. 2002; Hua and Newport 1998; Ritzl et al. 1998). This reserve of chromatin-bound MCM2-7 complexes is much larger than is needed to duplicate the genome, and supplementary origins remain dormant as long as replication forks that proceed from selected origins move freely. Under stressful conditions, however, dormant origins become essential to complete DNA replication and prevent genomic instability (Ge et al. 2007; Ibarra et al. 2008; Woodward et al. 2006). Whereas a reduction of 50% of chromatin-bound MCM2-7 is compatible with cell cycle progression (Ge et al. 2007), it leads to death when cells are exposed to low doses of hydroxyurea or aphidicolin (Ge et al. 2007; Ibarra et al. 2008). Subsequent studies have shown that supplementary origins are also necessary to prevent the pathologic consequences of endogenous replication blocks: Mice with reduced levels of MCM2-7 proteins are cancer prone (Chuang et al. 2010; Kunnev et al. 2010). Cells derived from Mcm4 mutant mice exhibit an increased frequency of stalled replication forks (Kawabata et al. 2011). Together, these studies show that supplementary origins are necessary to rescue stalled replication forks. Letessier et al. (2011) have now demonstrated that chromosomal breaks occur at fragile sites because these loci are late replicating and origin poor. At fragile sites, replication forks have to cover unusually long distances. Therefore, there is a probability that replication forks do not complete the duplication of fragile loci before entry into mitosis, and this probability increases if fork movement is slowed down by nucleotide depletion or polymerase inhibition (Letessier et al. 2011). Another study suggests that breaks at some fragile sites may stem from secondary DNA structures that induce replication fork stalling in a context where no supplementary MCM2-7 complexes are available to rescue stalled forks (Özeri-Galai et al. 2011). Both studies show that the density of usable replication origins is a key determinant of genome stability in S phase.

Role of FANCD2/I in the response to oncogene-induced DNA replication stress

The activation of oncogenes in an evolving population of tumor cells induces the expression of fragile sites and the formation of double-strand DNA breaks associated with DNA replication (Bartkova et al. 2005; Gorgoulis et al. 2005). One study has shown that inappropriate stimulation of S phase entry by human...
papillomavirus (HPV) E6/E7 or by cyclin E occurs without concomitant induction of nucleotide biosynthesis pathways to sustain DNA replication (Bester et al. 2011). E7 binds and degrades the retinoblastoma protein, leading to E2F activation, whereas E6 induces p53 degradation. It has been proposed that suboptimal levels of nucleotides in aberrant S phase cells may account for the increased frequency of replication fork stalling and for the formation of replication-associated DSBs (Bester et al. 2011). Individuals infected with HPV are at high risk of developing head and neck squamous cells carcinomas (HNSCCs). Fanconi anaemia patients are also predisposed to HNSCCs. It is noteworthy that FANCD2 counteracts DNA damage induced by (HPV) E7 activation (Park et al. 2010b). In cultured cells, the expression of (HPV) E7 oncoprotein induces FANCD2 foci formation and chromosomal aberrations (Spardy et al. 2007). In vitro, FA deficiency increases hyperplasia of HPV-positive keratinocytes (Hoskins et al. 2009). In vivo, the knockout of Fancd2 predisposes transgenic mice to (HPV) E7-induced HNSCCs (Park et al. 2010b). Conflicting clinical data, however, have been obtained regarding the potential increased susceptibility of Fanconi anaemia patients to HPV-induced carcinogenesis (Kutler et al. 2003; van Zeeburg et al. 2008). Nevertheless, these studies show strong connections between the FA pathway, the expression of fragile sites induced by (HPV) E7 and carcinogenesis. This is consistent with the notion that the FA pathway either prevents replication fork stalling within origin poor regions or resolves the problem of incomplete DNA replication at fragile sites.

FANCD2/I may coordinate the repair of un-replicated regions flanked by two stalled forks (Kawabata et al. 2011). Alternatively, a recent study has shown that FANCD2 is required for efficient initiation of replication origins in primary human cells (Song et al. 2010a). Hence, the paucity of origins at fragile sites may be further aggravated in FA cells. A third non-exclusive possibility is that FA proteins prevent formation of breaks in mitosis via the resolution of topological entanglements between partially replicated sister chromatids (discussed below).
Role of FA proteins in the resolution of ultrafine anaphase bridges

Abnormal replication intermediates formed at fragile sites have repercussions beyond the S and G2 phases of the cell cycle. Immunofluorescent staining of polo-like kinase 1 (PLK1) have revealed that ultrafine DNA bridges that connect sister chromatids form frequently in anaphase (Baumann et al. 2007; Chan et al. 2007). Ultrafine DNA structures are not detectable using conventional DNA dyes. Two classes of ultrafine anaphase bridges (UFBs) have been identified: centromeres-associated and non-centromeric/damage-induced, anaphase bridges. In mitosis, FANCD2 and FANCI form sister foci that localise specifically to the termini of UFBs induced by replication blocking agents, such as aphidicolin and mitomycin C (Chan et al. 2009; Naim and Rosselli 2009). Most FANCD2/I “sister foci” are already detectable in G2 cells and are located at fragile sites (Chan et al. 2009). Thus, it is likely that damage-inducible UFBs in anaphase originate from incomplete DNA replication. Consistent with this, nearly 50% of mice Mcm4 mutant cells that have less potential origins exhibit spontaneous FANCD2 sister foci in prophase (Kawabata et al. 2011). Interestingly, only 10% of FANCD2 sister foci detected in metaphase are connected by UFBs in anaphase, suggesting that a significant proportion of abnormal replication intermediates that recruit FANCD2/I are resolved before chromosome migration to the poles of the dividing cell (Chan et al. 2009). Unresolved UFBs would otherwise degenerate into double strand DNA breaks, yield chromosomal abnormalities and cause cytoreplication failure with the production of micronuclei and breaks, yield chromosomal abnormalities and cause cytokinesis failure with the production of micronuclei and binucleated cells. Formation of bulky anaphase bridges (stained with DNA dyes) in FA cells has been reported before (Qiao et al. 2004). The recent studies show that FA deficiency is accompanied with increased ultrafine anaphase bridges that derive from fragile sites, consistent with the notion that FA proteins rescue replication failure (Chan et al. 2009; Naim and Rosselli 2009; Vinciguerra et al. 2010).

Bloom’s syndrome protein (BLM) is recruited in anaphase to resolve DNA bridges, in association with its partner proteins RMII and the decatenase Topoisomerase IIIα (Chan et al. 2007). Similarly, some FA proteins may also participate in the resolution of topologically intertwined sister chromatids. FANCM decorates UFBs flanked by FANCD2 foci in telophase cells (Vinciguerra et al. 2010). Intriguingly, FANCM UFBs are BLM-dependent, but the two proteins do not colocalise. BLM UBFs staining tend to disappear at the time FANCM bridges form (Vinciguerra et al. 2010). This may reflect a BLM to FANCM “hand off” mechanism for the resolution of UFBs. Finally, two recent reports show that marks of replication failure can be transmitted to daughter cells. Incomplete DNA replication induced by aphidicolin or unresolved replication intermediates in BLM cells correlate with the formation of 53BP1 nuclear bodies in the subsequent G1 (Harrigan et al. 2011; Lukas et al. 2011). 53BP1 nuclear bodies are enriched at common fragile sites and contain a number of DNA damage response proteins (Harrigan et al. 2011; Lukas et al. 2011). These replication stress structures presumably shield DNA lesions that persist after mitosis until optimal repair conditions are met in the next cell cycle (Lukas et al. 2011). In conclusion, replication failure at fragile sites initiates a cascade of causally linked processes with repercussions up to the next cell cycle.

FA proteins repair DNA lesions induced by reactive aldehydes

The role for FA proteins at fragile sites is not sufficient to explain the severe clinical manifestations of Fanconi anaemia. Recent studies have provided answers to the long-standing question on the identity of endogenous DNA damaging molecules that can promote the appearance of FA symptoms. The Fanconi anaemia pathway appears to limit the toxicity of DNA adducts formed by endogenous aldehydes. Aldehydes are highly reactive products implicated, among other things, in amino acids, carbohydrates, vitamins and steroids metabolic processes (Voulgaridou et al. 2011). For instance, malondialdehyde and crotonaldehyde are both produced through lipid peroxidation and can induce interstrand crosslinks in DNA (Cho et al. 2006; Niedernhofer et al. 2003; Summerfield and Tappel 1984). Some studies have focused on identifying DNA damage response pathways necessary for tolerance to formaldehyde and acetaldehyde. Formaldehyde is widespread in the environment, in nutriments and in the metabolism of purines and some amino acids. The main alteration induced by formaldehyde is protein–DNA crosslinks (Voulgaridou et al. 2011). DT40 cells lacking Fanconi anaemia or homologous recombination proteins are hypersensitive to endogenous levels of formaldehyde in human plasma (Ribpath et al. 2007). Chinese hamster ovary cell lines bearing inactivating mutations of homologous recombination and Fanconi anaemia genes are also hypersensitive to acetaldehyde (Mechilli et al. 2008), the product of ethanol oxidation and an intermediate in carbohydrate metabolism. Acetaldehyde generates mainly mono adducts, and it can also produce ICLs (Brooks and Theruvathu 2005): In a basic environment composed of histones and polyamines, acetaldehyde reacts with deoxyguanine (dG) to form 1, N2-propano-2′-deoxyguanosine adducts, which can in turn react with dG on the complementary strand to form an
ICL (Fig. 4). Cellular exposure to acetaldehyde induces FANCD2 monoubiquitination (Marietta et al. 2009). To test the relevance of acetaldehyde-induced DNA damage in vivo, Langevin et al. (2011) have disrupted the main acetaldehyde detoxifying enzymes aldehyde dehydrogenase 2 (ALDH2) in Fancd2−/− mice. Aldh2−/− Fancd2−/− mice were born only from mothers bearing at least one wild-type Aldh2 allele, showing that the catabolism of acetaldehyde in utero was essential for embryonic development (Langevin et al. 2011). Stress overload with intraperitoneal injection of ethanol caused severe developmental defects in double-mutant embryos. After birth, exposure of Aldh2−/− Fancd2−/− to ethanol in drinking water induced severe bone marrow failure. Unexposed Aldh2−/− Fancd2−/− mice were born with subtle developmental defects and succumbed 3 to 6 months later from a disease similar to acute lymphoblastic leukemia (Langevin et al. 2011). These striking observations demonstrate that in addition to acetaldehyde detoxification pathways, Fanconi anaemia proteins constitute an essential line of defence against the genotoxic consequences of acetaldehyde. Based on the Aldh2−/− Fancd2−/− mouse model, a defect in the repair of acetaldehyde-induced lesions seems to account for most of the Fanconi anaemia symptoms. Reduced exposure to exogenous sources of acetaldehyde combined with therapeutic strategies to accelerate the catabolism of acetaldehyde will likely ameliorate the condition of Fanconi anaemia patients. Further studies will be necessary to decipher whether acetaldehyde is the most critical reactive metabolite for Fanconi anaemia or whether exposure to additional aldehyde-derived DNA damage formation.

DNA-interstrand crosslink
DNA-protein crosslink

1, N₂-PdG

DNA repair dependent on Fanconi anaemia proteins

Fanconi anaemia
• Developmental defects (foetal alcohol syndrome)
• Bone marrow failure
• Cancer proneness (alcohol consumption + mutation in ALDH2)

PdG can also be formed from crotonaldehyde, a product of lipid peroxidation (Marietta et al. 2009). The FA pathway coordinates the repair of DNA lesions induced by acetaldehyde. The accumulation of acetaldehyde-induced lesions may induce congenital malformations, bone marrow failure and leukaemia in Fanconi anaemia patients; foetal damage from excessive alcohol consumption during pregnancy; and oesophageal squamous cell carcinomas in ALDH2-deficient individuals (Brooks et al. 2009).
adducts must be reduced. Limited consumption of alcohol is also advisable for the 36% of East Asians that have an inherited deficiency in ALDH2 (Brooks et al. 2009).

Concluding remarks

Important connections between DNA replication and tumorigenesis have been established. Aberrant entry into S phase may occur without coordination with supportive metabolic programs, such as nucleotide biosynthesis pathways, and lead to chromosomal breaks, primarily at fragile sites, the latest origin-poor region of the genome to be replicated. Replication-blocking lesions induced by reactive metabolites, such as aldehydes, and exogenous DNA damaging agents aggravate difficulties intrinsic to the replication program. FA proteins appear to play a central role in the response to both endogenous and exogenous sources of replication obstacles. The question remains as to why FA-deficient cells appear uniquely hypersensitive to ICL-inducing agents. An important function of FA proteins is to ensure the maintenance of genome stability in cancer prone multicellular organisms. A small number of blocked replication forks do not necessarily induce cell death: In a mouse model that develops spontaneous tumors as a result of insufficient dormant origins, stalled replication forks poorly activate the ATR-Chk1 signalling pathway (Kawabata et al. 2011). One important challenge in the future will be to understand precisely how FA proteins ensure fragile sites stability in the S/G2 and mitotic phases of the cell cycle, which may be relevant for our understanding of the development of solid tumors in FA patients and in the general population. The protection of replication-associated double-strand DNA breaks from toxic repair by NHEJ may be sufficient to explain how the FA network maintains genome integrity. NHEJ components, however, are also required for the maintenance of genome stability. The existence of specific phospho-sites in DNA-PKcs that promote ICL repair suggests that it may be possible to control toxic NHEJ activities without compromising the entire NHEJ pathway in FA patients.

The identification of genotoxic aldehydes that account for disease symptoms in Fanconi anaemia is an important breakthrough that will foster the elaboration of new cancer prevention strategies. Future studies will be necessary to confirm whether acetaldehyde is the most hurtful metabolite in FA patients or whether protection against other aldehydes must be considered. As acetaldehyde induces several types of DNA lesions, mainly monoadducts, the identification of the specific acetaldehyde-induced lesion(s) that depend(s) on FA proteins for repair will shed new light on the FA pathway. After all, the main physiological function of FA proteins may not be to repair ICLs.

Nevertheless, FA proteins are necessary for cellular tolerance to chemotherapeutic, ICL-inducing agents. The complete understanding of the biochemical steps and associated factors involved in FA-mediated crosslink repair will provide new opportunities to improve the use of crosslinking agents in cancer therapy. The development of systems using mammalian or Xenopus extracts to study crosslink repair will greatly accelerate progresses in this field.

It is clear that studies of the rare genetic disease Fanconi anaemia have provided and will continue to provide important knowledge on how cells respond to endogenous replication obstacles, on the nature of these obstacles and on how cells can surmount chemotherapeutic treatments that cause replication failure. This knowledge will not only help improve the care of Fanconi anaemia patients but also contribute to reducing the burden of cancer in the general population.

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