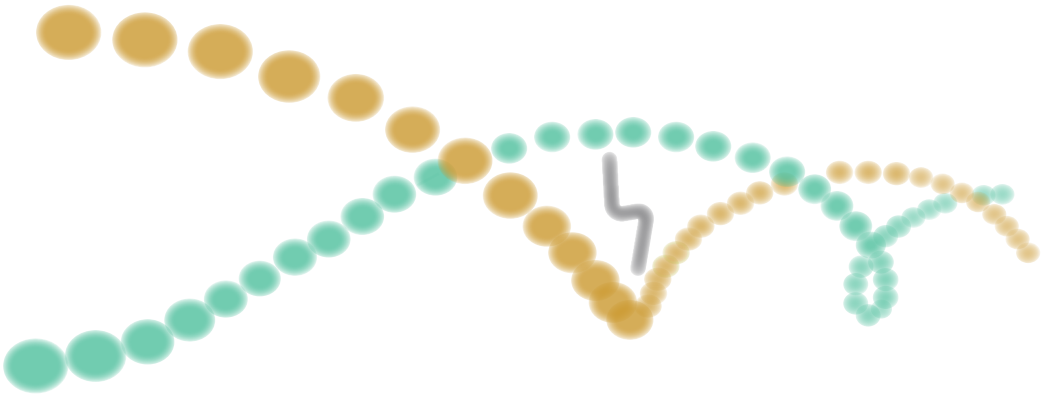

Fanconi Anemia

Mechanistic insights into a phenotypically diverse disease using divers model systems

Sandra Segura-Bayona

Fanconi anemia: mechanistic insights into a phenotypically diverse disease using divers model systems

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Sandra Segura-Bayona

Fanconi anemia (FA) is a rare recessive genetic disease characterized by developmental abnormalities, bone marrow failure and cancer predisposition. Cells from FA patients display hypersensitivity to DNA interstrand crosslink (ICL)-inducing agents, which manifests as reduced cell survival, chromosomal aberrations and disturbed cell cycle with a G2-phase arrest. The currently known 15 genes whose mutation has been identified as cause of FA are thought to function in a common pathway involved in the repair of ICLs, the FA pathway. The biochemical function of the 15 FA proteins has remained largely unknown, in part due to the fact that most FA proteins lack distinct functional domains. Deciphering the role of the FA proteins has been a major focus over the last years and has been dominated by studying ICL repair in a variety of model systems. In this review, I will discuss in detail four vertebrate model systems that have provided valuable insights into the function of the FA proteins in ICL repair. These model systems have greatly enhanced the knowledge of the molecular mechanism of ICL repair, the regulation of the FA proteins and the significance of the FA pathway in a whole-organism setting. Although much is still to be learnt, specifically whether and how the defect in ICL repair can be linked to the divers clinical FA phenotype, increasing our knowledge on how the FA pathway acts will further unveil mechanistic clues that will be key in designing therapeutics tailored to each of the defects found in FA patients.

Keywords: Fanconi anemia (FA), DNA damage, interstrand cross-link (ICL) repair, homology-directed repair (HDR), translesion synthesis (TLS), S-phase

Overview of Fanconi anemia

Pathobiology of Fanconi Anemia

Fanconi anemia (FA) is a rare genetic disease that affects 1 in 360 000 individuals (Auerbach, 2009). It is recessively inherited and genetically complex. Biallelic mutations (or hemizygous mutations in the case of the FANCB gene) in one of the 15 currently known Fanconi genes drive FA. Before the identity of these genes was known, patients were classified in complementation groups. The currently known 15 groups are named FA-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, -N, -O and –P (Table 1). After their identification, the genes affected in each complementation group were accordingly named FANCA, B, C, D1, D2, E, F, G, I, J, L, M, N, O and P. Some genes were already described before being identified as a FA gene. This results in more than one nomenclature for the genes FANCD1/BRCA2, FANCJ/BRIP1/BACH1, FANCN/PALB2, FANCO/RAD51C and FANCP/SLX4. In general, most FA patients suffer from developmental abnormalities, bone marrow failure and cancer predisposition. However, the severity of each of these phenotypes and the age at which the disease is diagnosed vary greatly. A mildly affected FA patient may only show hematopoietic phenotype, while a severely affected individual often shows clear developmental abnormalities at birth, may require a bone marrow transplantation within the first 10 years and eventually may not survive to adulthood (Kutler et al., 2003). Despite the fact that the phenotype is extremely heterogeneous, FA patients share some common traits. Developmental abnormalities give rise to congenital malformations, retarded growth, infertility and multiorgan defects. The most frequent skeletal abnormalities occur in the thumb and radius. The patients often suffer from bone marrow failure in childhood, which is in many cases associated with hematological malignancies such as progressive pancytopenia later in age. Cancer susceptibility emerges often from the second decade of age as increased incidence of acute myeloid leukemia (AML), head and neck squamous cell carcinoma (HNSCC), and other solid tumors (Auerbach, 2009; Neveling et al., 2009). Registered patient mutations distribute unequally among the 15 FA genes. Recently, the Rockefeller Fanconi Anemia Mutation Database listed 792 unique and 1408 total patient mutations. Prevalence of mutations among the FA genes varies (Figure 1). FANCA deficient individuals are the most abundant cases of FA. Among the classified FA patients, mutations in FANCA, FANCC or FANCG comprise more than 75% of the cases. Mutation carriers are ethnically diverse; nevertheless, consanguineous ethnical groups such as

Table 1. Fanconi anemia complementation groups and genes

Complementation group	Gene	Chromosome locus	Protein function and domains	Key references
FA-A	FANCA	16q24.3	FA core complex	(Lo Ten Foe et al., 1996)
FA-B	FANCB	Xp22.31	FA core complex	(Meetei et al., 2004)
FA-C	FANCC	9q22.3	FA core complex	(Strathdee et al., 1992)
FA-D1	FANCD1/BRCA2	13q12.3	HDR, promotes RAD51 loading onto DNA, stalled fork protection	(Howlett et al., 2002)
FA-D2	FANCD2	3p25.3	ID complex, repair promotion	(Timmers et al., 2001)
FA-E	FANCE	6p21.3	FA core complex	(de Winter et al., 2000a)
FA-F	FANCF	11p15	FA core complex	(de Winter et al., 2000b)
FA-G	FANCG	9p13	FA core complex	(de Winter et al., 1998)
FA-I	FANCI	15q26.1	ID complex, DNA binding	(Dorsman et al., 2007; Sims et al., 2007; Smogorzewska et al., 2007)
FA-J	FANCJ/BRIP1/BACH1	17q23.2	3'-5' DNA helicase	(Levitus et al., 2005)
FA-L	FANCL	2p16.1	FA core complex E3 Ub ligase	(Meetei et al., 2003a)
FA-M	FANCM	14q21.2	FA core complex and BLM complex recruitment to DNA, translocase activity, checkpoint activation	(Meetei et al., 2005)
FA-N	FANCN/PALB2	16p12.2	HDR, FANCD1/BRCA2 binding	(Reid et al., 2007; Xia et al., 2007)
FA-O	FANCO/RAD51C	17q22	HDR	(Vaz et al., 2010)
FA-P	FANCP/SLX4	16p13.3	Endonuclease scaffold, HDR	(Kim et al., 2011; Stoepker et al., 2011)

Spanish gypsies, Ashkenazi jews or white Afrikaner of South Africa established several founder mutations that result in a higher incidence of FA within these populations (Gulbis et al., 2010; Rosenberg et al., 2011).

Phenotypic heterogeneity lacks a clear pattern of association with the given genotypes, however there are some exceptions. Patients from FA-D1 and FA-N groups show early onset of tumors and strong cancer predisposition, which explains their average shortest lifespan among all FA individuals. FANCD1/BRCA2 biallelic mutation carriers display increased rates of leukemia and solid tumors (primarily medulloblastoma and Wilms tumor), which is also manifest in FANCN/PALB2 deficient individuals (D'Andrea, 2010; Tischkowitz and Xia, 2010). This enhanced cancer predisposition phenotype is consistent with the fact that monoallelic mutations in FANCD1/BRCA2, FANCN/PALB2 or FANCJ/BRIP1 are associated with breast cancer susceptibility in the general population (D'Andrea, 2010). In addition, the severity of the FA-D1 phenotype is consistent with the fact that FANCD1/BRCA2 knockout mice are embryonically lethal (Ludwig et al., 1997). Other than cancer risk phenotype in FA, patients from FA-D2 group appear on average more severely affected concerning their hematological problems. FANCD2 patient derived cell lines have some residual FANCD2 protein, suggesting that complete loss of function of FANCD2 may be lethal in humans (Kalb et al., 2007). It is likely that total loss of an allele and therefore protein expression results in a more severe phenotype than a mutated allele that results in a mutant protein or attenuated expression levels. Yet, a detailed analysis of these genotype-phenotype correlations has not been performed. For all FA complementation groups, null mutants have been found in some occasion, except for FA-D2, FA-I, FA-B and FA-D1 (Neveling et al., 2009). In addition, FA phenotypes diverge even within siblings that carry the same biallelic mutations (Koc et al., 1999; Schoof et al., 2000). These cases strengthen the notion that clinical heterogeneity is not only driven by a genetic cause. Factors other than the genetic alteration, such as gene or protein modification and environmental factors, play a role in the clinical phenotype of FA.

In contrast to the heterogeneous clinical phenotype of FA patients, the cellular phenotype is generally uniform. FA-cells display hypersensitivity to DNA crosslinking agents, such as diepoxybutane (DEB), mitomycin C (MMC), cisplatin or nitrogen mustard. These agents covalently connect both strands of DNA, mainly through guanines, creating DNA interstrand crosslinks (ICLs). These DNA lesions are extremely toxic for cells, resulting in a strong block of DNA replication and transcription. FA-cells also exhibit

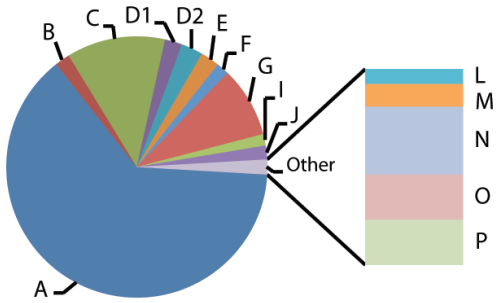

Figure 1. FA patient mutation distribution among the fifteen FA genes. Data listed in the Rockefeller Fanconi Anemia Mutation Database in 2012.

increased spontaneous chromosomal aberration rates such as appearance of breaks and radials, resulting in genomic instability. These chromosomal aberrations are enhanced when these cells are exposed to DNA crosslinking agents. This cellular phenotype is used by clinicians to confirm the FA diagnosis. In a chromosomal breakage assay, cells of potential FA affected individuals are scored for their ability to form MMC or DEB induced chromosomal aberrations in metaphase spreads (Castella et al., 2011; Oostra et al., 2012). In addition to their crosslinking sensitivity, FA-cells show a disturbed cell cycle and accumulate in G2-phase displaying features of premature senescence (Neveling et al., 2009). These observations have led to the hypothesis that the FA pathway is involved in the repair of ICLs. Therefore, much of the research on the function of the FA proteins has focused on their role in this repair process.

Identification of FA genes

The majority of FA groups, especially those that were first described, were classified using complementation assays (Joenje et al., 1994). In these assays, lymphoblastoid cells from unrelated FA patients were fused and the sensitivity of the fusion hybrid to crosslinking agents was assessed. Accordingly, fused cell lines able to overcome the hypersensitivity cellular phenotype were assigned to different complementation groups, whereas those unable to recover a wild type phenotype corresponded to the same group. These experiments provided a classification based on the genetic origin of the disease, as all the patients from a given complementation group were expected to have mutations in the same gene.

Functional rescue assays in the early days permitted cloning the cDNA for some of the FA genes. FA cells exhibit defects in dealing with MMC exposure resulting in MMC hypersensitivity. A cDNA expression library was used to functionally complement these defects in FA-C cells, which established FANCC as the first Fanconi gene (Strathdee et al., 1992). Soon FANCA, FANCG, FANCE and FANCF followed using similar methods (de Winter et al., 2000a; de Winter et al., 2000b; de Winter et al., 1998; Lo Ten Foe et al., 1996). FANCA, C, E, F and G were shown to assemble in a nuclear multimeric complex, the FA core complex (Medhurst et al., 2001). The next approach used to identify FA genes was positional cloning. Use of polymorphic marker exclusion identified FANCD2 in the 3p25 chromosome region (Timmers et al., 2001). FANCD2 colocalized in nuclear foci together with DNA repair factors (Garcia-Higuera et al., 2001) indicating for the first time that the FA proteins may have a direct role in DNA repair. A candidate approach revealed the breast cancer susceptibility gene BRCA2 as FANCD1 (Howlett et al., 2002). Purification of protein complexes in nuclear fractions through a FANCA antibody and subsequent mass spectrometry analysis identified several FA associated proteins (FAAPs), such as FAAP43, FAAP250, FAAP95, FAAP24 and FAAP100 (Meetei et al., 2003b). Analysis of FA patient cell lines with unknown mutation confirmed several of these FAAPs to be bona fide FA genes. FAAP43 was renamed as FANCL (Meetei et al., 2003a), FAAP95 as FANCB (Meetei et al., 2004) and FAAP250 as FANCM (Meetei et al., 2005). Simultaneously, genetic linkage analysis and FA-J cell line complementation by chromosome transfer pointed towards BRIP1, a DEAH helicase, as the FANCJ gene (Levitus et al., 2005). In the past five years, four additional FA genes were identified. FANCN/PALB2 was recognized as a FANCD1/BRCA2 interacting protein in a co-immunoprecipitation experiment and found mutated in several patients (Reid et al., 2007; Xia et al., 2007). FANCI was identified by various means; genome wide linkage analysis identified mutations in KIAA1794 in all the patients in the group FA-I (Dorsman et al., 2007). At the same time, an ionizing radiation screen followed by mass spectrometry showed KIAA1794/FANCI as a specific ATM/ATR kinase substrate (Smogorzewska et al., 2007) and as a binding partner of FANCD2 (Sims et al., 2007). The FANCO gene was identified by a Single Nucleotide Polymorphism (SNP) array in a single-family case as a FA-like disorder. The mutation found in these individuals appeared in RAD51C gene, a protein previously known for its role in homology

directed repair (Vaz et al., 2010). Mutations in SLX4 were found in a small group of individuals, thereafter defining FA-P group (Kim et al., 2011; Stoepker et al., 2011). The FANCP/SLX4 protein serves as a docking platform for several DNA endonucleases (Fekairi et al., 2009). Interestingly, a subset of the diagnosed FA patients lacks a mutation in any of the currently known FA genes and remains unclassified. This indicates that additional FA genes remain to be identified and that the FA pathway is even more complex than we envision at present.

Function of the FA proteins

The FA pathway proteins can be roughly divided in three groups. The FA proteins FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM, together with FAAP24 and FAAP100, form the Fanconi core complex (Figure 2). The FA core complex acts as a multisubunit E3 ubiquitin ligase, in which FANCL contains the catalytic E3 ligase domain. Together with the E2 enzyme UBE2T, the FA core complex mono-ubiquitinates both FANCD2 and FANCI upon exposure to DNA damage (Machida et al., 2006; Meetei et al., 2004). FANCI and FANCD2 form a dimeric complex that constitutes the second group of FA proteins, also named the ID complex (Figure 2). Ubiquitination of FANCI-FANCD2 localizes these proteins to the DNA and is therefore defined as a key step in the activation of the FA pathway (Wang and D'Andrea, 2004). Although FAAP24 and FAAP100 have not yet been found mutated in FA patients, they are important for FANCI-FANCD2 ubiquitination. The third group of FA genes contains FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, FANCO/RAD51C and FANCP/SLX4 (Figure 2). Mutations in these genes do not affect the ubiquitination of FANCI-FANCD2. The proteins in this third group all interact with other DNA repair proteins, and likely orchestrate the later steps in the repair process downstream of FANCI-FANCD2 ubiquitination, which include translesion synthesis (TLS) and homology-directed repair (HDR). As many of the FA proteins lack distinct functional domains, it has proven difficult to gain insight into their molecular function. Therefore, the precise role of most of the FA proteins within the DNA repair pathway still remains unclear.

Post-translational modifications, such as ubiquitination and phosphorylation, play a pivotal role in the FA pathway. FANCI-FANCD2 ubiquitination by the FA core complex represents an essential activating molecular switch observed when cells are exposed to DNA damaging agents. USP1/UAF1 is the deubiquitinating enzyme that inactivates the ubiquitinated FANCI-FANCD2 complex by removing ubiquitin (Nijman et al., 2005), which is crucial for FANCI-FANCD2 recycling and correct function of the FA pathway. FA pathway activation through ubiquitination in S-phase depends on ATR, the S-phase checkpoint kinase. ATR likely phosphorylates FANCI upon DNA damage or replicative stress, which is a crucial event for the mono-ubiquitination of both FANCI and FANCD2 and thus, for the activation of the FA pathway (Ishiai et al., 2008). ATR replication checkpoint activation also requires the core complex proteins FANCM and FAAP24 (Collis et al., 2008; Huang et al., 2010) and the helicase FANCJ (Gong et al., 2010). These modifications underscore the importance of regulatory networks in FA pathway activation in the DNA damage response.

FA cells are hypersensitive to DNA crosslinking agents, but largely resistant to other DNA damaging agents such as ionizing radiation or UV light (Kalb et al., 2004). Nevertheless, some FA cell lines have been reported to be sensitive to oxidative stress (Mukhopadhyay et al., 2006; Saadatzadeh et al., 2004) and the FA pathway is activated when cells are exposed to oxidative stress and redox imbalances (Pagano et al., 2012). FA patients body fluids and cells show accumulation of 8-oxoguanine and metabolites that indicate a peroxidant state (Degan et al., 1995; Pagano et al., 1997). In addition, the FA pathway is activated in response to stalled replication forks (Pichierri and Rosselli, 2004). These observations suggest a role for the FA pathway in genome stability mechanisms other than the repair of ICLs. Thus, further research may expand the roles of the FA pathway.

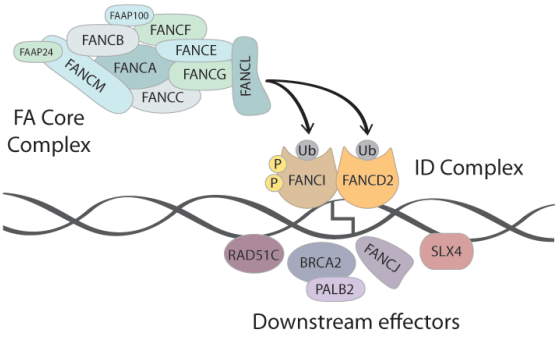

Figure 2. Schematic overview of the FA pathway. After ICL damage the FA core complex ubiquitinates the FANCI-FAND2 complex that results in chromatin localization of FANCI-FANCD2. Downstream effectors colocalize with ubiquitinated FANCI-FANCD2 and are involved in later steps of repair

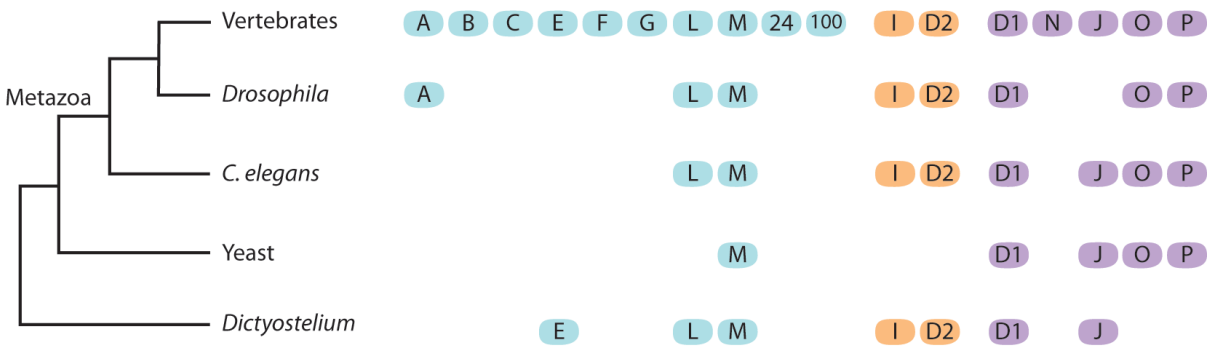

Figure 3. Evolutionary tree of the FA genes among diverse organisms. The FA functional subclassification is indicated with colors as in Figure 2.

Conservation of the FA pathway

The complex FA pathway as described in the previous paragraphs is only present in vertebrates. Prokaryotes seem to lack all FA genes while yeast comprises some homologs of FA proteins, such as Mph1/FANCM, Chl1/FANCJ, Brca2/FANCD1, Rad51/FANCO and Slx4/FANCP, but lacks important FA pathway components such as FANCD2 (Ward et al., 2012) (Figure 3). Nevertheless, studying the biochemical properties of the yeast FA protein homologs has provided insights into their possible function during DNA repair.

Metazoans including non-vertebrates such as insects and worms comprise a minimal FA pathway (Figure 3). This minimal pathway contains FANCL and FANCM, FANCI-FANCD2, and the downstream proteins FANCJ/BRIP1, FANCD1/BRCA2, FANCO/RAD51C and FANCP/SLX4. The factors from the minimal FA pathway may have evolved in the ancestral eukaryote, as they also are present in the amoeba *Dictyostelium* (Zhang et al., 2009). The other factors from the FA core complex such as FANCA, FANCB, FANCC, FANCE, FANCF and FANCG may have evolved later in the ancestral metazoan. In *Caenorhabditis elegans* a similar minimal FA pathway has been found consisting of K01G5.1/FANCL, DRH-3/FANCM, FCD-2/FANCD2, W02D3.10/FANCI, DOG-1/FANCJ and BRC-2/FANCD1/BRCA2 (McVey, 2010). This also holds true for *Drosophila*, which has the FA gene homologs FANCA (Zhang et al., 2009), FANCL, FANCM, FANCD2 (Marek and Bale, 2006), FANCD1/BRCA2 (Lo et al., 2003), CG13745/FANCI, Rad51/FANCO and Mus312/FANCP/SLX4 (McVey, 2010). These conservation patterns suggest simplified FA pathways with likely a similar ICL protecting function. In the future, homology search based on primary protein sequence may identify structural homologs, which due to genomic divergence lack sequence conservation. All FA proteins are highly conserved in vertebrates (Figure 3). Complete Fanconi protein networks are present in the main vertebrate model organisms such as zebrafish, frogs, chicken and mice.

The basis for a complete FA pathway only being present in vertebrates may have emerged from an evolutionary process of positive selection. Multicellular organisms involve several levels of complexity and are more dependent on genome stability, as an unstable genome could lead to cancer and other genetic disorders. These protecting mechanisms to ensure genome stability may include refined cell cycle checkpoints and DNA repair pathways, such as the FA pathway. Evolutionary research on the FA genes through the mammalian phylogeny provided evidence for selective pressure on this genome stability pathway (O'Connell, 2010), thus likely explaining the reduced genome divergence in the context of rapid developmental changes occurring in vertebrates.

The use of model systems to study the molecular details of FA

Although the role of the FA proteins in ICL repair has been a major focus over the last years, it is difficult to link all aspects of the FA phenotype to a defect in ICL repair. A model system that recapitulates all FA phenotypes could be used to gain insights into this issue. However, no perfect model organism for studying the FA disease has been found. Mice deficient for FA genes generally display reduced fertility and in some cases a cancer prone phenotype and growth retardation, but lack hematological defects and most congenital abnormalities found in human Fanconi anemia patients (Tischkowitz and Winqvist, 2011). An exception is the recently reported mouse model deficient for SLX4/FANCP, which

recapitulates many key features of FA including the hematological phenotype (Crossan et al., 2011). This indicates that the ICL repair defect is a key cause of the FA phenotype but whether it is the only problem in FA patients remains unanswered. Thus, the study into the molecular cause of FA is in a large degree dominated by studying ICL repair in diverse model systems. Each model system has its strengths and weaknesses, and insight from a variety of systems has contributed to the limited knowledge we currently have of the roles of FA genes in ICL repair.

Even though research in organisms that have a limited FA pathway, such as *C. elegans*, *Drosophila* or *Dictyostelium,* have shown crucial mechanistic points, most of the research on the role of FA proteins in the mechanism of ICL repair has been performed in vertebrate model systems. For this reason, in this review I will discuss in detail four model systems that have provided valuable insights into the function of the FA proteins in the molecular mechanism of ICL repair. I will first describe the *in vitro* model system based on *Xenopus laevis* egg extracts. Then I will describe how cell based systems, both from chicken DT40 and mammal origin, have contributed to our current knowledge. Finally I will describe the contribution made by mouse model systems.

I. Xenopus egg extracts

Protein extracts prepared from oocytes from Xenopus laevis frogs referred to as Xenopus egg extracts are a valuable system for studying eukaryotic DNA replication. It is the only system that allows regulated and efficient eukaryotic DNA replication *in vitro*. Several lines of evidence indicate that the major ICL repair pathway takes place in S-phase, the cell cycle phase where DNA replication takes place. First, FA deficient cells accumulate in late S/G2-phase after treatment with ICL-inducing agents (Akkari et al., 2000). Second, double strand breaks (DSB) are formed after exposure to DNA crosslinking agents, but this requires passage through S-phase. Third, the FA pathway is only activated in S-phase. (Rothfuss and Grompe, 2004). In the last five years the Xenopus egg extract system has emerged highly suited to study ICL repair. As these extracts are so efficient in DNA replication, the Walter laboratory (Harvard Medical School, Boston, USA) and the Hyrien laboratory (Ecole Normale Supérieure, Paris, France) focused on replication-dependent ICL repair. The Gautier laboratory (Columbia University, New York, USA) mainly focused in replication-independent ICL repair, a minor repair pathway that does not seem to require the FA proteins. The Hoatlin (Oregon Health and Science University, Portland, USA) and Sobeck (University of Minnesota, Minneapolis, USA) laboratories focused on FA protein recruitment to the DNA. The Xenopus egg extract system is suitable for studying several mechanistic steps in the repair of an ICL, therefore is highly appropriate to determine the specific roles of the FA proteins in this repair pathway.

The Walter laboratory showed in 2008 the complete repair of an ICL in the Xenopus egg extract system. They used DNA plasmids with a single site-specific ICL and showed that repair of the ICL was replication-dependent (Raschle et al., 2008). Repair of an ICL in this system involves convergence of two replication forks at either side of the ICL. Then lesion bypass occurs in several discrete steps. The replicative polymerase can extend both leading nascent strands up to a stalling site 20-40 nt away from the crosslink (Figure 4 step A). At this point, a polymerase switch is likely required to extend one of the nascent strands to within 1 nt from the crosslink (Figure 4 step B). Unhooking through dual incisions adjacent to the crosslink may require structure-specific endonucleases and results in a two-sided DSB in one sister (Figure 4 step C). Dual incisions allow the insertion of a cytosine opposite to the unhooked ICL (Figure 4 step D) and further extension of the nascent leading strand beyond the ICL (Figure 4 step E). This translesion synthesis (TLS) process has several candidates among specialized TLS polymerases, such as REV1, Polκ, Polη or Polζ/REV3-REV7 (Klug et al., 2012; Minko et al., 2008; Sharma and Canman, 2012). The polymerase for the insertion step is unclear, but the dCTP transferase REV1 has been suggested (Hicks et al., 2010). In Xenopus egg extracts, immunodepletion of Polζ abrogated the extension step (Figure 4 step E) in the process of ICL repair, indicating that Polζ is the polymerase responsible for this step (Raschle et al., 2008). The final steps in ICL repair may include homology directed repair (HDR) to restore the DSB present in the other sister (Figure 4 step F) and nucleotide excision repair (NER) to remove the adducted template base (Figure 4 step G). A recent study from the Hyrien laboratory supports the model of replication-coupled ICL repair (Le Breton et al., 2011) albeit with some differences in fork stalling positions. ICL repair in Xenopus egg extracts activated the ATR checkpoint, measured by Chk1 phosphorylation, and the FA pathway, measured by FANCD2 mono-ubiquitination (Raschle et al., 2008). Whether dual

fork collision occurs during ICL repair *in vivo* remains to be confirmed. However, based on the inter-origin distance and replication rate, the authors claim that it is very likely that at least part of the ICLs in the mammalian genome involve dual fork collision (Raschle et al., 2008). This study for the first time shows a direct link between the repair of ICLs and active progression of replication.

Next, the Walter laboratory set out to investigate the role of FA proteins using this assay. A direct assessment of the FA pathway role in ICL repair was made by immunodepleting FANCD2 from Xenopus egg extracts (Knipscheer et al., 2009). FANCD2 protein removal resulted in co-depletion of FANCI but did not affect replication efficiency. FANCD2 and its mono-ubiquitination were found to be crucial for replication-coupled ICL repair. The main known role for FANCD2 to that date was its recruitment to the chromatin when cells were exposed to DNA crosslinking agents (Taniguchi et al., 2002). Mapping the nascent products during ICL repair determined that the repair process was inhibited at the level of the insertion step (Figure 4 step D) in absence of FANCD2. Further analysis revealed that this was caused by inhibition of the incisions that unhook the ICL (Knipscheer et al., 2009). This suggested a specific role for ubiquitinated FANCI-FANCD2 in promoting the incisions flanking the crosslink and possibly also in the TLS step past the unhooked crosslink. This study was the first that demonstrated a direct role of the FA proteins in the process of ICL repair.

In an additional study from the Walter laboratory, they examined the role of RAD51, a protein known to play an important role in HDR, in ICL repair (Long et al., 2011). Dual incisions near the ICL create a DSB, which is likely repaired by HDR. A specific peptide inhibitor of RAD51 was used to prevent RAD51 nucleofilament formation and it was shown that this prevents the repair of the site-specific ICL plasmid in Xenopus egg extracts. Both incisions and lesion bypass were unaffected by RAD51 inhibition, but the reduction of fully extended products indicated an aberrant restoration of the sister chromatid containing the DSB. Consistent with this, RAD51 was shown to be required for strand invasion in ICL repair (Figure 4 step F). As FANCI-FANCD2 were previously shown to be important for incisions and therefore for DSB formation (Knipscheer et al., 2009); it was expected that RAD51 acted downstream of FANCI-FANCD2. Interestingly, the authors found that RAD51 was recruited to the plasmid before the DSB was

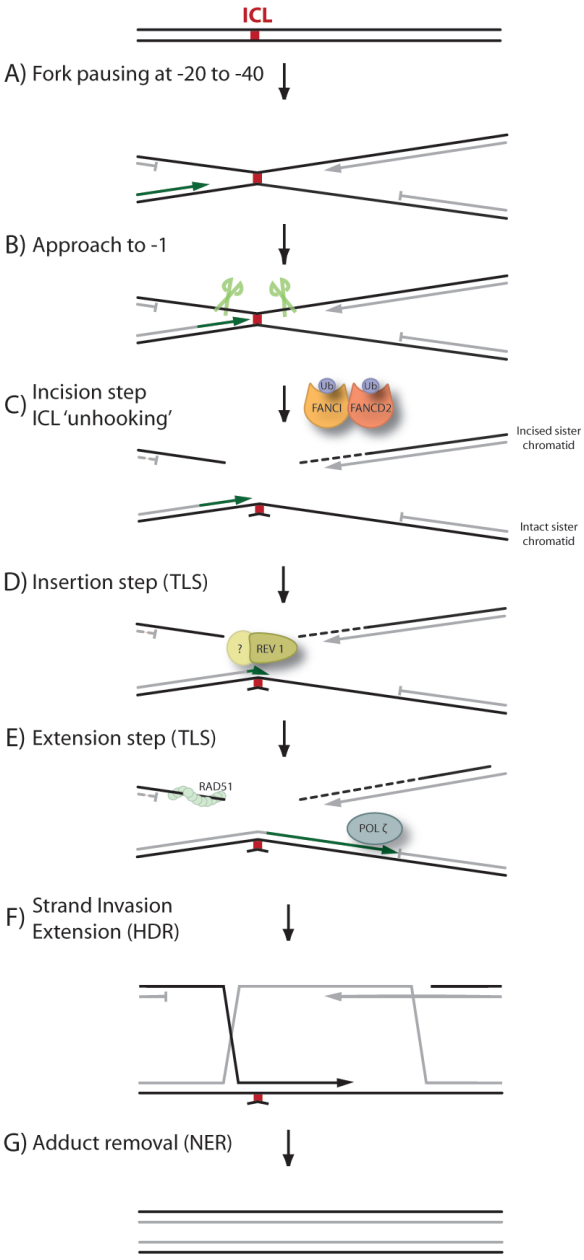

Figure 4. Model of replication-dependent ICL repair according to the findings using Xenopus egg extracts (Knipscheer et al., 2009; Long et al., 2011; Raschle et al., 2008). (A) Replication forks converge at the ICL. **(B)** A polymerase switch may extend one of the strands to within 1 nt to the ICL. **(C)** FANCI-FANCD2 promotes dual incisions to unhook the ICL. **(D)** REV1 likely inserts a nucleotide opposite to the unhooked ICL. **(E)** Polζ (REV3-REV7) extends the nascent strand beyond the ICL. **(F)** RAD51 nucleofilament formation trigger strand invasion to repair the sister chromatid DSB through HDR. **(G)** NER machinery repairs the DNA adduct.

initiated, possibly to prevent breakage of the ssDNA (Long et al., 2011).

Despite the fact that Räschle et al. (2008) were unable to detect significant repair when DNA replication was blocked, other studies from the Gautier laboratory on plasmids containing a defined

ICL in Xenopus egg extracts indicate that repair can also occur independent of DNA replication (Ben-Yehoyada et al., 2009; Williams et al., 2012). This repair pathway may be important for avoiding problems during transcription in quiescent and senescent cells. This replication-independent repair (RIR) of ICLs seems to be largely independent of the FA pathway, however, FA proteins might play an indirect role during G1-phase. They observed that ICLs activated a DNA repair checkpoint independently of active DNA replication. Depletion of FA proteins affected this checkpoint response, suggesting that the FA pathway may act upstream of the ATR checkpoint in RIR (Ben-Yehoyada et al., 2009). Due to distortion of the helical structure of the DNA, ICLs may be detected in the absence of replication and thereby induce checkpoint signaling outside S-phase. In addition, ssDNA-RPA intermediates may be formed during RIR thus triggering ATR checkpoint activation (Zou and Elledge, 2003). As depletion of some of the FA proteins such as FANCI does not inhibit RIR (Williams et al., 2012), while depletion of others such as FANCL partially inhibits RIR (Ben-Yehoyada et al., 2009), it remains to be established whether this FA dependent checkpoint activation is crucial for ICL repair outside S-phase. Interestingly, FANCD2 depleted Xenopus egg extracts showed no defect in ATR checkpoint activation (Knipscheer et al., 2009) indicating that this FA protein is not required for checkpoint activation in replication-dependent ICL repair.

In a follow-up study, the Gautier laboratory studied the role of translesion polymerases in replication-independent ICL repair. Depletion of the TLS polymerase Polκ diminished RIR, whereas depletion of Polζ/REV3-REV7 had no major effect (Williams et al., 2012). PCNA depletion experiments indicated that K164 PCNA mono-ubiquitination is likely responsible for Polκ recruitment to the ICL lesion outside S-phase (Williams et al., 2012). Whereas in Ben-Yehoyada et al. RIR was described as extensive repair DNA synthesis incompatible with NER processing (around 300 nt were substituted in each 2.7 kb plasmid), in Williams et al. RIR was assumed to be more similar to canonical NER processing, with substitution of approximately 30 nt. Both replication-dependent and -independent mechanisms require additional investigation, but it seems likely that the replication-dependent mechanism relies more on the FA pathway.

Several laboratories used Xenopus egg extracts to study FA proteins dynamics and recruitment to the chromatin. The Hoatlin laboratory showed that the FA proteins were recruited to MMC treated chromatin during DNA replication (Sobeck et al., 2006) and suggested that they play role in preventing the accumulation of DNA breaks. Next, FANCD2 mono-ubiquitination upon presence of specific DNA structures that mimic replication intermediates indicated that FA pathway activation might be a consequence of replication stalling, possibly as a result of DNA damage (Sobeck et al., 2007). Subsequently, they examined FANCM recruitment and regulation in Xenopus egg extracts. Phosphorylation and chromatin recruitment of FANCM were controlled by FANCD2, ATR and ATM (Sobeck et al., 2009); thus suggesting feedback signaling from upstream factors. The Sobeck laboratory recently showed dissociation of the FANCI-FANCD2 complex after FANCI phosphorylation (Sareen et al., 2012). These experiments indicated that the active state of these proteins might be the dissociated state instead of the FANCI-FANCD2 complex. In additional experiments the role of active DNA replication in FA protein recruitment was studied. While the FA core complex proteins and FANCI-FANCD2 were associated independently of active replication, downstream factors such as FANCD1/BRCA2, FANCJ/BRIP1 and FANCN/PALB2 required active replication for their recruitment (Shen et al., 2009). These experiments were performed in absence of ICLs and provide insights into a possible function of the FA pathway during DNA replication.

Xenopus egg extracts provide a cell-free system that supports efficient eukaryotic DNA replication and ICL repair under physiological conditions (Knipscheer et al., 2012; Lebofsky et al., 2009; Walter et al., 1998). This *in vitro* system has provided many mechanistic details on ICL repair never achieved before with any other system. Xenopus egg extracts are easy to manipulate through immunodepletion of proteins of interest or addition of specific inhibitors, and reversible regarding the addition of a functional recombinant protein after immunodepletion. This system is also suitable for studying protein functional domains through immunodepletion and subsequent addition of mutant recombinant proteins. Replication of chromatin or plasmids with desired sequences and lesions is supported by the Xenopus egg extract system. These reasons make of it a faithful model system for the understanding of replication-associated processes, repair and signaling.

II. DT40 avian cells

The chicken B lymphocyte line DT40 is well known for its easy-targeted integration of DNA in homologous loci, thus allowing efficient gene disruption. This has proven useful for reverse genetics, in which mutants of known candidate genes are functionally analyzed through their cellular phenotype. Homologous targeting in DT40 cells appears to be extremely efficient compared to any mammalian cell-based system, presumably because these lymphocytes have an efficient HDR mechanism involved in Ig gene conversion (Yamazoe et al., 2004). Genetic disruption in this system not only allows single, but also double and even triple knockouts, knock-in alleles and conditional knockouts. Thus, DT40 cells are suitable for epistasis studies that address the interaction between genetic alleles in a functional pathway. One complication is that DT40 cells deficient in some FA genes are less competent in HDR, thus the gene targeting efficiency decreases. To overcome this issue, conditional mutant cells are generated, for example using the tamoxifen-inducible Cre-LoxP system (Takata et al., 2009).

The genetic relation between the FA and HDR or TLS pathways has been examined using the DT40 system. The first studies targeting FA genes such as FANCC or FANCD2 in the DT40 system showed they were important for immunoglobulin gene diversification through HDR and TLS mechanisms (Niedzwiedz et al., 2004; Yamamoto et al., 2005). Subsequently it was shown that HDR and TLS related genes were required to counteract ICL-inducing agents. (Nojima et al., 2005). The link between HDR/TLS and the FA pathways has been examined using epistasis studies. In sensitivity assays for agents that cause ICLs the FA core complex gene FANCC was found to genetically interact with the HDR factors XRCC2 and XRCC3, the TLS polymerases REV1 and REV3, and the BLM helicase (Hirano et al., 2005; Niedzwiedz et al., 2004) suggesting that they act in the same pathway in ICL repair. In some processes TLS is promoted by ubiquitination of PCNA that recruits the appropriate TLS polymerase. However, FANCC was not epistatic with RAD18, an ubiquitin ligase responsible for PCNA modification, pointing to PCNA-Ub independent TLS pathway acting with the FA pathway during ICL repair (Hirano et al., 2005). A later study generated double mutants of FANCC and FANCD1/BRCA2. These cells did not show increased sensitivity to DSB-inducing agents compared to the FANCD1/BRCA2 single mutant, but did show additive sensitivity to ICL-inducing agents (Kitao et al., 2006). This suggested that FANCC and FANCD1/BRCA2 act in the same pathway to protect cells from DSBs but have some non-overlapping functions in relation to ICL repair. From a recent study using DT40 cells, FANCD1/BRCA2 was shown to genetically interact with several mediators of HDR including BRCA1, RAD52, SFR1, SWS1, and the five RAD51 paralogs (Qing et al., 2011) indicating that these factors require FANCD1/BRCA2 to contribute to HDR in response to cisplatin and DSB-inducing agents. All these data support the notion of FA pathway acting in concert with HDR and TLS in ICL repair.

Experiments in DT40 cells have been especially useful to dissect the activating molecular switch of the FA pathway through FANCI-FANCD2 ubiquitination (Figure 5). This work has mainly been carried out by the Patel laboratory (Medical Research Council, Cambridge, UK) and the Takata laboratory (Kyoto University, Kyoto, Japan). The role of FANCD2 mono-ubiquitination was assessed using FANCD2-ubiquitin and FANCD2-histone2A linear fusion proteins, both with mutated ubiquitin acceptor sites (Matsushita et al., 2005). These fusion proteins were both constitutively chromatin-bound and were able to rescue ICL sensitivity in a FANCD2 deficient mutant. This showed the main function of ubiquitination of FANCD2 is to target the protein to chromatin. Interestingly, these fusion proteins could not complement cells that were not only deficient in FANCD2 but also lacked FANCC, FANCG, or FANCL. This revealed functions of some components of the FA core complex apart from contributing to FANCD2 mono-ubiquitination. In addition, the Patel laboratory studied the role of the FANCD2 deubiquitinating enzyme USP1. USP1 deficient DT40 cells were hypersensitive to crosslinking agents (Oestergaard et al., 2007) even though they could still activate the FA pathway by FANCD2 mono-ubiquitination. The authors reasoned that persistent accumulation of mono-ubiquitinated FANCD2 at the chromatin prevented redistribution of FANCD2 to the sites of damage. In a subsequent study, they analyzed the assembly and activity of the FA core complex. By using DT40 strains disrupted in several FA core complex genes and knock-in of epitope tagged alleles, they showed that even though the FA core complex seems to be constitutively assembled, its E3 ligase activity is restricted to the chromatin-bound form (Alpi et al., 2007). The E2 enzyme UBE2T appeared to be constitutively present on chromatin, thus, the activation of the FA core complex may depend on its interaction with the E2 enzyme on chromatin after damage (Alpi et al., 2007). In a

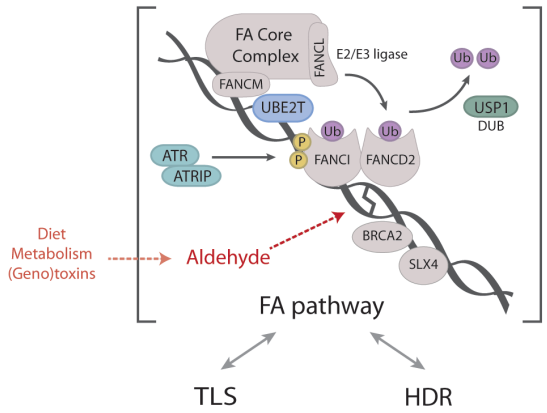

Figure 5. Model depicting the main findings elucidated using the DT40 cellular system. FANCI-FANCD2 ubiquitination is regulated by phosphorylation of FANCI. TLS and HDR play a role in FA pathway dependent ICL repair. Reactive aldehydes likely cause damage to the DNA that is repaired via the FA pathway.

follow-up study, they reconstituted FANCD2 mono-ubiquitination *in vitro* and found that not only the FA core complex and UBE2T were required, but also binding of FANCI, the interaction partner of FANCD2, enhanced FANCD2 mono-ubiquitination (Alpi et al., 2008).

The chicken cellular system has also been used to address the interplay between phosphorylation and FA pathway activation by ubiquitination. A study from the Takata laboratory targeted several ATM/ATR consensus phosphorylation sites (S/TQ motifs) on FANCI and examined FA pathway activation upon exposure to crosslinking agents (Ishiai et al., 2008). Phosphorylation of six conserved FANCI S/TQ motifs but not FANCI mono-ubiquitination was found crucial for FA pathway activation through FANCD2 mono-ubiquitination. FANCI phosphorylation is likely ATR/ATM mediated; as caffeine, an ATR/ATM inhibitor, abolished FA pathway activation. In a recent study, the Takata laboratory examined the role of the ATR interacting partner ATRIP to dissect the identity of the kinase that directly phosphorylates FANCI (Shigechi et al., 2012). In ATRIP deficient DT40 cells, ubiquitination of FANCI-FANCD2 was significantly reduced upon MMC exposure and damage foci formation was abrogated. In addition, *in vitro* kinase assays showed ATR ability to directly phosphorylate FANCI and trigger FA pathway activation. These data strongly indicate that the kinase that elicits FA pathway activation through FANCI phosphorylation is ATR.

The molecular function of several FA proteins has been studied using DT40 cells. Specifically, valuable insight into the function of FANCM and FANCP/SLX4 has been obtained. The Patel laboratory examined the function of FANCM, the vertebrate Hef ortholog that contains both helicase and nuclease conserved domains (Mosedale et al., 2005). They found that the conserved nuclease domain was dispensable for FANCM role in protecting against ICL-inducing agents. In addition, FANCM deficient cells failed to target the FA core complex to chromatin upon DNA damage, indicating an essential role for FANCM in damage-induced localization of the FA core complex. The Takeda laboratory (Kyoto University, Kyoto, Japan) characterized the role of FANCP/SLX4 in DT40 avian cells. Genetic disruption of FANCP/SLX4 resulted in cell death linked to extensive chromosome aberrations and significant crosslinking hypersensitivity (Yamamoto et al., 2011). Moreover, the UBZ domain of FANCP/SLX4 was found crucial for its recruitment to damage sites in crosslinked chromatin, which they suggest is mediated by interaction with mono-ubiquitinated FANCD2. As SLX4 interacts with several structure specific endonucleases, this may explain how endonucleases are recruited to the site of damage, namely through their interaction with SLX4 bound to FANCD2-Ub.

DT40 cells have also provided important insights into the role of the FA pathway in detoxifying endogenous metabolic threats. Although the FA pathway is known for its role in ICL repair, it has been unclear for a long time whether ICLs are the only endogenous damage this pathway neutralizes. DT40 cells deficient in FA genes showed hypersensitivity to formaldehyde and a recent breakthrough investigation confirmed that FANCD2 can counteract the toxicity of naturally produced aldehydes in mice (Langevin et al., 2011; Ridpath et al., 2007). It is presumed that these aldehydes may cause DNA damage (Figure 5), and both detoxification enzymes and DNA repair factors protect against this natural source of damage (Cheng et al., 2003; Lorenti Garcia et al., 2009). A related study focused on the effects of formaldehyde accumulation in DT40 cells by genetically targeting ADH5, the enzyme that metabolizes formaldehyde (Rosado et al., 2011). ADH5 disruption lacked a significant phenotype; however in a FANCL or FANCD2 deficient background, a synthetic lethal interaction was observed. These results further confirmed the role of the FA pathway in protecting against aldehyde toxicity.

The DT40 cell system allows clean genetic experiments and avoids residual protein expression present in mammal cells after RNAi silencing technologies. Use of gene-targeted DT40 cells elucidates functional genetic interactions through analyzing their cellular phenotype. This makes the DT40 cells a unique system among higher eukaryotes for thorough reverse genetic analysis.

III. Mammal cell lines

Mammal cell lines are thus far the most widely used model system for studying many aspects underlying FA. Insight into the function of the FA proteins has been gained using cells derived from patient tissue or cell lines in which FA genes are silenced by RNAi technology. The understanding of FA protein domains function and protein-protein interactions has been achieved by analyzing the phenotype of cells that stable express mutant proteins. In addition, cell lines have been used as a high-throughput screening platform for identifying novel genes implicated in ICL repair as well as protein interaction networks. Another highly useful technique for studying DNA replication or repair dynamics is the DNA fiber technique that allows the visualization of single-molecule DNA fibers from cells. This facilitates the comprehension of replication stalling, an important aspect in ICL repair. Finally, it is well-worth mentioning the development of GFP reporter assays monitoring the outcome of HDR, in some cases in the context of an ICL, that have provided much information about this process. These, and many other techniques have been used extensively over the past years to study ICL repair. In this chapter I will focus on some of the latest findings that have extended our knowledge on the molecular mechanism of the FA pathway in ICL repair.

The FANCM protein has been the subject of many investigations, in part because it was for a long time one of the only FA proteins with recognizable functional domains. FANCM turned into a controversial FA gene when the only reported patient was found also to carry additional FANCA biallelic mutations (Singh et al., 2009). When the patient-derived cell line was corrected for FANCA expression, FANCM cells were still hypersensitive to MMC but able to mono-ubiquitinate FANCD2, albeit not efficiently. Cells deficient in FANCM are MMC sensitive but, unlike many other FA cells, also camptothecin and UV sensitive, which indicates that FANCM might have roles beyond ICL repair (Rosado et al., 2009; Singh et al., 2009). FANCM has an N-terminal DEAH helicase domain and a C-terminal ERCC4-like nuclease domain that is largely dispensable for ICL repair (Singh et al., 2009) (Figure 6A). Purified FANCM is able to remodel Holliday junctions and reverse replication forks in the context of replicative stalling in an ATPase-dependent manner (Gari et al., 2008a; Gari et al., 2008b). This translocase activity was found important for MMC resistance but not necessary for FANCD2 mono-ubiquitination in cells (Xue et al., 2008), indicating that this catalytic activity of FANCM is dispensable for its assembly in the FA core complex. The observed translocase activity of FANCM *in vitro* agrees with *in vivo* results in mammalian cell DNA fibers, where FANCM promotes stalled replication fork recovery via fork reversal in the presence of UV and camptothecin (Luke-Glaser et al., 2010). Although this indicates that fork reversal by FANCM may play a role in ICL repair, this remains to be confirmed. Several factors have been found to interact with FANCM (Figure 6A). The binding partner FAAP24, also containing an ERCC4-like domain, interacts with the C-terminal part of FANCM (Ciccia et al., 2007; Kim et al., 2008). The MHF1-MHF2 heterodimer is constitutively located together with FANCM-FAAP24 in chromatin, mostly independently of other FA core complex factors (Yan et al., 2010). Both FAAP24 and MHF1-MHF2 are able to bind DNA and may target the FA core complex to chromatin. Like FAAP24, MHF1-MHF2 is important for FA pathway activation and for enhancing the replication fork remodeling activity of FANCM (Singh et al., 2010). Independently of the FA core complex, FANCM-FAAP24 interacts with HCLK2, which triggers efficient ATR checkpoint signaling after replication stress, including when ICLs are present (Collis et al., 2007; Collis et al., 2008) (Figure 6C). In the context of ICL repair, FANCM-FAAP24 is required for RPA foci formation and ATR checkpoint activation (Huang et al., 2010) (Figure 6C). In another study FANCM was shown to not only recruit the FA complex to DNA upon MMC treatment but also the Bloom's complex (BLM-TopIIIa-RMI1-RMI2) (Deans and West, 2009). Mutation of the Bloom's complex is associated with Bloom's disease, which suggests a crosstalk between this disease and FA (Figure 6C). All these studies support the fact that FANCM exhibits several activities that are required in the response to replication stress and diverse types of DNA damage including ICLs.

The FA protein FANCP, also named SLX4 or BTBD12, was recently identified as the human ortholog of budding yeast Slx4 (Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). Cells deficient in SLX4 displayed sensitivity to MMC and camptothecin, and

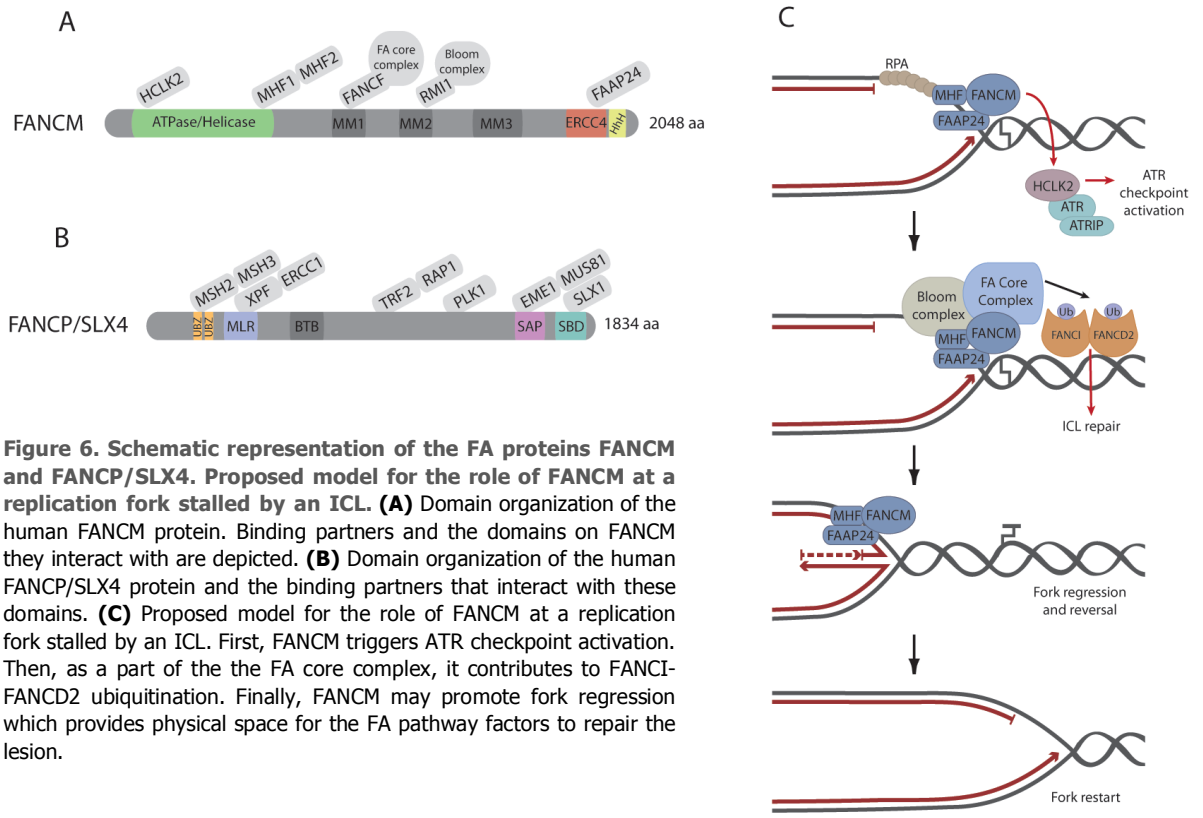

Figure 6. Schematic representation of the FA proteins FANCM and FANCP/SLX4. Proposed model for the role of FANCM at a replication fork stalled by an ICL. (A) Domain organization of the human FANCM protein. Binding partners and the domains on FANCM they interact with are depicted. **(B)** Domain organization of the human FANCP/SLX4 protein and the binding partners that interact with these domains. **(C)** Proposed model for the role of FANCM at a replication fork stalled by an ICL. First, FANCM triggers ATR checkpoint activation. Then, as a part of the the FA core complex, it contributes to FANCI-FANCD2 ubiquitination. Finally, FANCM may promote fork regression which provides physical space for the FA pathway factors to repair the lesion.

they also showed reduced DSB repair via HDR *in vivo*. Human SLX4 was shown to interact with several structure-specific endonucleases: SLX1, XPF-ERCC1 and MUS81-EME1 (Figure 6B). In addition, SLX4 contains two ubiquitin interaction domains (UBZ domains) that were shown to bind ubiquitin chains *in vitro* (Kim et al., 2011). FA-mutations found in SLX4 (Kim et al., 2011; Stoepker et al., 2011) either disrupt the UBZ domains or introduce stop codons that result in larger truncations affecting XPF- and/or MUS81-interacting domains. Interestingly, patient cell lines with UBZ domain deletions only display MMC sensitivity. Therefore it was suggested that SLX4 gets recruited to mono-ubiquitinated FANCD2 through its UBZ domains during ICL repair. In addition, the interaction of SLX4 with XPF-ERCC1, and to a lesser extent, with MUS81-EME1 were reported to be important for ICL repair (Fekairi et al., 2009), whereas the role of SLX1 in ICL repair is under debate. A recent study confirmed that the interaction between SLX4 and XPF is crucial for MMC resistance while the one between SLX4 and MUS81 is more important for camptothecin resistance (Kim et al., 2013). All these studies suggest that SLX4 is a scaffold protein that recruits multiple endonucleases acting on diverse DNA damage pathways. They also indicate that SLX4 interaction with XPF-ERCC1 has an important role in ICL repair.

Fanconi anemia-associated nuclease 1 (FAN1) was identified as a structure-specific nuclease important in ICL repair because it conferred resistance to ICL-inducing agents. It was suggested that mono-ubiquitinated FANCD2 recruited FAN1 via its UBZ domain to DNA damaged sites (Kratz et al., 2010; Liu et al., 2010). This finding made FAN1 a likely candidate to be involved in the dual incisions that unhook a crosslink in ICL repair. However, FAN1 depletion in human cells displayed the same extent of γH2AX foci compared to wild-type cells after being treated with ICL-inducing agents, indicating that FAN1 is not involved in the first incision that generates a DSB. FAN1 depleted cells did show reduced efficiency of HDR, suggesting a possible role for FAN1 in a later step of ICL repair that involves HDR (MacKay et al., 2010). Even though these data suggested that FAN1 mutations might cause FA, recent work has refuted this hypothesis. FAN1 null biallelic mutations have been found in several patients that suffer from developmental and congenital abnormalities but lack FA hematological phenotype and cellular FA hallmarks (Trujillo et al., 2012). In a recent study, FAN1 biallelic mutations have been pointed as the cause of chronic kidney disease (Zhou et al., 2012). This renal fibrosis pathology is suggested to arise from a defect in DNA damage response signaling independent of the Fanconi pathway.

Several new technologies that examine the DNA damage response in living cells have emerged. The Jasin laboratory (Memorial Sloan-Kettering Cancer Center, New York, USA) developed a system to detect HDR using a GFP reporter (Weinstock et al., 2006). The DR-GFP reporter system consists of a site specific DSB generated by I-SceI endonuclease that, when repaired by HDR, turns on GFP expression. In a variation of this reporter system the DSB is replaced by a site-specific ICL and ICL-induced HDR can be measured. Using this system they demonstrated that not only the upstream FA genes (Figure 2) were important for ICL-induced HDR, but also the structure specific endonuclease XPF-ERCC1 was implicated (Nakanishi et al., 2011). The downstream FA gene FANCD1/BRCA2 was shown to be equally important for ICL-induced HDR and DSB-induced HDR. Importantly the defect observed in FA cells was governed by active replication (Nakanishi et al., 2011), which further supports HDR to play an important role in replication-coupled ICL repair.

Single-molecule DNA fiber analysis in mammal cells was also used by the Jasin laboratory to study replication fork dynamics. They showed that FANCD1/BRCA2 has a role in fork protection independently of its role in HDR by stabilizing RAD51 filaments and preventing the degradation of the newly synthesized strands by the MRE11 complex (Schlacher et al., 2011). In a later study they showed that other FA genes such as FANCA or FANCD2 protected stalled replication forks in a manner independent of ICL repair (Schlacher et al., 2012). These results provide a more complete understanding of the role of FA proteins not only in ICL repair but also in the response to stalled replication forks in general.

Mammalian and especially human cell lines are the most closely related cellular system to study FA. As cell lines derived from FA patients need to be transformed for long-term viability this may affect the reproducibility between experiments performed in different cell lines. Use of primary cell lines may overcome these reproducibility limitations and give more physiologically relevant results. As described in this section, the use of these cellular systems has substantially contributed to dissect the molecular mechanism underlying FA and will likely be used to extend this knowledge in the future.

IV. Mouse models

Mouse models are a useful tool to study disease phenotypes *in vivo*. Both genetic and interventional studies can be conducted using mouse models, gaining insight into disease mechanisms and therapy response. In order to reproduce the pathophysiological features of Fanconi anemia, several mouse models deficient in the FA genes have been generated. However, the majority of these 'Fanconi' mice do not show strong phenotypic hallmarks of FA such as developmental defects, congenital malformations and hematological abnormalities. Nevertheless, these mice do display mild versions of some FA traits and thus provide us with means to study these in a whole-organism setting.

Mouse models have been generated for ten of the fifteen currently known FA genes (Table 2). The deubiquitinating enzyme for FANCD2, USP1, has also been targeted in mice. FANCD1/BRCA2 and FANCN/PALB2 null mice are embryonic lethal (Rantakari et al., 2010; Sharan et al., 1997). This severe phenotype is consistent with the observation that monoallelic mutations in FANCD1/BRCA2 and FANCN/PALB2 in humans are implicated in hereditary susceptibility to breast cancer (D'Andrea, 2010). This also suggests that FA patients with mutations in FANCD1/BRCA2 or FANCN/PALB2 do not contain null mutations but likely have some residual protein activity. Mice knockout for FANCC, FANCD2, FANCL, FANCP/SLX4 and USP1 show increased perinatal mortality or sub-Mendelian ratio of births (Table 2). The fertility of the viable mice is usually compromised due to hypogonadism and impaired gametogenesis (Table 2), consistent with the human phenotype. FA patients are often short and present congenital malformations. Only mice deficient in FANCD2, FANCL, USP1 and FANCP/SLX4 manifest growth retardation, whereas microphthalmia is displayed in some mutants such as FANCA, FANCC and FANCD2 (Table 2). FANCD2 mice have a modestly stronger phenotype compared to mice deficient in FA core complex genes (Houghtaling et al., 2003). It is noteworthy that the FANCP/SLX4 mutant mouse is the only one that displays significant congenital abnormalities including skull skeletal abnormalities, cerebral problems such as hydrocephalus and ocular abnormalities (Crossan et al., 2011). In addition, these are the only FA mice that faithfully mimic the hematological cytopenia phenotype (Crossan et al., 2011). Although hematological problems are not manifest in most of the FA mice, bone marrow

Table 2. Mouse models for FA and the associated phenotypes

FA gene	Mouse strain	Viability	Fertility	Growth defects	Congenital abnormalities	Hematological abnormalities	Hypersensivity to CL agents	Cancer predisposition	References
FANCA	Fanca$^{-/-}$	viable	reduced	pre-natal growth delay	microphthalmia	absent	present	increased for lymphoma	(Cheng et al., 2000; Wong et al., 2003)
FANCC	Fancc$^{-/-}$	viable, born with sub-mendelian frequency	reduced	absent	microphthalmia	absent	present	increased for sarcoma and adenocarcinoma	(Chen et al., 1996)
FANCD1/BRCA2	Brca2$^{-/-}$	embryonic lethal	na	developmental retardation	na	na	na	na	(Sharan et al., 1997)
FANCD1/BRCA2	Brca2$^{\Delta 27/\Delta 27}$	viable	normal	absent	absent	absent	present	increased for carcinoma and sarcoma	(Atanassov et al., 2005; McAllister et al., 2006; Navarro et al., 2006)
FANCD2	Fancd2$^{-/-}$	viable, born with sub-mendelian frequency	reduced	present	microphthalmia	absent	present	increased for adenoma and carcinoma	(Houghtaling et al., 2003; Parmar et al., 2010)
FANCF	Fancf$^{-/-}$	viable	reduced	absent	absent	absent	present	increased ovary tumors	(Bakker et al., 2012)
FANCG	Fancg$^{-/-}$	viable	reduced	absent	absent	absent	present	absent	(Koomen et al., 2002; Yang et al., 2001)
FANCL	Pog$^{-/-}$	viable depending on genetic background	reduced	present	absent	absent	not tested	not studied	(Agoulnik et al., 2002)
FANCM	Fancm$^{\Delta 2/\Delta 2}$	viable, reduced female birth	reduced	absent	absent	absent	present	increased hepatoma	(Bakker et al., 2009)
FANCN/PALB2	Palb2$^{-/-}$	embryonic lethal	na	developmental retardation	na	na	na	na	(Bouwman et al., 2011; Rantakari et al., 2010)
FANCP/SLX4	Btbd12$^{-/-}$	viable, born with sub-mendelian frequency	reduced	present	abnormal skulls, hydrocephalus, ocular problems	present: cytopenias	present	not studied	(Crossan et al., 2011; Holloway et al., 2011)
USP1	Usp1$^{-/-}$	viable, increased perinatal lethality	reduced in females, male infertility	present	absent	absent	present	not studied	(Kim et al., 2009)
FANCA; FANCC	Fanca$^{-/-}$; Fancc$^{-/-}$	viable	reduced	absent	absent	absent	present	absent	(Noll et al., 2002)
FANCC; FANCG	Fancc$^{-/-}$; Fancg$^{-/-}$	viable	not studied	absent	absent	present: BMF, anemia	present	increased for myeloid malignancies	(Pulliam-Leath et al., 2010)
FANCA; FANCG	Fanca$^{-/-}$; Fancg$^{-/-}$	viable	reduced	absent	absent	absent	present	absent	(van de Vrugt et al., 2011)

BMF: bone marrow failure, na: non applicable

hematopoietic stem cells (HSC) from these mice generally display reduced repopulating ability *in vitro*. In some cases FA double knockout mice have been developed. The Fanca$^{-/-}$Fancc$^{-/-}$ and Fanca$^{-/-}$Fancg$^{-/-}$ double mutants are phenotypically akin to the single mutants, indicating full epistasis between these genes (Table 2). The Fancc$^{-/-}$Fancg$^{-/-}$ double mutant has a hematological phenotype that includes bone marrow failure and anemia (Pulliam-Leath et al., 2010), which is not clearly observed in the single mutants. FA mouse models recapitulate the widespread cancer susceptibility that characterizes Fanconi patients. While FA patients mostly develop AML and HNSCC; tumor types in FA mice are less defined including adenomas, carcinomas, sarcomas and lymphomas (Table 2). The partial overlap between some FA mice and the human phenotype provides us with a useful model for understanding FA disease.

Recent work in mice demonstrated an interaction between the FA pathway and the catabolism of reactive aldehydes. The Patel laboratory (Medical Research Council, Cambridge, UK) was able to generate a mouse with a phenotype very similar to FA by deleting both ALDH2, which detoxifies acetaldehydes in cells, and FANCD2 (Garaycoechea et al., 2012; Langevin et al., 2011). Double mutant pups were only born when the maternal genotype was ALDH2 proficient (Aldh2$^{+/-}$), so the residual ALDH2 could compensate for the fetal deficiency in the detoxifying enzyme. When the double mutant embryos were exposed *in utero* to ethanol, an acetaldehyde precursor, the ratio of homozygous vs. heterozygous mice decreased and the homozygous mice that were born showed clear developmental defects. This indicated high toxicity of reactive aldehydes in embryos lacking these two genes. However, double homozygous newborn mice from unexposed mothers were healthy and only displayed subtle developmental abnormalities. Exposure of the viable homozygous mice to oral ethanol resulted in an anemic phenotype and a reduced proliferation of bone marrow cells. These cells showed increased levels of γH2AX, a DNA damage hallmark, indicating that these animals are subject to increased levels of DNA damage and/or show decreased capacity to repair DNA damage. The ethanol unexposed viable homozygous mice showed acute lymphoblastic leukemia in some months (Langevin et al., 2011). The ones that lacked spontaneous acute lymphoblastic leukemia developed aplastic anemia and loss of the HSC functional pool at later age (Garaycoechea et al., 2012), providing compelling evidence for synergistic function of FANCD2 and ALDH2 in the bone marrow to counteract naturally produced aldehydes. These data suggest that reactive aldehydes are a possible source of DNA damage that is repaired by the Fanconi pathway *in vivo*. Of note, these data were obtained from mice that accumulate high levels of reactive aldehydes and remain to be confirmed with endogenous levels.

Interventional studies in FA mice have also provided insight into the disease phenotype and its correction. Chronic exposure of FANCC deficient mice to a sublethal dose of a crosslinking agent was able to elicit anemia (Carreau et al., 1998). Although FA mouse models, with the exception of the FANCP/SLX4 deficient mouse, lack systemic hematological problems, the HSC pool generally has decreased repopulating ability and impaired progenitor proliferation *in vitro*. Several studies genetically corrected the murine FA deficient hematopoietic stem cells (HSC) *ex vivo* via lentivirus or retrovirus mediated gene transfer. Subsequently, they engrafted the corrected HSC in mildly irradiated conditioned recipients (Haneline et al., 2003; Rio et al., 2008). These corrected cells were able to reverse the chromosomal instability phenotype and restore the repopulating ability of the HSC pool *in vivo*, which is encouraging for future therapeutic approaches for FA patients.

The partial correlation between the mice and human FA phenotypes has shown to be useful for the study of some aspects of FA but limits the study of other aspects. Interestingly, the murine models that most closely recapitulate the FA phenotype are from complementation groups not very common in the human population, which hampers the mouse to human correlations. One of the reasons why the FA phenotype is not completely recapitulated in mice may be due to variations in the murine and human metabolism, which can cause differences in endogenous DNA damage. In addition, mice are bred in an environment free of exogenous genotoxic challenge and may therefore not be as vulnerable for deficiency in the FA pathway. However, that would indicate that the FA phenotype is mostly driven by exogenous damage. Whether and how endogenous aldehydes induce DNA damage remains to be seen. Further research into the phenotype of Fanconi mice and its relationship with the endogenous metabolic damage will help to increase FA pathophysiology understanding.

Conclusions and future prospects

Over the past decade, the model systems described in this review have contributed to most of the advances made in the field of the FA pathway and ICL repair. This progress has led to a convincing model for ICL repair where two replication forks collide at the ICL (Figure 4). The convergence of two stalled replication forks at both sides of the ICL triggers FANCI-FANCD2 ubiquitination and subsequent repair. Replication fork regression and reversal by virtue of FANCM (Figure 6C) may imply the stabilization of the stalled replication fork, thus suppressing the access of error-prone repair factors. In addition, fork regression may enable physical space for repair factors to faithfully process and remove the ICL. The repair process is initialized by incisions at both sides of the ICL. This unhooking allows insertion of a nucleotide opposite to the ICL and extension beyond the ICL, coordinated by TLS polymerases. Eventually, the two-sided DSB generated by the dual incisions is repaired by HDR. Although the two-fork collision model is the most accepted model at the moment, most of the steps and players could be similar in a one-fork collision model. The plausibility of this replication-dependent model is further supported by the fact that S-phase allows detection of ICLs as replication stalling and therefore may be crucial to promote repair.

Despite the many recent findings, several questions remain elusive. What is the molecular function of mono-ubiquitinated FANCD2 at the chromatin? Are there specific downstream factors that are recruited to mono-ubiquitinated FANCD2 and what is their role in ICL repair? The FANCI-FANCD2 modifications are essential for repair, but how they orchestrate downstream processes is not resolved. In addition, like other DNA repair pathways, ICL repair may be extensively regulated by post-translational modifications, thus additional regulating phosphorylation, ubiquitination or sumoylation events may emerge in the future. Another unanswered issue is which endonuclease initiates the incisions in either side of the ICL. Several candidates have been proposed, such as XPF-ERCC1, MUS81-EME1, FAN1, SLX4-SLX1 and SNM1A/B, but so far its identity is unknown. Depletion of these structure-specific nucleases in Xenopus egg extracts may clarify their role in ICL repair. In addition, mouse mutants of the structure-specific endonucleases may recapitulate some features of FA pathophysiology. Furthermore, the FANCM/FAAP24/MHF complex is suggested to act at distinct stages in the FA pathway but how that is coordinated also remains to be elucidated. As yet, we only understand a small fraction of how ICLs are repaired; many players in this repair pathway likely still await identification. For example, there are still unclassified FA patients and therefore additional FA proteins will likely be discovered indicating that the FA pathway is even more intricate than we currently foresee. Exome sequencing is emerging as a powerful tool to identify (new) FA mutations. Unbiased approaches such as mass spectrometry or shRNA screens will help to identify novel factors important for ICL repair or conferring ICL resistance that may be components of the FA pathway. A better understanding of the molecular details of the FA pathway will help to understand FA, but it will also improve treatment options for cancer patients. Knowing how the FA pathway orchestrates ICL repair and how FA proteins interact will be key in designing therapeutics tailored to each of the complementation groups. Regarding cancer, several chemotherapeutic agents act by inducing ICLs. Thus, chemotherapy can be applied more efficiently by looking at expression levels of proteins involved in ICL repair in cancer patients.

One of the major challenges for the future is achieving the molecular understanding of the FA disorder at a whole-organism level; it is time to bridge the gap between ICL repair and FA clinical phenotype. It seems reasonable that FA pathway emerged to counteract life-threatening DNA damage, which can be environmental and/or endogenous. We currently know most about the role of the FA pathway in the repair of ICLs. Even though ICLs may be formed *in vivo* by metabolic processes, it is still not clear whether ICLs are the (only) type of damage the FA pathway is counteracting. The recent identification of reactive aldehydes as a possible source of endogenous DNA damage starts to answer this question. The next step will be to define the nature of this endogenous damage and determine whether reactive aldehydes cause ICLs. Another major issue is how a defect in DNA repair can be translated into a clinical outcome such as FA. The maintenance of genomic stability by the FA pathway may be essential for cell proliferation. Thus, a DNA repair defect during specific stages of development may induce apoptosis and prompt specific congenital abnormalities. FA proteins might also have currently unknown repair-independent functions that contribute to the clinical outcomes of FA.

Ultimately, benefits from FA pathway molecular understanding should be transferred to the clinic. The

current therapeutic option for FA patients involves hematopoietic grafts from histocompatible siblings or donors in order to counteract the most common FA hallmark, bone marrow failure (BMF). Potential curative therapy perspectives have been generated with induced pluripotent stem (iPS) cells. Primary fibroblasts from FA patients were reprogrammed to a pluripotent state *in vitro* and genetically corrected, thus reverting the mutation causing FA (Raya et al., 2009). That would allow autologous cell transplantation of reprogrammed somatic cells subsequently differenced into various hematopoietic progenitor cells. Although gene therapy might imply the cure of BMF that most FA patients suffer from, several issues should be addressed before this can be transferred into the clinic. The efficiency of gene-delivery systems for both the genetic correction and the reprogramming has mainly been achieved using virus-mediated vectors; however the safety of those needs to be guaranteed to avoid insertional oncogenesis and a severe immune response. Implementation of more effective non-viral gene-delivery systems together with new protocols in gene therapy and somatic cell reprogramming may overcome this in the future. This together with increasing our insight into the molecular understanding of the FA pathway will contribute to effective and specific therapeutic options for FA patients.

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