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The Fanconi Anemia Pathway of DNA Repair and Human Cancer

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1. Introduction

The accurate repair of DNA damage and the maintenance of genomic integrity is a fundamental property of every cell. Amongst the different classes of DNA damaging agents, DNA interstrand crosslinks (ICLs) represent a class of DNA lesions wherein the two strands of DNA get cross-linked by covalent bonds. Unrepaired, such cross-linking will impede the progress of critical processes like DNA replication and transcription, resulting in a genomic instability-associated disorder called, Fanconi Anemia (FA).

Fanconi anemia is a rare genetic disorder that occurs at the frequency of 1 in 1:100,000 births. The clinical features of Fanconi anemia were first described by the Swiss paediatrician, Guido Fanconi, in the year 1927. The disease is characterized by low birth weight, developmental defects like congenital limb deformities, hearing failure, skin hyperpigmentation, gastrointestinal abnormalities and haematological defects like aplastic anemia, myelodysplastic syndrome (MDS) and bone marrow failure (BMF). During their life time, Fanconi anemia patients have a very high risk for developing leukemias and solid tumors, due to underlying genomic instability.

At the cellular level, cells deficient in the Fanconi anemia pathway show acute sensitivity to DNA interstrand crosslinking agents and the accumulation of chromosomal aberrations. This chapter will focus on the molecular mechanism underlying the Fanconi anemia pathway of ICL repair and the role this pathway plays in preventing human cancer.
2. Molecular control of ICL repair by the FA pathway

The FA pathway of DNA repair is activated when the DNA replication forks are stalled and they encounter an interstrand crosslinked DNA in the S phase of cell cycle. Human FA is caused by mutations in 16 FA gene products, identified so far. A central molecular event in the FA pathway is the monoubiquitination of FANCD2 at lysine 561 and FANCI at lysine 523, a process mediated by the FA core complex. The FA core complex (FANCA, FANCB, FANCC, FANC E, FANCF, FANCG, FANCL and FANCM) is a multi-subunit ubiquitin ligase. Mutations in any of the constituting subunits cause impaired ICL repair and FA. Upon monoubiquitination, FANCD2 recruits the nucleases, FAN1 and SLX4 (FANCP), which together with XPF/ERCC4 (FANCQ) incise on either side of the crosslink. Finally, homologous recombination (HR) repair by the DNA repair proteins FANCD1 (BRCA2), FANCN (PALB2), FANCJ (BRIP1) and FANCO (RAD51C) in conjunction with translesion repair restore the fidelity of the original DNA double helix. Apart from above described bonafide FA genes, there is a growing list of FA-core complex associated proteins, such as FAAP20, FAAP24, FAAP100, MHF1, MHF2, USP1, and S phase checkpoint proteins like ATR and CHK1. As yet, mutations in such accessory proteins have not been uncovered in FA patients, but, they are nevertheless important in controlling the various steps of the repair process. In the first section of this chapter, the molecular regulation of the Fanconi anemia (FA) pathway of DNA repair will be discussed.

2.1. Replication-coupled interstrand crosslink repair

DNA interstrand crosslinking occurs when nucleotides on opposing DNA strands undergo a covalent linkage. The resultant crosslinked DNA poses a physical impediment for the movement of the DNA replication and transcription apparatus. Hence, ICL agents are highly toxic and it is estimated that even 1-2 crosslinks can be lethal in repair-deficient yeast strains [1]. Chemicals such as mitomycin C, cisplatin, diepoxybutane, metabolic by-products like acetaldehyde, formaldehyde, malondialdehyde and acrolein containing electrophilic groups are capable of causing DNA interstrand crosslinking.

In seminal work by Raschle et al, an elegant cell-free repair assay with Xenopus egg extracts was used to elucidate the exact order of events during ICL repair [2]. A plasmid containing a crosslink was mixed with Xenopus egg extracts and DNA repair was monitored. In this model, two opposing forks collide with crosslinked DNA to activate ICL repair. It was observed that as the leading strand of a replication fork approaches a crosslink, it pauses at the distance of 20-40 nucleotides from the crosslink due to a block posed by the MCM helicase that traverses ahead of the moving replication fork. The 5′ end of the lagging strand also stalls at variable distance from the crosslink. The nascent leading strand that has paused at 20-40 nucleotides then advances to within one nucleotide distance of the ICL after the eviction of the stalled-MCM helicase by BRCA1 [3]. Dual incisions follow that cut on either end of the crosslinked oligonucleotide, to mediate the ‘unhooking’ step. This reaction then generates two sister chromatids with different kinds of DNA lesions: one sister chromatid contains the crosslink whereas the other sister chromatid has a break. The sister chromatid carrying the crosslink is repaired by translesion polymerases that bypass the crosslink. On the other hand, the broken
sister chromatid is repaired by RAD51-dependent strand invasion into the repaired DNA strand.

This cell-free assay was used to demonstrate that the FA core complex-dependent FANCI/FANCD2 monoubiquitination is a critical step for mediating the nucleolytic incisions and translesion synthesis past the lesion. Also, it was shown that recombination acts downstream of the FANCI/FANCD2 loading step [4, 5].

DNA ICL repair can be subdivided mechanistically into the following key steps: Recognition of lesion, FA core complex-mediated FANCI/FANCD2 monoubiquitination, crosslink unhooking, lesion bypass by translesion synthesis of the sister chromatid carrying the excised nucleotide and HR repair of broken sister chromatid (Figure 1 and Figure 2).

2.2. Recognition of lesion

A stabilized DNA replication intermediate consisting of an ICL-bound stalled-replication fork acts as that activating signal for the FA pathway of repair [6]. Since most of the FA core complex proteins lack recognizable functional domains, the proteins involved in FA pathway activation and lesion recognition remained a mystery until the discovery of FANCM.

One of the first proteins capable of recognising the ICL-bound stalled-replication fork is the FANCM-FAAP24-MHF1 complex. FANCM is a large 230 kDa protein, which complexes with FAAP24 through its C-terminal domain. The FANCM-FAAP24 complex binds to synthetic substrates like ssDNA, splayed arm, and 3' flap DNA structure, which mimic intermediates during replication or repair [7]. FANCM has a functional DEAH-type helicase domain with DNA-dependent ATPase activity and it translocates on dsDNA in an ATPase-dependent manner to promote the migration of Holliday branches and replication fork branch points [8] [9] [10]. FANCM-dependent translocation stimulates the accumulation of RPA, the ssDNA binding protein [11]. In turn, RPA recruitment is required for ATR loading and activation of the ATR-dependent checkpoint. Thus, the depletion of FANCM or FAAP24 causes defective ATR-mediated checkpoint signalling leading to impaired CHK1, p53 and FANCE phosphorylation after DNA damage [12] [13]. FANCM and FAAP24 also regulate FA core complex relocalization to chromatin during ICL repair. Hence, the depletion of FANCM or FAAP24 with SiRNA cause impaired FANCD2 monoubiquitination and FANCD2 focus formation [14].

Unlike the other FA core complex proteins, FANCM and FAAP24 are constitutively localised on chromatin through their interaction with the histone-fold containing complex MHF1 and MHF2. The MHF complex stimulates DNA binding and replication fork remodelling by FANCM. The depletion of MHF1 or MHF2 caused the destabilization of FANCM, impaired chromatin localization of the FA core complex, reduced FANCD2 monoubiquitination and focus formation and resulted in the accumulation of chromosomal aberrations [15] [16]. Recent structural studies have revealed that the MHF complex senses branched DNA by binding to a pair of crossover DNA duplexes providing mechanistic insights on how the MHF complex stimulates FANCM translocation activity at such a DNA structure [17].
2.3. Role of ATR in ICL repair

ATR or Ataxia telangectasia mutated related is a master regulator of the S-phase checkpoint. In response to different classes of DNA replication stresses, ATR activation is dependent on the presence of RPA-coated single-stranded DNA (ssDNA) containing regions [18]. ATR coordinates checkpoint activation with the completion of DNA repair by phosphorylating CHK1. Defective ATR function results in crosslinker hypersensitivity, impaired FANCD2 monoubiquitination and the accumulation of massive genomic instability in the form of radial chromosomes [19, 20].

Many FA proteins undergo phosphorylation by ATR and these phosphorylation events are necessary for a functional FA pathway. ATR phosphorylates FANCD2 at threonine 691 and serine 717, at clustered SQ/TQ (serine/threonine-glutamine) motifs on FANCI and at ser 1449 on FANCA. In response to replication stress, CHK1 is also activated by ATR and it directly phosphorylates FANCE subunit at threonine 346 and serine 347. All these phosphorylation events are essential for FANCD2 monoubiquitination [21-24]. Interestingly, FANCM which is implicated in ATR activation, itself undergoes ATR-dependent ser 1025 phosphorylation. FANCM phosphorylation at this site controls integrity of the FA pathway, prevents premature mitotic entry and is required for ATR-dependent checkpoint activation [25].

Figure 1. Major steps of the DNA Interstrand crosslink repair pathway
indicate the existence of a feedback loop wherein the initial signal constituting the ICL-bound stalled-replication fork is recognised by FANCM, which activates ATR. In turn, ATR phosphorylates a number of substrates such as FANCA, FANCI, FANCD2 and lastly FANCM itself, triggering the complete activation of the FA pathway that leads to FANCD2 monoubiquitination.

2.4. The FA core complex

Historically, FA complementation groups were assigned after the pair-wise fusion of patient-derived cell lines followed by assessment of crosslinker sensitivity. Genes mutated in each complementation group were cloned and re-introduced back into mutant cell lines and the ability to rescue crosslinker sensitivity was assessed. Based on this analysis, about 80% of Fanconi anemia patients were assigned to the subtypes FANCA or FANCC [26].

Using a combination of techniques like immunoprecipitation and immunofluorescence, some FA proteins were found to associate to each other, leading to the concept of a FA core complex [27]. It is now known that the FA core complex is constituted by a group proteins mutated in
The assembly of the FA core complex depends on protein-protein interactions between the components. One of the first interactions reported between FA core complex proteins was the binding between FANCA and FANCC [28]. FANCA also interacts with FANCL and this interaction is dependent on FANCG, FANCB and FANCM. FANCB interacts with FANCL and a complex of these proteins binds FANCA [29]. FANCE interacts with FANCD2 [30] and FANCG interacts with FANCA [31, 32]. FANCC nuclear localization depends on interaction with FANC and FANCA requires nuclear localization of FANCC [34].

The accessory protein, FAAP100 is essential for the stability of the core complex and directly interacts with FANCB and FANCL to form a stable sub complex [35]. Another accessory protein, FAAP20 binds to FANCA and ensures the functional integrity of the FA core complex. The depletion of FAAP20 causes hypersensitivity to crosslinking agents, chromosomal aberrations and reduces FANCD2 monoubiquitination [36].

Recently, a modularised organisation has been ascribed for the FA core complex and the catalytic module composed of the FANCL-FANCB-FAAP100 proteins was identified as the minimal subcomplex essential for the ubiquitin ligase function [37]. On the other hand, the other two modules composed of FANCA, FANCG, FAAP20 module and FANCC, FANCE, FANCF modules were proposed to provide non-redundant functions that facilitate the catalytic module to bind sites of DNA damage [38].

### 2.5. The FANCD2/FANCI complex

A major conundrum in the FA field was solved with the discovery that the FA core complex monoubiquitinates FANCD2 (Ub-FANCD2) at lysine 561 [39]. FANCD2 monoubiquitination is DNA damage-dependent and Ub-FANCD2 localizes to sites of DNA damage to form FANCD2 nuclear foci.

Since most of the FA core proteins described above lacked any enzymatic domain, the next big search was for the FANCD2 ubiquitin ligase that catalyses the monoubiquitination reaction. FANCL was later identified as the key ubiquitin ligase responsible for catalysing the monoubiquitination of FANCD2 [40]. It was the only protein with known enzymatic activity in the form of a ubiquitin ligase activity and it contained a PHD-type ring finger at the C terminal domain and an RWD (RING finger proteins, WD-repeat proteins, and yeast DEAD-like) domain responsible for substrate binding [41-43]. The PHD domain of FANCL interacts with the ubiquitin conjugating enzyme, UBE2T. As expected, UBE2T-depleted cells accumulate abnormal radial chromosomes due to impaired FANCD2 monoubiquitination and defective ICL repair [44]. UBE2T itself undergoes monoubiquitination and inactivation after DNA damage, in a process stimulated by FANCL. Thus, UBE2T was identified as the E2 of the FA
pathway and it was proposed that UBE2T has a self-inactivation mechanism that is important for the negative regulation of the FA pathway.

The identity of the protein responsible for Fanconi complementation group I solved another missing link in the FA field. FANCI was identified as a protein essential for FANCD2 mono-ubiquitination [45-47]. FANCI is a FANCD2 paralogue and it heterodimerizes with FANCD2 to form the so-called FANCI/FANCD2 complex or the ID complex [45]. Just like FANCD2, FANCI also undergoes monoubiquitination and surprisingly, the monoubiquitination of both proteins are inter-dependent on each other, which suggested a unique regulation for ubiquitin conjugation. Upon treatment with crosslinking agents, both FANCI and FANCD2 form nuclear foci. The solved crystal structure of FANCI and FANCD2 revealed a saxophone-shaped crystal structure, with single-strand and double-strand binding regions, indicating that the complex can bind to DNA structures that arise after replication forks encounter crosslinking lesions [48]. Surprisingly, the FANCI/FANCD2 regulatory and monoubiquitination sites mapped to the interface of FANCI/FANCD2 binding. In order to explain how the ubiquitin ligase can access the buried ubiquitination site, it was proposed that the monoubiquitination reaction probably acted on monomeric proteins [48].

When the binding activity of the ID complex was tested towards several DNA substrates, it was found that ID complex had better affinity for branched substrates such as splayed arm, Holliday junction, 5'-flap, 3'flap and static fork structures as compared to simple dsDNA, ssDNA or 5'tailed or 3'-tailed structures [49]. Recently, using purified proteins it has been shown that the ID complex is a poor substrate for ubiquitination by UBE2T and FANCL, unless branched or duplex DNA is added to the reaction. Also, mutations in FANCI that inhibit its DNA binding also prevent FANCD2 monoubiquitination. Conversely, FANCI can undergo the monoubiquitination reaction, in a manner independent of FANCD2 [50, 51]. Using Xenopus egg extracts, it was proposed that the ID complex might represent the inactive form and monoubiquitination might break apart the complex into active monomers which then exhibit distinct DNA substrate specificities [52]. According to an evolving model, FANCI and FANCD2 may also function separately at different stages of the repair process. Future research is needed to clarify the function and precise regulation of ID complex formation.

2.6. Negative regulation of FA pathway by USP1

The deubiquitinating enzyme, USP1 is an important regulator of the FA pathway because it deubiquitnates FANCD2 [53]. USP1 depletion increases FANCD2 monoubiquitination both at the steady-state as well as after DNA damage. Unexpectedly, despite there being an increase in Ub-FANCD2 levels, USP1 depletion results in increased crosslinker sensitivity, impaired HR repair, chromosomal aberrations and constitutively chromatin-bound FANCD2. This has led to the model that USP1 is required for recycling and releasing Ub-FANCD2 from chromatin [54, 55]. USP1 exists as a stoichiometric complex with the activator subunit UAF-1 (USP1-associated factor) [56]. The UAF1 contains a tandem repeat of SUMO-like domains at its C-terminus and one of the domains (SLD2) binds directly to the SUMO-like domain interacting motif of FANCI. Thus, UAF1/USP1 proteins get targeted to the FANCI/FANCD2 heterodimer [57]. Both Usp1 and Uaf1 deficiency in mice caused an increase in chromosomal aberrations,
crosslinker sensitization, defective HR and impaired FANCD2 focus assembly. These results indicate that FANCD2 monoubiquitination levels have to be tightly regulated for the proper functioning of the FA pathway [58, 59].

2.7. FANCD2 chromatin localization and nuclear focus formation after DNA damage

The monoubiquitination of FANCD2 is necessary but not sufficient for FANCD2 chromatin retention and nuclear focus formation. This is because monoubiquitination of FANCD2 can be uncoupled from focus formation upon the depletion of certain genes, suggesting the existence of additional layers of regulation in the FA pathway.

For example, the depletion of BRCA1 does not impair FANCD2 monoubiquitination, but only FANCD2 nuclear focus formation [60]. Interestingly, depletion of KU70 overrode the requirement for BRCA1 in FANCD2 recruitment to DNA damage foci, indicating that NHEJ proteins may negatively regulate FANCD2 focus formation. FANCD2 chromatin retention and focus formation are also defective in the absence of XPF-ERCC1 suggesting that a specific DNA structure created by the XPF-ERCC1 mediated-incision might stabilize the chromatin association of Ub-FANCD2 [61]. USP1 and UAF-1 depletion also give rise to impaired FANCD2 focus formation, but increase Ub-FANCD2. Phosphorylated H2AX is also essential for FANCD2 focus formation [62]. Recently, the RUNX family of transcription factors were demonstrated to control FANCD2 chromatin localization and focus formation, but not FANCD2 monoubiquitination. In a non-transcriptional but DNA damage-dependent manner, RUNX proteins interact with the FANCI/FANCD2 heterodimer. The disruption of RUNX proteins in mice gave rise to FA phenotypes such as BMF, MDS and crosslinker sensitivity [63]. Moreover, in at least two FA patients genomic deletions in the region bearing RUNX1 have been identified [64, 65]. In another example, FANCD2 chromatin retention and focus formation are regulated by FANCJ, independent of FANCD2 monoubiquitination [66]. It is possible that multiple proteins co-operatively regulate the FANCD2 focus formation step, because once Ub-FANCD2 lodges itself at the site of DNA damage, it ‘licenses’ the incision step by orchestrating the recruitment of DNA cleaving nucleases.

2.8. Unhooking of the crosslink

The incision of the parent DNA strand on either end of the crosslink is referred as the ‘unhooking step’ of ICL repair. Several structure-specific endonucleases have been implicated in the incision process such as the FAN1 nuclease, SLX4-SLX1 heterodimer, XPF-ERCC1 complex, MUS81-EME1 heterodimer and the SNM1 nuclease.

The ‘unhooking’ step of ICL repair is dependent on FANCD2 monoubiquitination and focus formation [67] because the ubiquitin domain of FANCD2 can recruit both FAN1 and SLX4. In a ShRNA (short hairpin RNA) screen for crosslinker resistance, FANI was identified as a DNA repair nuclease that undergoes recruitment through its UBZ domain (ubiquitin binding domain) by binding to Ub-FANCD2. FANI has both 5'-3' exonuclease activity and 5' flap endonuclease activities. Depletion of FAN1 causes crosslinker sensitivity and defective ICL repair [68-70].
In addition to FAN1 nuclease, ICL processing also involves the SLX4 protein, which complexes with several proteins such as the MUS81-EME1, XPF-ERCC1 heterodimers and the SLX1 nuclease. SLX4 is mutated in FA patients belonging to the complementation group FANCP and results in crosslinker sensitivity and chromosomal aberrations [71-73]. Similarly, ERCC4, the gene that encodes for XPF, undergoes biallelic mutations in FA patients, resulting in XPF being designated as FANCQ [74].

The SLX4 complex cleaves 3’flap, 5’flap and replication fork structures and promotes symmetrical cleavage of static and migrating Holliday junctions, identifying it as a Holliday junction (HJ) resolvase [75]. The ubiquitin-binding zinc finger domain (UBZ) of SLX4 mediates its interaction with Ub-FANCD2 leading to its recruitment to DNA damage sites [76]. In more detailed structure-function analysis, it has been found that the N-terminus of SLX4 protein that only binds XPF-ERCC1 is sufficient to confer DNA crosslinker resistance [77, 78]. SLX4 enhances XPF-ERCC1 nuclease activity in vitro by 100-fold and stimulates dual incisions around a DNA crosslink embedded within a synthetic replication fork [79].

According to another line of evidence, XPF-ERCC1 might be sufficient to make the dual incision during ICL repair. In elegant in vitro biochemical studies, the XPF-ERCC1 complex makes an incision 5’ to a psoralen lesion on a Y-shaped DNA in a damage-dependent manner. Subsequent to the first incision, it creates a second incision specific to the 3’-end side of the ICL. The ICL-specific 5’ and 3’ incisions result in the separation of the two crosslinked DNA strands resulting in ‘unhooking’ [80]. Another nuclease that collaborates with XPF-ERCC1 is the mammalian homolog of yeast Pso2 exonuclease, the human SNM1A. hSNM1 exhibits a 5’-3’ exonuclease activity and initiates ICL repair by creating a favourable substrate for TLS through its nucleolytic action [81]. In summary, multiple nucleases engender the incisions required for ICL repair to proceed, although the relative contribution of these nucleases and the order of their recruitment are not fully clear.

2.9. Downstream of FANCD2 monoubiquitination- FANCD1 (BRCA2), FANCJ, FANCO and FANCN

The Fanconi proteins FANCD1 (BRCA2), FANCJ (BRIP1), FANCO and FANCN (PALB2) are loosely referred to ‘downstream proteins’ in the FA pathway because they act downstream to the FANCD2 monoubiquitination step. Mutations in these proteins give rise to more severe phenotypes in FA patients especially with regards to cancer onset.

FANCD1: The biallelic inactivation of FANCD1 or BRCA2 in Fanconi anaemia patient families created tremendous excitement at the time of the discovery, because it linked FA with a gene closely associated with hereditary cancer susceptibility [82]. Also, it strengthened the idea that the FA and BRCA pathway are intimately linked for the successful repair of ICL damage.

BRCA2 is a central protein of HR repair of DNA damage. In response to double strand breaks, BRCA2 mediates the delivery of RAD51 to ssDNA and facilitates the displacement of RPA from ssDNA. RAD51 is a protein that forms a nucleoprotein filament on ssDNA and invades DNA duplex to search for sequence homology. It is suggested that monoubiquitination of
FANCD2 is needed for BRCA2 loading to DNA damage foci, indicating a functional coupling between FANCD2 monoubiquitination and HR [83].

FANCJ is also called as BRCA1-associated helicase or BRIP1. FANCJ-depleted cells show crosslinker sensitivity and chromosomal instability after mitomycin C treatment [84, 85]. Using a chromatin-IP (immunoprecipitation) based method, recruitment of FANCJ and FANCN to DNA crosslinked sites was found to be FA core complex-independent, but DNA replication-dependent [86].

FANCJ has both DNA-dependent ATPase and helicase activities. The helicase activity of FANCJ is a target for mutational inactivation in patients and it depends on the ATPase activity [87]. FANCJ preferentially binds and unwinds forked duplex substrates and 5’ flap substrates which arise during normal replication and repair. Although FANCJ was isolated as a BRCA1-binding protein, the repair function of FANCJ is independent of its BRCA1 binding because a FANCJ mutant deficient for BRCA1 binding can still rescue the crosslinker sensitivity of FANCJ-deficient chicken DT-40 cells [88]. On the other hand, genetic studies have revealed that instead of BRCA1-binding, FANCL–MLH1 binding is essential for FANCJ to be able to correct crosslinker sensitivity of FANCJ-null cells [89].

RAD51C (FANCO) is essential for HR-mediated repair of lesions associated with replication and controls the intra-S checkpoint through CHK2 activation [90]. RAD51C mutation resulted in a Fanconi-anemia like disorder in a family with several congenital abnormalities characteristic of FA [91]. RAD51C is a part of a complex that also contains PALB2 and BRCA2. FA and cancer-associated RAD51C mutants that show reduced complex formation with PALB2 also have a reduced capacity for HR repair [92].

Partner and localizer of BRCA2 or PALB2 mutations were detected in the Fanconi complementation group N patients. FANCN-deficient cells are sensitive to mitomycin C, are defective for BRCA2 loading and RAD51 focus formation and have reduced HR activity [93, 94]. PALB2 was originally identified as a BRCA2-interacting protein and it physically links BRCA1 and BRCA2 to form the so-called BRCA-complex [95, 96]. PALB2 focus formation after DNA damage was itself dependent on BRCA1. PALB2 is important for BRCA2 localization to sites of DNA damage and in supporting BRCA2 function during HR. Clinically relevant point mutations that either disrupt PALB2-BRCA1 or PALB2-BRCA2 binding fail to support HR [97]. Thus, an intact BRCA1-PALB2-BRCA2 pathway is essential for HR and in the suppression of FA phenotypes.

2.10. Translesion synthesis: after unhooking of the crosslink

Translesion synthesis (TLS) is an inherently error-prone pathway that promotes DNA repair by allowing the bypass of a stalled-replication intermediate [98]. Although this process can increase genomic instability in the form of point mutations, the TLS step is required during ICL to generate the intact template for HR. Consistently, genomic analysis of FA patient samples revealed a ‘hypomutability’ phenotype with respect to point mutations [99].

TLS is achieved because the canonical replicative polymerase is replaced by the translesion polymerase that can accommodate DNA lesions into a larger active site. Several classes
of human translesion polymerases like Rev1, Pol zeta, Pol eta, Pol kappa, Pol iota, and Pol nu participate in replication-dependent ICL repair and extent of bypass depends upon the structure of the crosslinking agent as well as the extent of nucleolytic processing of the crosslink [100].

Of the various translesion polymerases, Rev1 and the translesion polymerase eta form foci at sites of damage in a PCNA-dependent manner. After DNA damage, PCNA is monoubiquitinated in a RAD6/RAD18-dependent manner at lysine 164. PCNA is a sliding clamp that carries the replication polymerase delta along DNA during replication. When PCNA undergoes monoubiquitination, it switches the canonical DNA polymerase into the translesion polymerase eta, which allows for lesion bypass due to a larger active site. Monoubiquitinated PCNA also stabilizes the recruitment of another translesion polymerase, Rev1. Rev1 is a deoxycytosine monophosphate (dCMP) transferase that can insert a cytosine opposite the unhooked ICL. Rev1 recruitment and focus formation is also promoted in a PCNA-independent manner because the FAAP20 subunit of FA core complex binds to ubiquitinated Rev1 through its Zinc finger 4 domain and stabilizes Rev1 nuclear foci [101]. Intriguingly, PCNA monoubiquitination has TLS-independent role in promoting the monoubiquitination of FANCI and FANCD2 in a FANCL-dependent manner [102]. These findings indicate multiple levels of crosstalk between the FA and TLS pathways.

2.11. ICL repair: HR (HR)

The by-product of the ‘unhooking’ step is the generation of a broken strand of DNA that is repaired by the FA pathway using HR. HR is initiated by a DNA end-resection reaction that creates the 3’ overhangs.

During ICL repair, the MRN complex and CtIP participate in the end resection reaction. ATR phosphorylates the MRN complex which is required for the resection of the double strand created after the excision of the ICL to create 3’ overhangs. Recently, CtIP has been found to undergo recruitment to DNA repair sites in a FANCD2 monoubiquitination-dependent manner to start the end resection reaction. The interaction between CtIP and Ub-FANCD2 is required for the formation of mitomycin C-induced CtIP foci and RPA phosphorylation. It is proposed that Ub-FANCD2 channels ICL repair into the error-free HR pathway by tethering CtIP to damaged chromatin. Thus, CtIP prevents illegitimate recombination during ICL repair [103, 104]. In another study, FANC2 binding to CtIP has been observed to promote replication fork-restart [105].

After end resection, RAD51 plays an important role in HR repair. Although RAD51 functions downstream of FANCD2 monoubiquitination, RAD51 recruitment to stalled -replication forks happens independently of FANCI/FANCD2 and before double strand break (DSB) formation. The depletion of RAD51 from Xenopus egg extracts completely disrupted ICL repair by HR [4].

More recently, the role of FA proteins in HR has been firmly established using the TR-GFP assay. In this assay, ICL formation is achieved by conjugating a triplex-forming oligonucleotide to the crosslinking agent psoralen. Using this system, a profound defect in ICL-induced HR
was observed in FA patient cells, but only upon reporter replication [106], providing evidence that FA proteins are essential for replication-coupled ICL repair by HR.

According to another line of thinking, one of the principal functions of FANCD2 is to channel DSBs away from error-prone NHEJ into error-free HR. Accordingly, the concurrent disruption of FANCC and KU70 suppressed crosslinker sensitivity, reduced chromosomal breaks and reversed defective HR in chicken DT40 cells [107]. Similarly, the DNA repair defects of *C. elegans* FANCD2 mutants can be rescued by simultaneously eliminating the NHEJ pathway, leading to the conclusion that FA-defective phenotype may be consequence of promiscuous end-joining reactions catalysed by NHEJ [108]. However, similar results were not obtained in mice, where the depletion of KU or 53BP1, another NHEJ factor, exacerbated genomic instability in cells lacking FANCD2 [60]. Regardless of whether NHEJ inhibition is the sole function of the FANCD2 monoubiquitination or not, at the least, it can be concluded that channelling DSBs away from toxic NHEJ into error-free HR is one of the important downstream outputs of an activated FA pathway.

### 2.12. Endogenous sources of DNA interstrand crosslinks

The DNA repair defects associated with the FA pathway have been studied *in vitro* mostly after the exogenous addition of crosslinking agents like cisplatin or mitomycin C. However, FA patients show their disease manifestations without evidence for prior exposure to exogenous crosslinkers. This indicates that human cells are inadvertently exposed to endogenous genotoxic agents and the FA pathway preserves the genomic integrity of cells in the presence of such stresses.

In recent studies, reactive aldehydes have emerged as one of the endogenous agents capable of generating lesions that have to be repaired by the FA pathway. One such example is acetaldehyde, an intermediate by-product of alcohol metabolism. Acetaldehyde is enzymatically catabolised into acetate by the acetaldehyde dehydrogenase, ALDH2. In the absence of ALDH2, acetaldehyde accumulates and binds DNA to form N2-ethylidene-dG adducts. In Aldh2-knockout mice that have been exposed to 8% ethanol for 14 months, increased genotoxic stress in the form of N(2)-ethylidene-dG DNA adduct accumulation is evident in the oesophagus, tongue and submandibular gland [109-111]. Furthermore, epidemiological studies have demonstrated a strong correlation between long-term drinking and a predisposition for oral and esophageal cancers in people bearing the ALDH2*2* polymorphism (a dominant negative isoform of ALDH2).

To understand whether the FA pathway protects cells against the genotoxic effects of aldehydes, mice double-deficient for ALDH2 and FANCD2 were generated. Aldh2 and Fancd2 double knock-out mice exhibited lethality at the embryonic stage. However, when these embryos were transferred into ALDH2-catabolism efficient mothers (*Aldh2+/−*), the mice were born to term, but had developmental defects and leukemic predisposition. Aged-double mutant *Aldh2-/-Fancd2-/-* mice that did not develop leukemia spontaneously developed aplastic anemia, another characteristic feature of human FA patients, together with a drastic 600-fold depletion in haematopoietic stem cell pools [112]. Similar results were obtained in
chicken DT-40 cells, where ADH5 (formaldehyde-catabolising enzyme) and FA pathway factors exhibited a synthetic lethal genetic interaction [113]. The developmental phenotypes and embryonic lethality associated with Aldh2-/-Fancd2-/- embryos could be rescued by transferring such embryos into aldehyde catabolism-efficient wild-type mothers. This suggested that maternal aldehydes emanating from ALDH2-deficient mothers can be transferred via the placenta into the growing foetus resulting in embryonic lethality [114]. When such ‘rescued’ Aldh2-/- Fancd2-/- neonates are analysed after birth, they still had severely depleted hematopoietic stem cell pools. This indicated that both fetal and maternal aldehyde detoxification systems are important to counteract the genotoxic effects of aldehydes in the growing embryo. Together, the above studies provided a glimpse on how the repair of aldehyde-mediated damage of the haematopoietic stem cell pool might be one of the underlying functions the FA pathway and can explain the haematopoietic defects of human FA patients.

3. NON-ICL repair function of the FA pathway

It has recently emerged that the FA pathway can also function in an ICL-repair independent manner for the maintenance of genomic integrity. In this section, we will discuss how the FA proteins participate in alternative genome-maintenance pathways.

One of the main ICL repair-independent functions of FANCD2 is that it co-operates with BRCA2 and RAD51 to protect stalled-replication forks from nucleolytic degradation [115]. FANCD2 also co-ordinates the re-start of stalled-replication forks in concert with the BLM helicase and recruits the FAN1 nuclease to promote the re-start of forks [116, 117]. Consistently, FA proteins FANCI and FANCD2 localize to stalled-replication forks in mass spectrometric studies [118]. Apart from FANCD2, other FA proteins FANCM, FAAP24, MHF1 and MHF2 were also found to play an important role in stabilizing stalled replication forks. The ATPase-dependent FANCM translocase activity is needed for replication fork stability. Cells expressing translocase-deficient FANCM showed altered global replication dynamics and stalled replication forks that result in the formation of spontaneous DSBs and 53BP1-marked nuclear bodies called as 53BP1-OPT domains in the G1 phase of cell cycle [119]. Similarly, loss of Fancc exacerbated genomic instability by impairing fork progression during DNA replication in a tumor-prone mouse model that had ~60% loss of dormant origins [120].

FANCI and FANCD2 also bind indirectly to minichromosome maintenance (MCM) proteins that are present in nascent DNA after replication arrest. FANCD2 was found essential for cells to restrain DNA synthesis in the presence of reduced pool of nucleotides. In an ATR-dependent but monoubiquitination-independent way, FANCD2 is required for general replisome surveillance mechanisms [121].

FA proteins may also promote genomic integrity in a transcription-dependent manner. For instance, Fancd2-deficient mice are susceptible to squamous cell carcinomas of the skin in response to Ras oncogene induction. Ub-FANCD2 activates the transcription of a tumor suppressor TAp63 to prevent skin carcinogenesis [122]. FANCD2 also interacts with NFκB in
the TNF-alpha promoter region. Defective FANCD2 causes the activation of TNF-alpha and the production of inflammatory cytokines [123]. Inflammatory cytokines, in turn, are an important source of reactive oxygen species to increase the DNA damage exposure of FA-defective cells.

The FA pathway is also required for maintaining the genomic integrity of B cells during class switch recombination (CSR). FANCA is required for the induction of transition mutations at A/T residues during somatic hypermutation and to prevent short-range recombination downstream of DSB formation during CSR [124].

The FA pathway co-operates with BLM to maintain genomic integrity during mitotic progression. FA pathway-dependent BLM targeting to non-centromeric abnormal structures induced by replication stress has an important role in mitotic progression because it prevents micronucleation and reduces aneuploidy in daughter cells [125]. FA-pathway deficient cells also express a higher number of UBFs (ultra-fine bridges) as compared to wild-type cells. This was attributed to the higher rate of cytokinesis failure in FA-impaired cells resulting in binucleated cells [126] (Figure. 3).

4. The Fanconi anemia pathway as an anti-cancer barrier

In the last section of this chapter, we will examine how the FA proteins suppress tumorigenesis. There are three lines of evidence linking FA pathway disruption and human cancer. Firstly, FA patients have a heightened risk for developing leukemias and solid tumors in their life time as compared to the general population. Secondly, mouse models deficient for FA genes spontaneously develop tumors. Lastly, FA gene mutations have been uncovered from cases of human sporadic cancers, suggesting that they could be drivers of genomic instability in human cancers.
4.1. Cancer Incidence in FA patients

Due to variability in clinical manifestations between FA patients, phenotypes such as BMF, haematological malignancies and solid tumors were used to subdivide patients. In general, anywhere between 20%-80% of FA patients experience BMF. About ~30% of FA patients get cancers, although this number can vary depending on the patient’s risk for getting BMF. For example, the high-BMF risk category have a lower chance of getting cancers because they may not live long enough, whereas the patients in the lowest BMF risk group were likely to live long enough and get leukemias or solid tumors [127]. The risk for getting all types of cancers including leukemia and solid tumors is 50-fold higher in the FA population as compared to the non-FA population [128]. In a literature survey published in 2003, cancer incidence was measured in 1300 FA patients for the years in between 1927-2001 [129]. While the median age for cancer development was ~68 years in the normal population, it significantly dropped to a median age of only 16 years for the FA population. Of the patients who developed tumors, about 60% had tumors of haematological origin and 40% were solid tumors. The risk for getting leukemia in the FA population is 800-fold higher than the general population [130, 131]. Haematological cancers, in particular, acute myeloid leukemia and myelodysplastic syndrome accounted for ~50% of cancers in FA patients by age 40. FA-related leukemias were myeloid in 94% of the cases and only 6% of the leukemias were lymphoid in nature. This pattern strongly differed from spontaneous leukemias, where ~84% were lymphoid in nature.

Amongst the solid tumors, ~50% were squamous cell carcinomas of the head and neck, anogenital region, skin, vulvar region, oesophagus and cervix and 10% were liver tumors. When compared to the general population, the risk is for getting all solid tumors is 48-fold higher in the FA population and in the range of 100-1000 fold higher for head and neck cancers and for cancers of the esophagus, liver, vulva, and cervix [130]. Solid tumors had a slower incidence at younger age, but the probability of getting a solid tumor dramatically increased to 76% by age 45, suggesting that older FA patients had a significantly higher risk for developing solid tumors.

It is noteworthy that there is considerable variability between FA patients with respect to the spectrum of cancers manifested and the age of cancer-onset. In general, mutations in FA core proteins result in a milder cancer phenotype as compared to mutations in downstream genes. For example, FA patients belonging to the FANCD1 (BRCA2) subgroup have a much higher risk for an earlier onset of malignancies. The cumulative probability of getting any malignancy such as leukemia or solid tumors in a FANCD1 subtype patient was ~97% by age 5.2 years, indicating that amongst FA patients, FANCD1 subtype was the most severe with respect to their risk for developing malignancies [132, 133]. Similarly, patients of the subtype FANCD1, FANCO and FANCI show greater predisposition for developing breast cancers, whereas patients of the subtype FANCO are likely to get ovarian cancers. In summary, cancer spectrum and the age of cancer-onset are reflective of the distinct roles played by the FA proteins during ICL repair.
4.2. FA pathway disruption predisposes knockout-mice to cancers

Immediately following the discoveries of mutations responsible for human FA, a number of groups generated mice defective for FA proteins. The first mouse models to be generated were the *Fancc*-/— mice and *Fanca*-/— mice (exons 4-7 deletion). These mice had normal viability and no developmental defects, but impaired fertility. *Fancc*-/— and *Fanca*-/— cells had characteristic crosslinker sensitivity and exhibited several-fold higher accumulation of chromosomal aberrations after treatment with crosslinking agents [134, 135]. Another mouse model for Fanca bore deletions in exons 1-6 and these mice showed more severe phenotypes like prenatal growth retardation, craniofacial abnormalities, and crosslinker sensitivity which were all typical features of human FA patients [136]. *Fancg*-/— mice also showed hypogonadism, impaired fertility and cells isolated from these mice had an accumulation of spontaneous chromosomal aberrations, increased sensitivity to mitomycin C [137, 138]. Together, the characteristics of *Fancc*-/—, *Fanca*-/— and *Fancg*-/— mice strongly resembled each other, supporting the premise that they function in a common pathway. Yet, phenotypes evident in human FA patients such as typical haematological manifestations and increased risk for spontaneous cancers could not be recapitulated in these mouse models. It was also hypothesised that the benign environment in typical mouse facilities did not provide sufficient exogenous stress to precipitate FA phenotypes. Hence, *Facc*-/— knockout mice were challenged with clastogens such as mitomycin C. Upon crosslinker exposure, *Facc*-/— mice showed progressive pancytopenia and died within a few days [139]. Interestingly, double-deficient mice defective in Fancc and Fancgg, in C57BL/6J background started displaying haematological manifestations seen in FA patients, such as BMF, acute myeloid leukemia (AML), MDS, and complex chromosomal rearrangements that were not seen in the single KO mice. Hence, this mouse model was considered a close mimic of human FA, especially with respect to onset of haematological malignancies [140].

*Fancd2*-/— mice are viable, but show prenatal and post-natal growth retardation. The severity of the phenotypes in *Fancd2*-/— mice is mouse strain-dependent and mice generated in the C57BL/6J background had more severe phenotypes that those in 129S4 background. *Fancd2*-/— mice shared phenotypes common with *Fanca*-/—, *Fancc*-/— and *Fancg*-/— mice such as impaired fertility, increased chromosomal aberrations and sensitivity to MMC. In addition, *Fancd2*-/— mice also exhibited unique features such as microphthalmia and perinatal lethality. *Fancd2*-/— mice were particularly tumor-prone, and showed epithelial cancers in several tissues that eventually caused their mortality. The tumors found in *Fancd2*-/— mice included ovarian cancers, hepatic adenoma and adenocarcinoma, gastric cancers, mammary B cell lymphoma, lung adenocarcinoma and bronchoalveolar carcinoma [141]. Tumor formation was further accelerated when *Fancd2*-/— mice were crossed with *p53*+/— mice. The tumor spectrum found in the double mutant mice included mammary and lung adenocarcinomas, cancers rarely seen in the *p53*+/— heterozygotes [142]. More recently, mice double-deficient for Fancd2 and Aldh2 were generated. Due to aldehyde-mediated genotoxicity, such mice showed leukemic predisposition and BMF [143]. The above studies reiterate that FA phenotypes are strongly influenced by the type and extent of genotoxic stress exposure. Only when cells accumulate
unrepaired crosslinks beyond a certain threshold, FA phenotypes such as developmental abnormalities, BMF, haematological malignancies and solid tumors are manifested.

Another example, where the absence of FA core proteins results in increased cancer incidence are *Fancf*-/-/ mice and *Fancm*-/-/ mice. *Fancf*-/-/ mice showed increased incidence of ovarian cancers whereas *Fancm*-/-/ mice showed tumors such as lymphomas, histiocytic sarcoma, hepatoma and lung carcinomas [144, 145].

Apart from core FA proteins, mouse models were also generated for the downstream proteins involved in the unhooking step or in HR. Mice deficient for SLX4 recapitulated several key features of FA. These mice were born at submendelian ratios, had fertility defects and were prone to blood cytopenias. *Slx4*-/-/ cells show increased chromosomal aberrations and crosslinker sensitivity [72]. Slx4-deficient mice also developed epithelial cancers and died by 100 weeks with haematological cancers and solid tumors such as squamous cell carcinomas, hepatocellular carcinoma and ovarian tumors. Thus, SLX4 deficiency closely mimicked the clinical features of human FA.

According to human genetic data, the heterozygosity of HR proteins cause increased susceptibility to breast, ovarian and other cancers whereas biallelic mutations in HR genes cause FA. A homozygous deletion of HR genes, however, results in an embryonic lethal phenotype. Consistently, mice bearing homozygous deletion of repair proteins involved in HR such as BRCA2, PALB2 and Rad51C show embryonic lethality with gross chromosomal aberrations [146, 147]. But, when hypomorphic strains were derived for these mice, an FA phenotype could be observed. For example, mouse expressing a hypomorphic *Palb2* allele showed reduced fertility, MMC hypersensitivity and chromosomal breakage when treated with mitomycin C [148]. Similarly, thymic lymphomas were observed in mice expressing a truncated form of BRCA2 [149]. Homozygous deletion of exon 27 of *Brca2* prevents its interaction with RAD51. Hence, mice carrying homozygous deletion of exon 27 of *Brca2* were generated and several phenotypes in these mice recapitulated phenotypes observed in human FA patients of the complementation group FANCD1. *Brca2*Δ27/Δ27 mice showed haematological defects such as compromised progenitor cell function, reduced hematopoietic stem cell self renewal, spontaneous accumulation of chromosomal aberrations and sensitivity to crosslinking agents. In addition, *Brca2*Δ27/Δ27 mice were more prone to epithelial tumors such as squamous cell carcinomas of gastric origin and mammary gland, endometrial cancers, sarcomas and lung cancers [150, 151].

### 4.3. Fanconi anemia pathway in human sporadic cancer

The discovery that bi-allelic mutations in BRCA2 manifests as FA was a major conceptual advance because it linked FA with a gene responsible for familial cancers. FA phenotypes such as cancer predisposition could now be directly linked to HR and DNA repair [82, 152]. However, only about 25% of human hereditary cases of breast cancer are owing to BRCA1 or BRCA2 mutations. Therefore, it was hypothesized that mutations in BRCA1-related FA pathway genes such as FANCJ, FANCN and FANCO could be responsible for BRCA1/BRCA2-negative breast and ovarian human cancers. Amongst these, there is strong evidence that germ-
line mutations in BRIP1 increase the susceptibility to familial ovarian cancer [153]. Similarly, germ-line mutations in FANCO (RAD51C) increase susceptibility to ovarian cancer and breast cancer [154-157]. PALB2 (FANCN) mutations have been uncovered in 1-4% of inherited breast cancer patients [158, 159]. Polymorphic FANCA mutations were also detected in high-risk non-BRCA1/BRCA2 breast cancer patients in a French Canadian population [160]. FANCM has also emerged as a breast cancer susceptibility gene in a Finish population and FANCM mutations confer strong predisposition for triple-negative breast cancers [161]. PALB2, FANCC and FANCG mutations have also been associated with increased risk for pancreatic cancer [162-164]. In summary, a small but significant proportion of familial cancers are driven by mutational inactivation of FA genes.

There is also accumulating evidence that genes of the FA pathway may undergo inactivation by epigenetic silencing in some sporadic human cancers. FANCF promoter methylation and inactivation was observed in lung and oral cancers and promoter methylation correlated with smoking and alcohol use [165]. FANCF promoter methylation and inactivation was also observed in ovarian cancer patients [166]. Epigenetic inactivation of FANCA was observed in non-small cell lung cancer due to microRNA miR-503 dependent de-regulation [167]. An early-stage inactivation of FANCC due to promoter methylation was observed in dysplastic lesions of the head and neck [168]. FANCA and BRCA2 promoters were recurrently methylated in laryngeal squamous cell carcinoma [169]. FANCC and FANCL promoters were hypermethylated in sporadic acute leukemia [170]. PALB2 promoter was methylated in sporadic breast and ovarian cancer [171], FANCF promoter was methylated in sporadic breast cancer [172] and ovarian cancer [173, 174] cervical cancer [175] and in AML [176]. In an unknown manner, FANCD2 expression was reduced in human uveal melanoma and influenced spontaneous sister chromatid exchange and RAD51 focus formation [177]. Cancer-associated alterations in the form of deletions were recovered for FANCC in breast cancers [178]. Put together, the inactivation of FA genes might be a common feature in several sporadic cancers.

Lastly, cancer genomics has enabled the analysis of the cancer genome in a high-throughput manner. When cancer genomes are queried for mutational inactivation of the 16 FA genes (FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCD1, FANCN, FANCO, FANCP, FANCO), nearly 25-30% of solid tumors show a mutational inactivation of one of genes (http://www.cbioportal.org/public-portal, [179, 180] (Figure. 4).

Given that an intact FA pathway is required for the successful repair of ICLs, the inactivation of any of the FA genes can potentially lead to genomic instability during cancer progression. At the same time, genomic instability due to disrupted FA pathway can be exploited for therapy because such cancers by targeted by chemotherapeutics known to cause DNA interstrand crosslinking like Cisplatin. Further functional characterisation of cancer-associated FA mutations is required to provide clearer knowledge on the role of the FA pathway in sporadic human cancer.
5. Conclusion

Even though FA was described several decades ago, a thorough knowledge on the functional aspects this pathway has emerged only in the last 15 years. Mechanistic studies have revealed that FA proteins orchestrate the complex process of ICL repair. Unrepaired crosslinks are a major source of genomic instability and responsible for several FA patient phenotypes. But, according to recent studies, FA proteins may also fulfil DNA repair-independent roles to maintain genomic integrity. As even more proteins are being ascertained as accessory factors of this pathway, the study of FA is becoming complex, but exciting.

The most serious complication for FA patients at a young age is aplastic anemia, but, this haematological complication can be mostly treated by stem cell transplantation. An even greater concern for FA patients is their risk for getting leukemias and solid tumors at an older age. The observation that reactive aldehydes are a major source of ICLs has given hope to FA patients that reducing aldehydes levels can perhaps reduce cancer incidence. If environmental or metabolic sources of ICL agents are discovered, then such risk factors can be mitigated to reduce cancer burden in FA patients.

We now know that an intact FA pathway and enzymatic detoxification of aldehydes act together to provide a double-tier protection against crosslinking lesions. Given that ~500 million people world-wide have polymorphisms that make their aldehyde-detoxification pathways inefficient, studying the FA pathway is likely to gain more importance from a human health perspective. Importantly, several known human carcinogens like tobacco smoke, nitrosamines from diet and alcohol can result in potentially genotoxic interstrand crosslinking lesions within cells.
Indeed, the likelihood for getting sporadic human cancer has increased at an alarming rate over the past few decades. Given that the FA pathway lies at the interface of genome maintenance and human cancer, further studies on this pathway can lead to novel strategies for cancer prevention.

**Abbreviations**

Acute myeloid leukemia (AML)
Ataxia telangectasia-mutated related (ATR)
Bone marrow failure (BMF)
Double strand break (DSB)
Double-stranded DNA (dsDNA)
Fanconi Anemia (FA)
Homologous recombination (HR)
Interstrand crosslink (ICL)
Monoubiquitinated FANCD2 (Ub-FANCD2)
Myelodysplastic syndrome (MDS)
Non-homologous end joining (NHEJ)
Single-stranded DNA (ssDNA)
Translesion synthesis (TLS)

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