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Review

Causes and consequences of chromosomal instability in Fanconi anemia. Alterations at the cellular and organism level.

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Abstract: Fanconi anemia (FA), a chromosome instability syndrome, is caused by inherited pathogenic variants in any of 22 FANC genes, that cooperate in the FA/BRCA pathway. This pathway regulates the repair of DNA interstrand crosslinks (ICLs) through homologous recombination. In FA proper repair of ICLs is impaired, and accumulation of toxic DNA double strand breaks occurs. In order to repair this type of DNA damage, FA cells activate alternative error-prone DNA repair pathways, that may lead to the formation of gross structural chromosome aberrations of which radial figures are the epitome and origin of subsequent aberrations like translocations, dicentrics and acentric fragments.

The deficiency in DNA repair has pleiotropic consequences in the phenotype of patients with FA, including developmental alterations, bone marrow failure and an extreme risk to develop cancer. The mechanisms leading to the physical abnormalities during embryonic development have not been clearly elucidated, however FA has features of premature aging with chronic inflammation mediated by pro-inflammatory cytokines, that results in tissue attrition, selection of malignant clones and cancer onset. Moreover, the effect of the FA/BRCA pathway in germinal cells, evidenced by infertility in patients with FA attests of chromosomal instability and cell death also occurring in the germinal compartment.

Keywords: Chromosomal instability; FA pathway; Radial figures; TGFβ pathway; MYC; p53; Bone marrow failure; cancer; physical abnormalities; infertility.

1. Introduction

Fanconi anemia (FA) is a rare disease with an incidence of 1-5 per million of births and is the most commonly inherited bone marrow failure syndrome [1]. FA is caused by the failure of the Fanconi anemia/breast cancer (FA/BRCA) pathway [2], so far 22 genes called *FANCA* to *FANCW* participate in this pathway, germline pathogenic variants in any one of these genes are the origin of this disease [3]. Pathogenic variants show an autosomal recessive inheritance for 20 of these genes, autosomal dominant inheritance has been shown for one gene (FANCR/RAD51), and X-linked inheritance for another gene (*FANCB*). Inherited pathogenic variants (PV) in the *FANCA*, *FANCC*, or *FANCG* genes account for approximately 90% of FA cases, whereas the other 19 FANC genes account for 10% of the remaining cases [3]. FA/BRCA pathway is involved in the proper functioning of various cellular processes, one of its most important functions is during the repair of DNA interstrand crosslink (ICL), lesions that covalently join the two DNA strands and impair DNA replication and

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transcription. Failure of the FA/BRCA pathway has consequences at various levels of complexity, 1) at the chromosomal level by the presence of numerical and structural chromosomal instability; 2) at the cellular level there is an increased cell death, alteration of the cell cycle, high sensitivity to oxidative damage and to DNA cross-linking agents, both exogenous such as chemotherapeutic drugs like Cis-platinum, Mitomycin C or Diepoxybutane, as well as endogenous aldehydes product of cell metabolic activities [4,5]; 3) at the clinical level, patients with FA present three typical characteristics: developmental disorders, bone marrow failure and an increased risk of cancer [6].

2. Basic defect of Fanconi anemia and its consequences at the chromosomal level.

2.1 Involvement of FA/BRCA pathway in DNA repair.

The protein product of the FANC genes collaborate in the FA/BRCA pathway that is in charge of DNA replication fork protection and ICLs repair [1]. ICLs are dangerous lesions that prevent the opening of the double stranded DNA for transcription and replication. For ICLs repair the cell needs to call several DNA repair converging mechanisms, and the FA/BRCA pathway works as a staging that coordinates the progression of the repair process, resulting most of the time in error-free repair. FANC proteins individual functions during this assemblage appear in Table 1.

Table 1. Fanconi anemia genes involved in FA/BRCA pathway [1]

FANC Gene/Alias	Cytogenetic Location	Function of the FANC protein
FANCA	16q24.3	FA core complex
FANCB	Xp22.2	FA core complex
FANCC	9q22.32	FA core complex
FANCD1/BRCA2	13q13.1	Homologous recombination. Enable RAD51 to displace RPA from ssDNA.
FANCD2	3p25.3	Monoubiquitylated complex ID recruits the downstream repair proteins and facilitates repair of DNA interstrand crosslinks
FANCE	6p21.31	FA core complex; bridge between the FA core complex and FANCD2
FANCF	11p14.3	FA core complex
FANCG/XRCC9	9p13.3	FA core complex
FANCI	15q26.1	Monoubiquitylated complex ID recruits the downstream repair proteins and facilitates repair of DNA interstrand crosslinks
FANCJ/BRIP1	17q23.2	FA core complex
FANCL	2p16.1	E3 ubiquitin-protein ligase, monoubiquitination of FANCD2
[2]*FANCM*	14q21.2	FA core complex. Acts by sensing stalled fork by ICLs and recruiting the core complex proteins to the site of ICL
FANCN/ PALB2	16q12.2	Homologous recombination
[2]*FANCO/RAD51C*	17q22	Resolution of D-loop Structures through Holliday Junction Intermediates and Homologous DNA Pairing and Strand Exchange.
FANCP/ SLX4	16p13.3	Cooperate with FANCQ-XPF to generate endonucleolitic incisions to unhook the ICL.
FANCQ/ XPF	16p13.12	DNA endonuclease, involved in homologous recombination; responsible of 5′ incision for removing ICLs
[2]*FANCR/RAD51*	15q15.1	Interact with the ssDNA-binding protein RPA and RAD52 homologous; pairing and strand transfer of DNA
[2]*FANCS/BRCA1*	17q21.31	Homologous recombination
FANCT/UB2T	1q32.1	E2 ubiquitin-conjugating enzyme, associates with FA core complex, with FANCL, catalyze monoubiquitination of FANCD2
FANCU/ XRCC2	7q36.1	Homologous recombination
FANCV/REV7	1p36.22	Translesion DNA synthesis

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FANCW/RFWD3	16q23.1	RING-Type E3 Ubiquitin Transferase

[1]Cytogenetic location was obtained from OMIM. [2]Can be called Fanconi anemia-like genes, mainly due to the absence of bone marrow failure in the patients [2,7,8]

The FA/BRCA pathway is activated for repairing ICLs during the S phase of the cell cycle, when the replisome finds an ICL and two convergent replication forks become stalled [9]. The ICL repair process can be divided in modules of activity of the FA/BRCA pathway [10] (Figure 1).

1) FANCM and its interacting partners FAAP24, MHF1and MHF2 [3], detect the lesion on the DNA when two replication forks converge in an ICL [9]. Replisome complexes are unloaded rendering stalled replication forks with single stranded DNA (ssDNA) regions that are covered by Replication Protein A (RPA), this leads to activation of the ATR/CHK1 signaling that triggers DNA damage checkpoints [11]. The best described scenario for triggering ICL repair, implicates the convergence of two replication forks at the ICL site [9], the leading strand on one side of the ICL stops 20-40 nucleotides before the ICL, then the CMG helicase is removed from the stalled fork, aided by the ubiquitin E3 ligase TRAIP, ATPase p97 and FANCS/BRCA1 protein, the fork advances to nucleotide 1 respect to ICL and waits for the opposite fork to reach the ICL in a similar manner. Once FANCM and its interacting partners are in close proximity of the ICL, their key function is to recruit the members of the next module, the FA core complex, to the chromatin [12].

2) The FA core complex best described function is as an E3-ubiquitin ligase that is integrated by the proteins FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FAAP100, FAAP20, FAAP24 and FANCT. Three subcomplexes can be recognized in the core complex, *a)* the FANCB-FANCL-FAAP100 (BL100) important for the integration of all components of the FA core complex; *b)* the FANCA-FANCG-FAAP20 (AG20) subcomplex important for the nuclear localization of the entire multimer; and *c)* the FANCC-FANCE-FANCF (CEF) subcomplex, in charge of bridging the FA core complex with the members of the module 3 (its target), the FANCI-FANCD2 (ID2) complex [3]. Once assembled, the entire FA core complex exerts its E3-ubiquitin ligase activity, and through its FANCT subunit, adds a ubiquitin group to FANCI at lysine 523 and to FANCD2 at lysine 561.

3) The FANCD2-FANCI heterodimer, frequently called the central complex, is recruited to the stalled fork, where FANCI is tri-phosphorylated by the ATR-kinase [13], stimulating the FA core complex mediated monoubiquitination of both FANCI and FANCD2. The tri-phosphorylation of FANCI also inhibits the deubiquitinase activity of the USP1-UAF1 complex over the ID2 complex until ICL repair and replication are completed [13]. The ubiquitinated ID2 complex protects the replication forks and regulates the activity of the proteins involved in the processing of the ICL, enabling the recruitment of the proteins of the fourth module.

4) Proteins acting downstream in the FA/BRCA pathway include BRCA2/FANCD1, BRIP1/FANCJ, PALB2/FANCN, RAD51C/FANCO, RAD51/FANCR, BRCA1/FANCS, XRCC2/FANCU, XPF/FANCQ, SLX4/FANCP, REV7/FANCV and RFWD3/FANCW, all of them are committed to remove the ICL and maintain genomic integrity through various types of DNA repair. The ubiquitinated ID2 complex recruits the FANCP/SLX4 scaffold protein, which in turn coordinates the endonucleolytic activity of FANCQ/XPF, this endonuclease will make DNA incisions on both sides of the ICL and will unhook it [14]. After ICL unhooking, three different types of lesions are generated: a single strand break (SSB) and an adduct in one of the chromatids, while in the sister chromatid a double strand break (DSB) is generated, where the endonucleases made the incisions to unhook the ICL (Figure 1). All these lesions are repaired by different DNA repair pathways that act coordinated by the FA/BRCA pathway.

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The SSB is repaired by translesion synthesis (TLS), through polymerases REV1 and the polymerase ζ complex (REV7/FANCV-REV3), an error-prone polymerase that uses as template the complementary strand with the adduct; while this allows the replication progress, the low fidelity of this polymerase can introduce errors in the nucleotide sequence [15]; this polymerase is necessary for ICL repair as part of the FA/BRCA pathway, indeed, its recruitment to ICL repair intermediates is performed by ubiquitinated PCNA and FA core complex [16]. The unhooked adduct in the opposite strand is repaired by nucleotide excision repair (NER); FANCQ/XPF protein participate in both, FA/BRCA and NER pathways, however FA patients with mutations in this gene, do not share the phenotype with Xeroderma Pigmentosum patients, making evident that is a multitask protein [17] During ICL repair FANCQ/XPF make the incisions around the ICL and NER protein XPV is recruited by FANCD2; FA proteins FANCM and FANCT have been implicated in the regulation of NER, these data show the crosstalk between FA/BRCA and NER pathways in the ICL repair [7].

Finally, the ICL-associated DSB is processed by the proteins downstream of the FA/BRCA pathway (FANCD1/BRCA2, FANCN/PALB2, FANCS/BRCA1, FANCJ/BRIP1, FANCO/RAD51C, FANCR/RAD51) this set of proteins are recruited by ubiquitinated ID2 complex and perform an homology-directed repair, using the recently restored sister chromatid to perform the error-free homologous recombination (HR) repair; when the lesion is repaired the cell is able to continue the cell cycle. Interestingly, some of the downstream proteins have also recently being shown to have functions upstream of the ubiquitinated FANCI-FANCD2 complex. For example, FANCS/BRCA1 is required for positioning FANCD2 at the ICL site, whereas FANCD1/BRCA2 and FANCJ promote the FANCD2 chromatin localization [7], importantly, heterozygous for pathogenic variants in genes of module 4 are at high risk for developing breast and ovarian cancer [2,18].

5) After ICLs repair the activity of the replication fork is restarted, the finisher module includes the deubiquitination of FANCD2/FANCI activated complex, leading to the re-start of the DNA synthesis by the canonical DNA polymerases. The deubiquitination is performed by the USP1-UAF1 complex resulting in the release of the ID2 complex from the chromatin to finish the ICL repair cycle [10].

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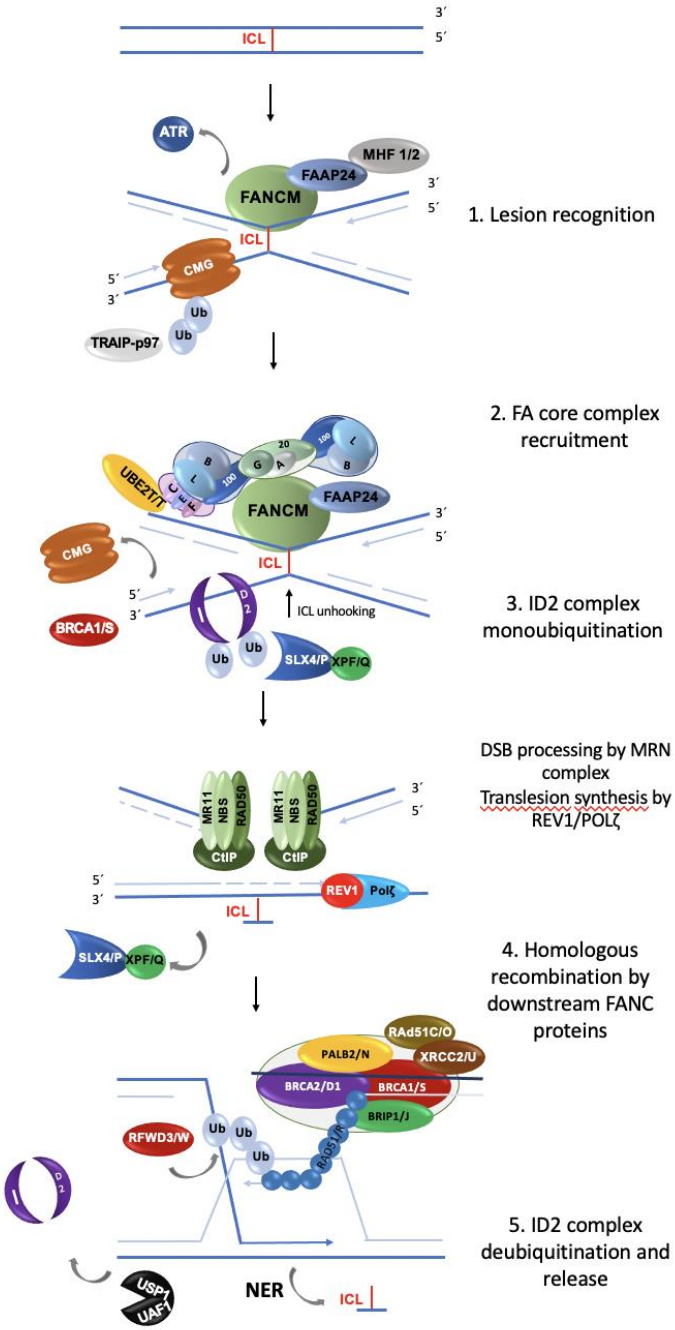

Figure 1. Summary of the activity of FANC proteins in the FA/BRCA pathway. The main function of this pathway is the removal of DNA interstrand crosslink (ICL), 22 FANC proteins participate in: 1. **Lesion recognition**. FANCM and its partners recognize ICLs during the convergence of two replication forks and promote ATR activation; the CMG helicase complex is unloaded to allow the approach of the leading strands to the ICL. 2. **FA core complex recruitment**. FANCM and its partners recruit the FA core complex and UBE2T/FANCT (the "upstream" proteins), to exert their E3-ubiquitin ligase activity and monoubiquitinate the FANCI and FANCD2 heterodimer (also known as the "ID2 or central complex"). 3. **ID2 complex monoubiquitination**. The monoubiquitinated central complex activates the endonucleolitic function of FANCP-SLX4-FANCQ/XPF resulting in the unhooking of the ICL from one of the DNA strands and the generation of a DSB. Both DNA ends of the DSB are processed by the MRN/CtIP complex to form a 3′ overhang. In the opposite strand, the unhooked ICL has now become an adduct; in order to bypass it, the REV1- polymerase ζ complex (including FANCV protein) performs translesion synthesis of the new strand. 4**. The processed DSB is repaired by homologous recombination**. The "downstream" FANCD1/BRCA2, FANCN/PALB2, FANCS/BRCA1, FANCJ/BRIP1, FANCO/RAD51C, FANCR/RAD51 proteins coordinate to coat the processed DNA strand of the DSB with RAD51/FANCR and paralogs RAD51C/FANCO, to invade the newly polymerase ζ-synthesized double strand of its sister chromatid, using it as a template to recover the

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original nucleotide sequence. 5. The cycle finishes with the **deubiquitination and unloading of the ID2 complex** by USP1-UAF1, and the removal of the ICL-adduct by the NER (nucleotide excision repair) pathway.

2.2. Repair of Double Strand Breaks

DSBs are generated as a byproduct during the processing of ICL by the FA/BRCA pathway. DSBs are considered one of the most toxic lesions for the cells misrepair may originate mutations and chromosomal abnormalities that may lead to cell death or tumorigenesis, therefore the accurate repair of this type of lesion is essential for maintaining genomic stability and cell viability.

Processing and repair of DSBs depends on several factors, such as the phase of the cell cycle in which the damage occurs, their origin (associated to replication fork stalling or replication-independent), and the number of DSB events in the same cell, among others. The two major repair mechanisms for DSBs are HR and non-homologous end joining (NHEJ), for the later a canonical NHEJ (cNHEJ), as well as an alternative pathway, also called microhomology-mediated end joining (MMEJ), have been described (Table 2). Homologous recombination uses an intact homologous sequence as a template for the repair of DSBs, for this reason HR is carried out during the post-replicative period of the cell cycle, which includes the S and G2 phases, when a sister chromatid is available; the free DNA ends have to search for the homologous sequences, thus requiring extensive DNA resection and processing. On the contrary, the ligation of non-homologous ends performed by NHEJ requires minimal or no sequence homology and allows ligation of DNA ends with minimal processing.

In normal cells, NHEJ efficiently binds the correct ends of DNA without the formation of chromosomal aberrations, although the original DNA sequences flanking the DSB may not be exactly restored, due to small losses of nucleotides that occur during the DNA end-processing and are needed for successful NHEJ; however if multiple DSBs occur simultaneously, the activity of NHEJ, which can be independent of template and homology, may lead to ligation of wrong DNA ends and generate gross chromosomal rearrangements [19].

Table 2. Characteristics of the major double-strand break repair mechanisms [19–21]

	Non-homologous end-joining	**Microhomology mediated end-joining**	**Homologous recombination**
Timing	Fast	Fast	Slow
Template dependence	Independent	Independent	Dependent
Homology usage	0-4 bp	2-20 bp	>100 bp
End resection	no	yes	yes
Cell cycle phase	G1, S, G2	G1, S, G2	S-G2
Accuracy of repair	Mostly accurate, error prone	Frequently error prone	Highly accurate

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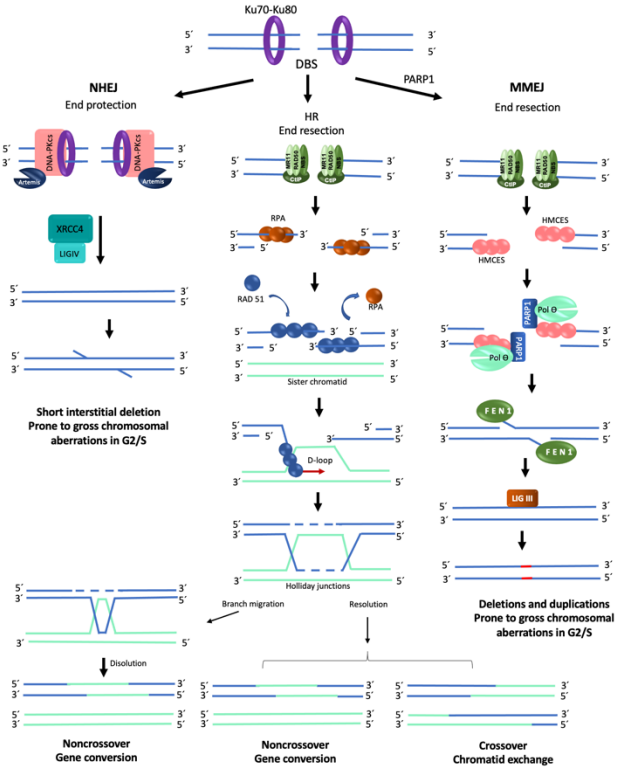

* RPA or HMCES (5-Hydroxymethylcytosine binding, embryonic stem cell-specific protein)[21,22]

Figure 2. Major mechanisms of double-strand break repair. NHEJ, Non-Homologous End Joining. DNA ends are protected by KU70/KU80, which prevents their end-processing. When the ends are incompatible, a segment of up to 4 nucleotides is located and Artemis eliminates the remaining incompatible segments, and ligation is carried out by LIGIV-XRCC4. This pathway can repair a DSB without chromosome modification, however during S / G2 and in the presence of several DSBs, it is considered prone to generate chromosomal alterations. **HR, Homologous Recombination**. This pathway is only available during the S/G2 phases of the cell cycle since it requires a homologous template; HR is the best choice to maintain sequence fidelity because in general it repairs in an error-free manner. An ssDNA 3' overhang is produced by the action of the MRN-CtIP complex; it is first covered by RPA proteins which are later replaced by RAD51 to form the nucleoprotein strand that will carry out the invasion of the DNA of the sister chromatid in order to use it like a template to restore the continuity of the original nucleotide sequence. **MMEJ, Microhomology-Mediated End Joining**. PARP1 prevents KU70/KU80 binding to DNA ends and allows the recruitment of MRN-CtIP to initiate end-resection creating a short 3' overhang. This overhang is preferentially covered by HMCES which channels the damage to be repaired by MMEJ instead to HR;. PARP1-POLQ search for microhomology of 2-20 bp and align the strands; resulting flaps are eliminated by XPF/FANCQ-FEN1. Alternatively POLQ can direct DNA synthesis to add nucleotides to make DNA ends compatible, this end processing generates in situ deletions and duplications. In addition, if this pathway is active during S/G2 and several DSBs coincide in time and space, gross chromosomal aberrations are formed, because, like NHEJ, they do not require long stretches of homology to ligate the DNA ends [21,23].

2.2.1 Homologous Recombination

In a normal cell, the FA/BRCA pathway continues "downstream" after the generation of a DSB, using the homologous recombination repair pathway to join the DNA ends in an error free manner.

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HR is restricted to S and G2 phases of the cell cycle, using the sister chromatid as template to recover the original nucleotide sequence [19] however, in any phase of the cell cycle, Ku70-Ku80 are abundant proteins that binds to the broken DNA ends and protects them [20]. Since DSBs in S-G2 phases are preferentially repaired by HR, the Ku70-Ku80 heterodimer is removed by proteins that will process the DNA ends. A first step in this processing is mediated by the MRN complex (MRE11–RAD50–NBS1), that aided by CtIP (CtBP-interacting protein) will introduce an endonucleolytic nick up to 300 bp away of the DSB site [19]. Next the 3’ to 5' MRN exonuclease activity extends the nick forming a 3 'overhang, this process finally ends up displacing proteins Ku70-Ku80 and elicits the entrance of the late DNA end resection proteins EXO1, BLM-DNA2 (exonuclease1, Bloom syndrome helicase-endonuclease2) [19,24]. These proteins facilitate unwinding of the DNA and digestion of the 5 'strand to lengthen the 3' overhang; this allows the entry of the RPA protein complex, to protect the single stranded DNA (Figure 2) [19].

The mediators of HR, FANCD1/BRCA2, FANCS/BRCA1, FANCN/PALB2 and FANCJ/BRIP1 act by displacing RPA from the single stranded DNA and loading of FANCR/RAD51 and its paralogs FANCO/ RAD51C and FANCU/XRCC2 into the ssDNA, this leads to the formation of a nucleoprotein filament with the capacity to invade the sister chromatid and search for homologous sequences for restoration of the original sequence interrupted by the DSB. This nucleofilament assists the base-pairing when the complementary sequences in the sister chromatid have been found (synapsis). Additional FANC proteins mediate the homology search , including FANCW/RFWD3 a ubiquitin E3 ligase that regulates the turnover of RPA by FANCR/RAD51, initially promoted by FANCS/BRCA1. Interestingly, FANCS/BRCA1 has also been shown to promote DNA end resection, RAD51 loading and collaborate in the homology search mediated by FANCR/RAD51, highlighting the multitask roles of FANCS/BRCA1 [19,24](Figures 2 and 3).

When the synapsis is stabilized, the dissociation of FANCR/RAD51 promotes DNA synthesis, a displacement-loop (D-loop) is formed that engages the DNA polymerase δ (Pol δ) [12,21,23,25] to incorporate nucleotides and synthesize new DNA for completing the homologous recombination process. The heteroduplex DNA, forms Holliday junctions, which are resolved by helicases and endonucleases that depending on its dissolution or resolution, conduces to restore the original sequence by gene conversion, or sister chromatid exchange, avoiding chromosomal translocations, as expected by an error-free repair [26,27] (Figure 2).

Importantly, HR preferentially uses the sister chromatid as a template due to its perfect homology and close proximity, though the use of the homologous chromosome is also possible, however this alternative is less efficient and is prone to generate regions of homozygosity in the next cell generation. Once the DSB is repaired, FANCD2-Ub have to be extracted from the lesion by the deubiquitinase complex USP1-UAF1 and p97 to finish HR repair[10,28].

Accompanying the processing of the DNA ends, ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia related) proteins have also been activated by MRN and RPA respectively, to regulate the DNA damage response of the cell. All known “downstream” FANC proteins have important functions in HR and some of them also in the control of the start and finish of the repair cycle, as well as in the choice of the repair mechanism to process the DSB repair [21].

2.2.2 Non-Homologous End Joining

NHEJ is the dominant pathway for the repair of DSBs in the human cells [20]. Processing of a DSB by NHEJ is notably different from HR. As observed in Table 2, the abundance and availability of its components throughout all the cell cycle, and the speed of DSB repair kinetics (15-30 minutes) explain the dominant role of cNHEJ in the preservation of the genome integrity. The initiation of the classical cNHEJ requires the union of the Ku70 / 80 heterodimer to the broken ends of the DNA, this protects the DNA from the exonuclease activity of proteins like MRN or EXO1.

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Ku70/Ku80 is also a platform for the recruitment of other DNA repair proteins, such as DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and its cofactor with exonuclease activity called Artemis. The Ku70/Ku80-DNA-PKcs complex makes a first synapse between the two DNA ends followed by a second synapse, a closer approach that is operated by the proteins DNA ligase IV (LIG4) , XRCC4/XLF, PAXX, DNA polymerases λ and μ, Aprataxin and PNK-like factor (APLF), all of them also in charge of performing the end processing by removing some nucleotides from the broken ends so that ligation can be carried out between either, blunt DNA ends or DNA ends with a very short resection that leads to small single stranded DNA overhangs (Figure 2a). This resection (≤4pb), although small, can change the information in the damaged site, explaining part of the errors associated with DNA repair by cNHEJ [19–21]. The NHEJ simplicity explains in part its high speed, enabling the re-ligation of DSBs shortly after they were formed. High speed in NHEJ partially compensates for the lack of homology usage, if there are no multiple DSBs, the proximity and topology of the two original DNA ends increases the probability of its re-ligation; however when more than two DNA ends coexist, the template-independent ligation that characterizes NHEJ increases the probability of generating gross chromosomal rearrangements [29].

2.2.3 Alternative End Joining (Microhomology Mediated End Joining).

When HR or NHEJ, the most widely used routes for DNA repair are not available, the MMEJ route is selected by the cell. The distinctive characteristic of MMEJ is the use of very short homologous sequences, 2-20 bp to elicit the re-joining of the two DNA ends. MMEJ is an error prone pathway with characteristics similar to the classical NHEJ that however includes DNA end processing. This mechanism starts with the DNA end resection using PARP1 (poly(ADP-ribose) polymerase 1), CtIP and the MRN complex that creates a 15–100-nucleotide 3′ overhang, this ssDNA is coated by proteins RPA or HMCES (5-Hydroxymethylcytosine binding, embryonic stem cell-specific protein) [22]; the ssDNA make a short displacement until it finds a microhomology up to 20 bp, then proteins HMCES are unloaded through the DNA polymerase θ (Pol θ)-associated helicase activity and the displaced 5′ ssDNA flaps are removed by FEN1 (flap endonuclease 1). When no homology is found, the Pol θ polymerase activity is turned on to add nucleotides to provide the necessary microhomology to stabilize the joint between the two free DNA ends; either because it removes nucleotides to match existing microhomology regions in DNA ends, or because it inserts nucleotides to create microhomology regions, MMEJ is prone to introduce deletions and duplications [21,22]. In HR defective cells Pol θ is enhanced, suggesting that this type of repair may act when the DNA ends cannot be repaired by the canonical NHEJ [21]. The covalent sealing of the two DNA ends, is performed by either DNA ligase I or DNA ligase III [30] (Figure 2). The high increase in chromosome translocations observed in c-NHEJ mutants that use this repair pathway, is indicative that this process tends to carry out the union of non-homologous segments and therefore to produce SCA [22,30]. The use of MMEJ and suggests that this alternative mechanism of DSB repair, function as a backup when HR and cNHEJ fails, and reveals the no tolerance for unprotected DNA ends by the cells [29].

2.2.4 DSB repair pathway choice

Some calculations suggest that the integrity of the genome in human cells is put at risk 10 DSBs are simultaneously induced [31], therefore choosing the best DNA repair pathway, in order to maximize the efficiency of preserving the genome integrity, is critical for the survival of any cell. Several pathways and sub-pathways have been implicated in the repair of DSBs, here we consider the three main pathways: HR, NHEJ and MMEJ.

The phase of the cell cycle in which a DSB occurs is one of the most important and defining characteristics for DNA repair pathway choice. HR repair is not active in G1 because sister chromatids are not available, therefore DSBs appearing in this phase will be channeled to NHEJ or MMEJ. When a DSB occurs in S-G2 phases, HR is the preferential pathway for its repair, even if the

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end joining mechanisms are active, because it is the best way to preserve the integrity of the DNA sequence. Although the exact mechanism behind the DSB repair pathway choice remains elusive, proteins driving the initial steps of DSB processing are the candidates to determine the selection of the best pathway[21].

Nucleolytic processing of the DNA-ends is a critical step during DSB repair pathway choice (Figure 2). During the G1 phase of the cell cycle an active suppression over the end resection machinery, specifically MRN, is performed by the proteins 53BP1 and the shieldin complex, thus restricting HR to S-G2 phases, and leaving the Ku70/Ku80 heterodimer without competitors during its DNA end-protection activity. During the postreplicative phases (S and G2), the FANCS/BRCA1 protein antagonizes 53BP1 and in collaboration with MRN and CtIP promotes DNA end resection, an important modification needed for the start of both HR and MMEJ [32]. The histone H4K20me is a posttranslational modification that serves as a docking site for 53BP1 and allows NHEJ activity. The FA/BRCA pathway, via FANCD2, restrains the accumulation of 53BP1 by regulating the activity of TIP60, an acetylase of the histone H4 that increases the presence of H4K16ac and H2AK15ac in the site of DNA damage and avoids the accumulation of H4K20me (the docking site for 53BP1). Failure of the FA/BRCA pathway leads to 53BP1 accumulation and favors NHEJ, leading to chromosomal aberrations [10].

The HR, NHEJ and MMEJ pathways are all active during S-G2, therefore for channeling the repair of DSBs into HR during S-G2 is necessary the repression of NHEJ activity; recently, a specific inhibitor of NHEJ in the post replicative phase has been proposed, CYREN (cell cycle regulation of NHEJ), which binds the Ku70/Ku80 complex and regulates the DNA repair pathway choice by inhibiting NHEJ and promoting HR when a sister chromatid is available, thus allowing error free recombination [33].

2.3. FA/BRCA failure and chromosomal aberrations

The heterogeneous clinical phenotype observed in the patients with FA contrasts with their highly constant cellular and cytogenetic phenotype. The homogeneous cellular phenotype observed in FA indicates that failure in any stage of the FA/BRCA pathway results in the incapacity for repairing ICLs and DSBs in an error-free manner. Misrepaired DSBs in particular, that arise after initial ICL processing, are the main source of chromosomal aberrations (CA) present in FA cells, and this sensitivity has been critical for the diagnosis of FA, that is largely based in the detection of CA observed in cultures treated with Diepoxybutane (DEB) and mitomycin C (MMC) [34].

The most common CAs observed in metaphase spreads from patients with FA are chromatidic or isochromatidic breaks, deletions, duplications, fragments and gross chromosomal aberrations, such as translocations, dicentric chromosomes, radial figures, and other complex rearrangements (Figure 3) [35]. All these structural chromosomal aberrations (SCA) can be accompanied by numerical alterations, such as aneuploidies (gains or losses of whole chromosomes) and polyploidization. It is relatively common in FA to find tetraploid cells and mitotic figures with endorreduplicated chromosomes, with four instead of two chromatids. FA cells are also known to have alterations in the duration of the cell cycle phases (explained below), or in the transition from one phase into another, these might provoke new DNA replication cycles in the absence of mitosis and cytokinesis, leading to endorreduplicated chromosomes in the next mitosis (Figure 3a) [36]

SCAs formation involves breaking and rejoining of DNA molecules, therefore DSBs are considered to be the origin of SCA [37]. DSBs can arise from exposure to radiation or virus infection, but also from failures in DNA replication. Importantly for FA, DSBs may come from the processing of ICLs

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originated by endogenous aldehydes [5] or exogenous exposure to bifunctional alkylating agents such as chemotherapeutics MMC, Cis-Platin or DEB [35].

2.3.1 Breaks

In FA cells, chromosome breakage is the result of initiated but unfinished ICL repair. Most of the breaks in FA cells are of the chromatid type (Figure 3b,3c), indicating that they were formed during the post-replicative period of the cell cycle, and therefore only one chromatid is affected even though the chromosome is already composed by two sister chromatids. When a break of the chromosomal type is detected, it can be inferred that a DSB occurring during the G1 phase is the cause; nonetheless, the FA pathway operates in the S-G2 phase, therefore it is more likely that these breaks are the result of two very close DSBs, one in each chromatid, and can be considered isochromatidic breaks. In addition, chromosomal breaks are less common than chromatid breaks when metaphase spreads from FA are analyzed.

The FA/BRCA pathway is not functional in FA cells, therefore the presence of chromosome breaks, when FA cells are treated with ICL inducing agents, suggests that endonucleases alternative to the canonical FA/BRCA pathway unhook the ICL and generate a DSB. This DSB however is not channeled to HR by the downstream modules of the FA/BRCA pathway and might remain unrepaired; when a cell reaches metaphase, the sites of these unrepaired DSBs can be visualized as chromatid breaks. Of note, the piece of broken chromatid usually remains adjacent to its chromosome as a result of the structure of mitotic chromatin and cohesin proteins that hold together the sister chromatids and prevents their separation until anaphase [38] (Figure 3c).

2.3.2 Structural chromosome aberrations

In healthy cells, DSBs are mainly repaired by DNA repair pathways not prone to form gross chromosomal rearrangements, like HR. In FA cells, the presence of translocations, dicentrics and radial figures makes evident the relevance of FA/BRCA pathway in the protection against SCAs, since all of these originate by ligation of two or multiple broken DNA ends with little or absent homology. The evidence accumulated so far suggests that FA cells use NHEJ and probably also MMEJ for the repair of DSBs arising from ICL processing, however, these DNA repair pathways are error-prone when multiple DSBs coexist in the vicinity of chromatin domains. If the end joining pathways NHEJ and MMEJ operate during S-G2 phases of the cell cycle, when the replicated chromosome is composed by two sister chromatids, the joining of one of these chromatids with a non-sister chromatid from a different chromosome (homologous or non-homologous) will lead to SCA (Figure 3).

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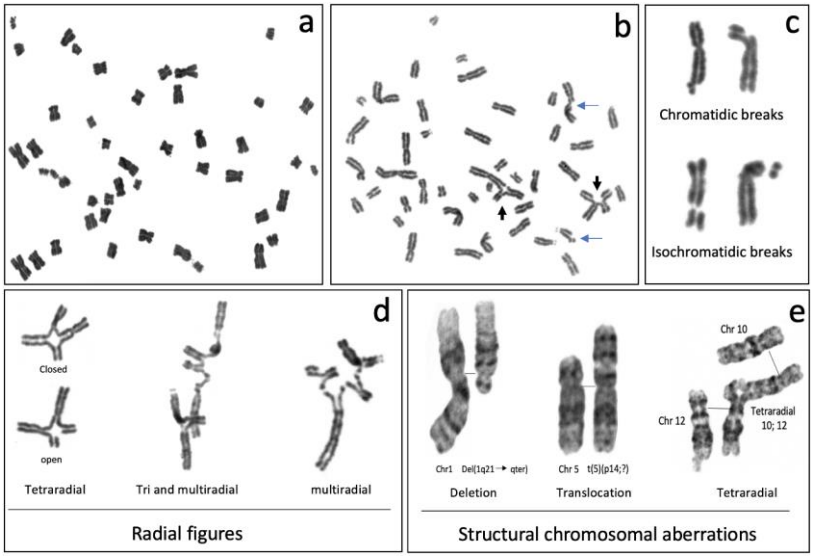

Figure 3. Representative metaphases and common structural chromosome aberrations from FA cells treated with Diepoxibutane. a) Endoreduplication; b) Metaphase with structural chromosomal aberrations, blue arrows show chromatidic breaks and black arrows show radial figures; c) Breaks, the images show how the broken fragments are kept very close to the chromosome that originated them because there is a cohesion of chromatids in metaphase; d) Radial figures; in the first column can be observed that the tetraradial can be closed when the four DNA ends of the two non-homologous chromosomes are rejoined , or open when only two of the four DNA ends were rejoined; f) GTG banded chromosomes reveal other gross structural chromosomal aberrations that may be found in FA cells, such as deletions and translocations; In the radial figure, normal chromosomes 10 and 12 are aligned with the tetraradial, to highlight the trajectory of the rearrangement.

Radial figures are formed when at least two DSBs from non-sister chromatid meet; in these two DSBs there are four DNA ends. If the four DNA ends are joined by an error prone DNA repair machinery, a closed tetraradial can be generated. However, an open tetraradial figure will be originated if only two DNA ends are rejoined (Figure 3d). A triradial figure has the pre-requisite of three DSBs, one of them in a chromatid of the receptor chromosome and two more (of the isochromatidic break type), in the second chromosome to join one chromatid of the receptor chromosome; in this way, polyradial figures require several DSBs for their formation (Figure 3d). In FA cells, radial figures form between non-homologous chromosomes (Figure 3e) [39], both spontaneously and induced by MMC or DEB, indicating the importance of the FA/BRCA pathway in preventing these harmful chromosomal abnormalities. When proteins in both the HR and NHEJ pathway are inactivated, an increase in the frequency of radial figures can be observed [40], suggesting the MMEJ pathway highly contributes to its generation.

Translocations, dicentric chromosomes and chromosome deletions are frequently found in FA cells; and may directly be originated during the abnormal processing of ICLs in FA cells or, more probably, be a consequence of the extremely abnormal segregation that radial figures undergo during mitosis. Depending on the type of radial chromosome, the transition through anaphase will result in the segregation to the daughter cells of translocated chromosomes, dicentric chromosomes, acentric fragments and deleted chromosomes (Figure 4). Importantly, large numbers of cells can succumb to cell death by the accumulation of gross genomic imbalance, i.e. radial figures can lead to anaphase bridges and mitosis blockage, or chromosome fragments can lead to micronuclei

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formation if the cell reaches cytokinesis, this can create a vicious circle of chromosomal instability that can eventually result in the emergence of neoplastic clones. Of note, each cell with only one radial figure can give rise to 4 different daughter cells, carrying different non-clonal chromosomal alterations, this makes it clear that a cell with several SCA will generate daughter cells with karyotypes different from the progenitor cell, generating a wide genotypic diversity (Figure 4).

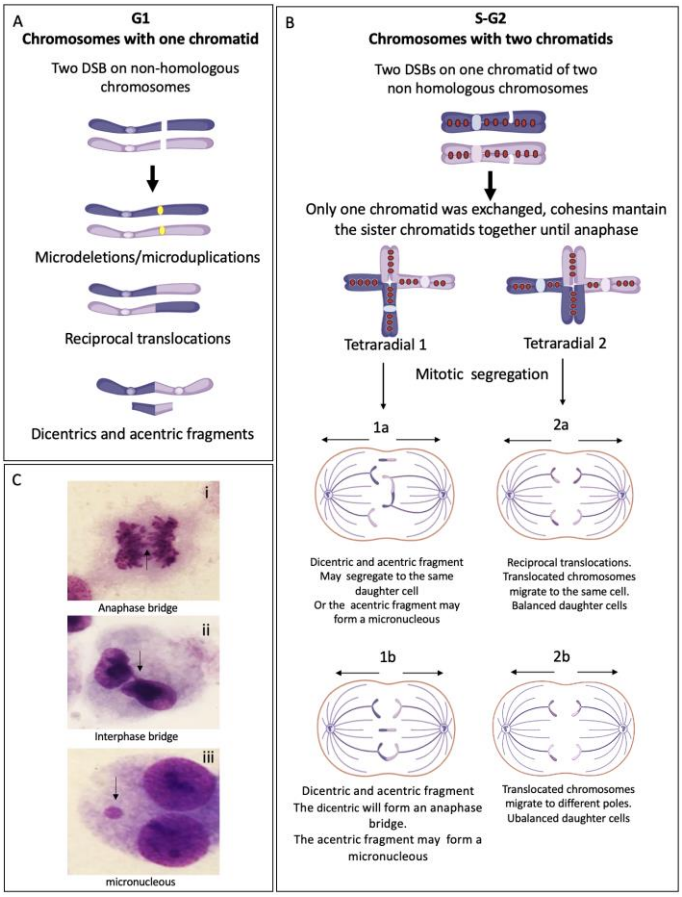

Figure 4. End Joining Repair pathways outcomes when more than one DSB is present in the same space-time and the rejoining is between non-homologous chromosomes. A. G1 chromosomes have only one chromatid, when two chromosomes have DSBs, repair by NHEJ and MMEJ can perform the reunion of the two original chromosomal fragments, without error or leaving microdeletions or microduplications; while if two fragments from different chromosome are joined, translocations or dicentrics + acentric fragments are generated. **B.** S/G2 chromosomes have two sister chromatids linking each other by cohesins, if only one sister chromatid has a DSB, the interchange of segments during repair generates gross structural aberrations such as translocations or radial figures, which may have several configurations depending on the rejoined fragments, here we show two possible tetraradial figures with different outcomes after segregation. 1.The segregation of a closed tetraradial with two DNA ends rejoining chromatids with centromere: a dicentric 1a) In this type of segregation, both normal chromosomes segregate in a daughter cell and the dicentric chromosome moves together with the acentric fragment to the second daughter cell. 1b) The normal chromosomes segregate each to a different daughter cell, the dicentric is attached to both centrosomes of the mitotic spindle, an anaphase bridge is formed, with a high probability of breaking at some point, generating chromosomes with deletion or translocation. In any type of segregation, the acentric fragment can form a micronucleus. 2. Segregation of a tetraradial with two DNA ends rejoining segments without centromere. 2a) both chromatids with the translocated centromere segregate to the same pole, the result is one daughter cell with balanced translocation and one daughter cell normal. 2b) The translocated chromosomes segregate to different daughter cells, both will have unbalanced translocations. **C**. Cells from a FA patient showing i. Anaphase bridge, ii. Interphase bridge, resulting of the segregation failure of a dicentric. iii. micronucleus, frequently formed by an acentric fragment that could not join the mitotic spindle.

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Because SCA are hallmarks of the FA cells phenotype, the analysis of the number and type of chromosomal aberrations is used in the diagnosis, an approximate 10-fold increase in the DEB-induced frequency of chromosomal aberrations, and the presence of radial figures, are indicative of FA (Figure 5). In some patients, the diagnostic chromosome breakage test for ruling out FA, might turn out to be inconclusive due to the presence of a subpopulation of cells that are not sensitive to DEB or MMC, and behave as normal cells. In these cases, the presence of a revertant mosaicism should be sought. Mosaicism in the context of FA refers to the existence, in a single patient, of two hematopoietic cell populations, one sensitive and one resistant to ICL-inducing agents. Mosaicism appears due to the reversion of one of the original germline pathogenic variants causing FA, is calculated to be present in up to 20% of the patients with FA and can have multiple origins, including gene conversion, back mutation, second-site mutation and others. The presence of mosaicism has clinical implications, if the reversion occurs early in the primitive hematopoietic stem cells it might lead to increased blood cell counts and improved aplastic anemia as well as a reduction in the incidence of bone marrow failure and hematologic neoplasias [41].

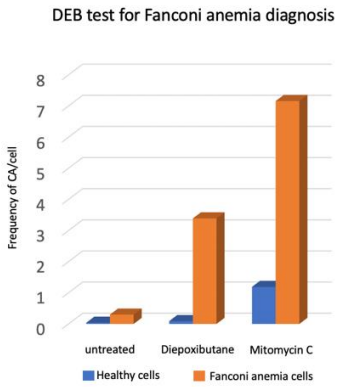

Figure 5. Response of lymphocytes from FA patients (n=18) and healthy subjects (n=117), to treatment with ICL inducing agents, diepoxybutane [0.1 mg / ml] and mitomycin C [40ng / ml]. Gross chromosomal aberrations such as radial figures, translocations, deletions and duplications were commonly observed in FA cells [42] Although the two challenge agents are effective, the use of DEB for diagnosis is preferred, because the results are less variable and the difference between non-FA vs FA cells is generally clearer.

It has also been shown that progression towards MDS and AML in FA is associated with the presence of clonal and non-clonal chromosomal alterations. This agrees with the theory of CIN as a driver of the evolution to cancer. In fact, as can be seen in Figure 4, in FA cells chromosomal instability constantly generates cells with different karyotypes due to non-clonal chromosomal alterations. These gross changes in the karyotype are important, since each cell has a specific genome reorganization that generates a new genome system, susceptible to being selected and moving towards a macro-cellular evolution, a previous step to micro-cellular evolution that will present chromosomal clonal changes and alterations at the gene level [43]; both non clonal and clonal chromosomal abnormalities are valuable biomarkers for detecting progression to cancer. In fact, some specific SCA for FA such as the gain of 1q23-32 (minimal region) and 3q36-29 (minimal region), and other commonly found in FA and non-FA patients, like the monosomy 7 or loss of chromosome 7q31-qter (minimal region), are recurrent anomalies with clinical value for the diagnosis of FA and as factors associated with clonal evolution [40,41].

Chromosomal instability affects not only somatic, but also germ cells. The adverse consequences of not having a functional FA/BRCA pathway in gonadal function can be associated with important processes. Firstly, ICL mis-repair would lead to gross chromosomal rearrangements that will affect the mitotic divisions that germ cells undergo before entering into meiosis (millions in the male germ line), this would lead to cell death of cells carrying chromosome breakage and

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genomic imbalances, that in FA can be in full-scale, as suggested by the infertility observed in most FA patients. Secondly, if cells with balanced chromosome rearrangements (translocations or inversions) move into the prophase I of meiosis, the pairing that the homologous chromosomes undergo during zygotene might be impaired, thus preventing the synaptic events needed for recombination of the homologous chromosomes, or generating complex meiotic figures during pachytene, which subsequently could stop the meiotic division and trigger apoptosis [44]. The third process that might impair gonadal function in FA patients, is the involvement of FANC proteins in the proper development of meiosis, both in the process of programmed DSBs and in meiotic recombination [45]

3. Fanconi anemia cell decision in the face of genomic damage.

3.1 The control of the cell cycle checkpoints in FA cells

The DNA repair deficiency that characterizes FA, leads to accumulation of unrepaired DNA damage with cellular and physiological consequences [46]. When facing DNA damage, every cell has to make the decision whether to divide or not based on the amount of DNA damage that the cell is harboring. Some calculations suggest that a certain threshold of DSBs can be tolerated and when this is exceeded, cells activate apoptotic mechanisms [47].

The normal and timely progression through the cell cycle is controlled by several cell cycle checkpoints functioning during the G1, S and G2 phases [21,48], these checkpoints monitor the integrity of the DNA molecule, whereas an additional M-phase checkpoint monitors for the appropriate chromosome alignment and aneuploidy before chromosome segregation (Figure 6A) [49]. A defect in the G1 phase has been described in FA HSPCs [50], but no defects have been described in other tissues or cell lines derived from patients, however, early work suggested an impaired S-phase checkpoint in FA cells that would allow an accelerated S-phase completion at the expense of DNA damage accumulation [51], and an exacerbated G2 checkpoint, that would be used by FA cells so as to gain time for repairing the DNA damage that was left to pass the S-phase. This phenotype is easily identified when FA cells are exposed to increasing concentrations of ICL-inducing agents: an almost extinct S-phase and a prominent G2 peak.

The prominent cell cycle arrest to which FA cells are subjected has been ascribed to p53, a cell cycle master regulator [50]. p53 is a transcriptional factor whose better-known function is to fine-tune the expression of genes controlling cell cycle arrest and genes controlling apoptosis [52]. p53 can undergo post-translational modifications, mainly phosphorylations, that shape its affinity for certain domains in the promoters of its target genes [53]. p53 is one of the main proteins sensing the amount of DNA damage and has even been suggested that its amount and target affinity respond to the amount of DNA damage that a cell is harboring, leading to the assumption that less DNA damage leads to a transient activation of p53 and cell cycle arrest mediators, whereas big amounts of DNA damage lead to p53 stabilization and activation of pro-apoptotic targets thus conducting to cell demise [54,55].

For the case of FA, multiple reports suggest a direct role for p53 hyperactivation in the typical bone marrow failure that threatens FA children [50,56–58]. HSPCs from FA patients have increased levels of p53 and its target p21, which seems to avoid the proliferation of hematopoietic progenitors harboring excessive DNA damage, thus leading to a poor supply of the mature hematopoietic cells needed for normal blood functions. However, when the activity of p53 is nullified, the bone marrow from FA mice is exhausted due to unrestrained HSPCs proliferation that leads to

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exhaustion of the HSPC pool [57]. The ATM and CHK1 kinases are other cell cycle checkpoint regulators that have been shown to be overexpressed in FA cells, both kinases have the capacity to phosphorylate p53, and ATM has the capacity to phosphorylate CHK1 (though its canonical target is considered to be CHK2) in response to DNA damage [59,60]. Thus, indicating that a strong cell cycle arrest is induced in FA cells in basal conditions. Of note, an attenuation in the characteristic cell cycle arrest of FA cells has been observed in certain FA patients by downregulation of CHK1, which however allows the division of cells with unrepaired DNA damage [61].

Until recently, bone marrow failure in FA has been thought as a consequence of excessive growth suppressive pathways, including hyperactivation of the TGFβ pathway [62], a potent growth inhibitory pathway. However, the mere existence of FA patients suggests that mechanisms allowing their survival must exist and counteract the growth suppressive activities of p53 and TGFβ pathways. Recently, single cell RNA sequencing of primary HSPCs from FA patients showed that overexpression of the MYC oncogene occurs in a subset of FA HSPCs and appears to be a counteracting force against the growth suppressive activities of TGFβ and p53, since inhibition of MYC expression reduces the proliferative capacity of FA HSPCs. MYC overexpression in FA cells, however, is a double edge sword that allows the progression of FA cells through the cell cycle but at the same time increases their replicative stress [63].

Additional mechanisms allowing the escape of FA cells from the strong cell cycle checkpoints have recently starting to be elucidated, these include the checkpoint recovery, a whole machinery of phosphatases, led by PPM1D/WIP1, that dephosphorylates ATM, CHK1, p53 and the histone γH2AX [46]. When this cascade is dephosphorylated; the cell can ignore the DNA damage and divide despite the presence of broken chromosomes. Of note, when bulk FA samples are studied, overexpression of both checkpoint and checkpoint recovery genes can be observed [64], however the recent single cell RNA sequencing studies have the potential to deconvolute the heterogeneity that FA cells might have regarding their response to DNA damage, and both cells arrested or poised for cell division despite DNA damage could be detected (Figure 6B).

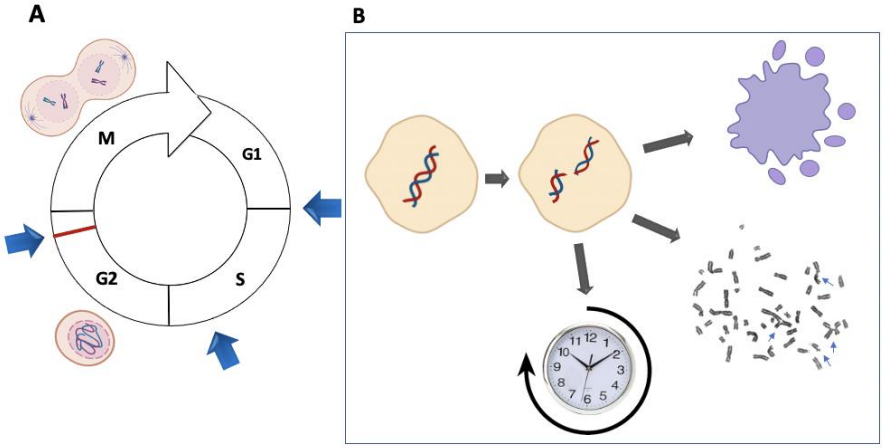

Figure 6. FA cells take cell fate decisions in their transitions through the cell cycle. (A) The G1 checkpoint verifies that the cell has the requirements for starting DNA replication, the S phase checkpoint verifies the accurate and timely replication of DNA, the G2 checkpoint verifies that all the chromosomes are correctly replicated and without DNA damage, the M phase checkpoint verifies that chromosomes are in appropriate numbers and correctly aligned to the mitotic spindle before chromosome segregation. (B) The cell cycle checkpoints are safeguarding moments in which cell fate decisions are taken. For DNA repair deficient cells, such as FA cells, these points are critical since the decision has to be taken whether to activate apoptosis, divide with unrepaired DNA damage or enter into the senescence program.

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3.2 Aging versus cancer in FA

FA is a premature aging disorder since diseases considered of the aged appear in FA patients at a remarkable younger age than in the general population [65], these include aplastic anemia, myelodysplastic syndrome, acute myeloid leukemia [66,67], osteopenia/osteoporosis, diabetes [68] and premature ovarian insufficiency [45,69].

Aging in the tissues from FA patients is associated to ineffective cell divisions, especially in the HSPCs compartment, leading to impaired supply of new functional cells and tissue attrition (Figure 7A); however, division of cells with unrepaired DNA damage can still occur, enticing the appearance of malignant/premalignant clones that can develop into cancer and out-take the tissue (Figure 7B) [65]. Importantly, aging and cancer are considered opposing processes, on the one hand aging develops over the lifespan of a tissue and results from accumulation of detrimental mutations that impair the correct execution of cellular functions. Aged tissues are characterized by accumulation of senescent cells and increased apoptotic rates; cancer on the other hand results from accumulation of mutations that confer a survival and proliferative advantage, with unrestrained cell division capacity these cells can generate a tumor.

Paradoxically, and although cancer and aging are considered antagonists, age is the most significant risk factor for cancer development with the majority of cancers being diagnosed after the age of 65, however in patients with FA cancers typically appear at a remarkable young age [70–72]. This dichotomy between aging and cancer stresses the relevance of a functional FA pathway, that becomes situated at the crossroads between appropriate tissue maintenance and cancer.

The hallmarks of aging are grouped into three main categories: 1) primary hallmarks, considered to be the origin of cellular damage; 2) antagonistic hallmarks, considered to be compensatory or antagonistic responses to the damage, antagonistic hallmarks initially mitigate the damage but might eventually become deleterious themselves; and 3) integrative hallmarks, responsible for the functional tissue decline associated with aging [73]. At the cellular level, FA cells meet several hallmarks of aging, some of them have been very well characterized in FA, whereas some others, although potentially present in FA, have remained understudied.

Primary hallmarks of aging include genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis (the mechanisms that maintain correctly folded proteins and correct protein turnover) [73]. For all of these, genomic instability is the most prominent primary hallmark of aging in FA [65], whereas telomere shortening has been shown to be subtle [74] and epigenetic alterations and defective proteostasis remain poorly studied in FA.

Antagonistic hallmarks of aging include deregulated nutrient sensing, mitochondrial dysfunction and cellular senescence [73]. Mitochondrial dysfunction has been described in FA and is gaining relevance [75], whereas cellular senescence in FA remains a matter of debate [53,76].

Finally, integrative hallmarks of aging, considered the culprits of the phenotype, are the exhaustion of tissue specific stem cells and altered intercellular communication [73]. Exhaustion of the stem cell pool becomes evident in FA as the dramatic decline of HSPCs takes place at young ages [50], whereas defective intercellular communication, involves changes in communication between tissues. An example of aging-associated alteration in intercellular communication is inflammation [73,77]. Acute inflammation events are commonly triggered by pathogen infections, excessive DNA damage (for example during chemotherapy), UV radiation and physical trauma [77]. Another type of inflammation, known as sterile inflammation, is considered to be low-grade and chronic, independent of pathogen infection, specifically associated with aging and also known as “inflammaging” [77,78].

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Acute inflammation is a transient response to infection or tissue damage that is beneficial and facilitates tissue repair, however sterile inflammation is a chronic sustained process, probably promoted by incomplete resolution of the initial stimuli and might ultimately result in tissue remodeling and dysfunction. Sterile inflammation is thought to result from exposure to various endogenous and environmental insults throughout the entire lifespan of a person [73,77,78].

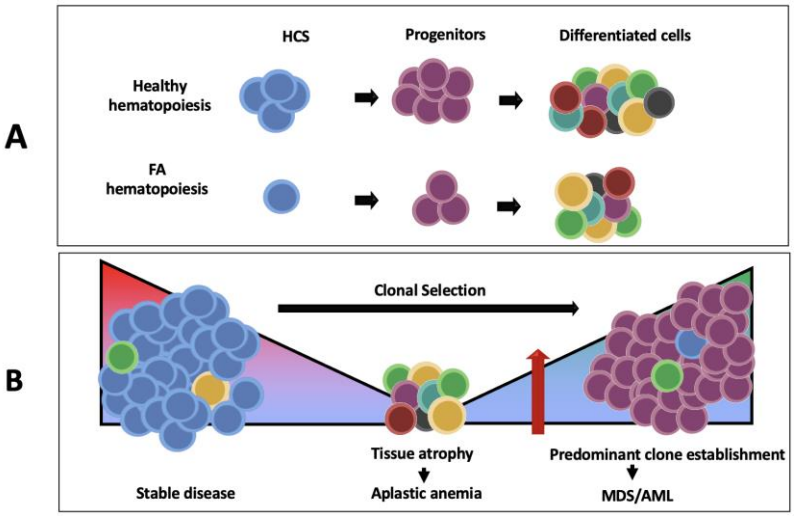

Figure 7. HSPCs in FA are subject to a strong selective pressure. (A) The reduced number of hematopoietic stem cells in the bone marrow of patients with FA leads to reduced numbers of progenitor cells and differentiated cells. (B) Young patients with FA usually have a more stable disease, however DNA damage and environmental stressors might lead to a dramatic reduction in the number of HSPCs, this sweeping of fragile HSPCs might potentially select apoptosis-resistant hematopoietic clones with acquired somatic mutations that eventually could give rise to premalignant hematopoiesis, such as MDS, or AML.

Several agents can activate sterile inflammation including debris from macromolecules, microbial components or extracellular and cytoplasmic DNA fragments, collectively known as damage-associated molecular patterns (DAMPs). DAMPs can activate innate immune cells (neutrophils, macrophages and dendritic cells) and non-immune cells (epithelial cells, endothelial cells and fibroblasts) through the transmembrane pattern-recognition receptors of the Toll-like receptor (TLR) family. TLRs in turn activate the NF-kB transcription factor that upregulates various pro-inflammatory cytokines, including TNFα, IL-1β, IL-12 and Interferons [79].

Although DAMPs-mediated sterile inflammation has not been coined in FA, a pro-inflammatory phenotype has been very well described, and includes increased production of pro-inflammatory cytokines, including TNFα and IFNγ, and increased C-Reactive protein (CRP) [80–82]. Importantly, the inflammation observed in FA, either acute by infections, or sterile, can trigger HSPCs senescence or apoptosis, both processes are strong tumor suppressors and prevent the damaged FA cells from undergoing division [82,83]. However, if tissue regeneration is not efficient or at an appropriate rate, these two processes can derive into depletion of HSPCs, tissue degeneration and function loss, all of which are aging hallmarks. This lead to hypothesize that recurrent inflammatory events in patients with FA might contract the HSPC pool [84], or that a constant low-grade chronic sterile inflammation, which remains unexplored in FA, might contribute to tissues attrition (Figure 8A).

In FA however, adaptation of HSPCs to the harsh bone marrow microenvironment can lead to survival and selection of clones resistant to the pro-apoptotic and pro-senescent mechanisms. For example, the inflammatory episodes mentioned above can serve as “selective sweeps” that get rid of non-fitted HPSC and permit the evolution of clones with the capacity to tolerate the stressors [85,86]. The environmental challenge therefore creates an opportunity for selection and emergence

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of HSPCs with somatic mutations or epigenetic alterations, in this process aberrant HSPCs will replicate with more success than their competitors and can give rise to malignant progeny that can overtake the bone marrow (Figure 7B) [87].

This selection can fine-tune, similar to aging, a clonal drift in the composition of HSPCs populations in the FA bone marrow, this drift is typically characterized by a decline in the frequency of lymphoid committed HSPCs, and an increase in the frequency of myeloid committed HSPCs [87,88]. Differences in the response and tolerance to DNA damage might operate behind this drift in HSPCs clones, but these differences remain unexplored in FA.

Although the above-mentioned adaptations would extend the survival of HSPCs under hostile microenvironmental conditions, the clonal progeny might acquire subsequent abnormalities in mechanisms controlling growth and differentiation, thus diverting from the original clone and give rise to MDS and AML. This takes relevance since up to 40% of children and young adults with FA will exhibit signs of clonal evolution in the bone marrow [84], while up to 15% to 60% of patients with FA, depending on the cohort, might developed MDS/AML [70,71,89]. These clones can be a frequent finding in the bone marrow aspirates of FA patients, even before having any morphological signs of MDS or AML progression [90], however additional chromosomal abnormalities below the microscopic detection limit might add to actual frequency of clones.

Cytogenetic and next generation sequencing analysis of MDS and AML bone marrow samples from patients with FA have identified gross chromosomal abnormalities. The most frequent findings include partial duplication of chromosome 1q (1q+, 44.8%), partial duplication of chromosome 3q+ (41.3%), duplications in 21q+ (20.7%), monosomy of chromosome 7 or deletion of chromosome 7q- (17.2%), and 11q+ (13.8%); whereas mutations are more commonly in the genes *RUNX1* and *RAS* [90,91].

Although some of the chromosome abnormalities mentioned above are shared between patients with FA and MDS/AML from the general population, mutations in MDS/AML oncogenes and tumor-suppressor genes classically found in MDS/AML samples are rarely found in FA. On the other hand, chromosomal lesions that seem to be specific to FA include 1q+ and 3q+; of note 1q+ has been observed in the BM of patients with FA in all MDS/AML stages and even in normocellular bone marrow or hypoplastic bone marrow without signs of transformation, suggesting that 1q+ clones might confer a survival advantage to the HSPCs from FA patients without being a part of the malignant transformation process [90,91].

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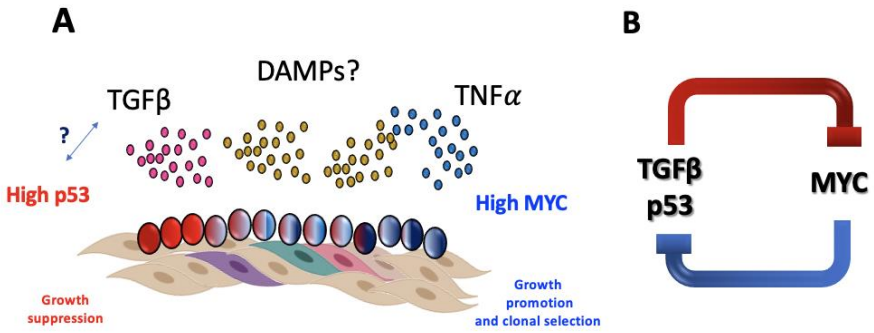

Figure 8. HSPCs in different functional states co-exist in the FA bone marrow and respond differentially to microenvironmental stimuli. (A) Overexpression of different pro-inflammatory cytokines has been described in FA and can have opposite effects on responsive HSPCs. On the one hand, TGFβ inhibits the proliferation of FA HSPCs, it is currently unknown if TGFβ and p53 are functionally linked in FA HSPCs, however both proteins suppress proliferation and have increased levels in the FA bone marrow. On the other hand, TNFα promotes the proliferation of FA HSPCs, at the expense of DNA damage, by activating the expression of the oncogen *MYC*. TNFα is considered a DAMP that promotes sterile inflammation, however the existence of additional DAMPs in the bone marrow of FA patients remains unexplored. (B) MYC has opposite transcriptional activities to p53 and TGFβ, its overexpression in FA HSPCs suggests a counteracting force against the growth suppressive activities of TGFβ and p53.

4. Clinical consequences of FA/BRCA failure.

Fanconi anemia is a well-recognized entity in humans characterized by 3 main features: 1- developmental alterations, 2- bone marrow failure and 3- an increased risk to develop cancer. The clinical presentation among patients is highly heterogeneous, not all patients develop all features and there is important variability in the severity of each documented feature.

The complete FA pathway is only present in mammals, but can be found in a reduced version in other organisms [92] The effects of an altered FA/BRCA pathway are not universal within mammals. Although a clear phenotype is recognized in humans, mice models do not recapitulate the complete human FA phenotype [93] This species differences have proven to be an obstacle to model several FA features, and mechanistic studies that explain phenotypic outcomes are scarce.

4.1 Development alterations

A recent literature review of published cases, that analyzes the reported physical features of the largest number of confirmed patients with FA, found that almost 80% of them had at least one physical feature, the more frequent ones were: short stature, upper limb radial ray abnormalities, skin pigmentation changes, renal malformations and central nervous system findings [6]. Morphogenesis is a highly regulated process, there are critical moments during development where different organs can be particularly sensitive to insults [94]. It was recently found that aldehydes

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derived from normal cellular metabolism can form ICL in the DNA that have to be processed by the FA/BRCA pathway [95].

Aldehydes are also a byproduct of alcohol metabolism, alcohol has been shown to rearrange chromosomes and kill cells [96] and its teratogenic effects are clearly shown in children born to mothers who have ingested alcohol during pregnancy manifesting as fetal alcohol spectrum disorders (FASD), where developmental issues and malformations are important features.
It could be speculated that the hardship found by patients with FA during development to take care of the ICL resulting from endogenous acetaldehydes could contribute to the physical phenotype of patients with FA. It has been hypothesized that the overlapping features in FA and FASD are the consequence of aldehyde susceptibility of somatic stem and progenitor cell populations [97]. ALDH2 genotype has been proposed to be a phenotype modifier in FA, the A allele has been found to be associated to early bone marrow failure progression as well as an extensive malformation phenotype [98]. Moreover, a more severe phenotype has been observed in individuals with an ALDH2-AA genotype from three different sibling pairs with FA (same *FANC* gene genotype and similar genetic background among siblings)[99].

Anthropometric features are key components of the classical FA physical phenotype. Short stature is reported in half of the patients and low birth weight is also a commonly reported feature of patients with FA [100]. Evidence in mice supports the hypothesis that growth retardation and short stature in humans with FA could be due to the loss of pluripotent stem cells during embryogenesis [101]. FA mice (fancd1 and fancn) die early during embryonic development due to increased apoptosis [101]. Nevertheless, short stature has not been found to be associated to D1 and N groups in humans, but has been linked to genotypes of downstream genes [6]. The association to genes from the downstream part of the FA/BRCA pathway has also been found for a small head; microcephaly, described in almost 30% of published cases [6], may reflect the importance of appropriate DNA repair in neural progenitors undergoing rapid replication cycles during central nervous system development [102,103].

Upper limb radial ray abnormalities are also a pivot feature that brings the FA diagnosis into the minds of clinicians. It has been estimated that up to 1% of patients with congenital thumb malformations have FA [104]. The upper limb phenotype in patients with FA is extremely variable: most patients have normal structures, but for the 40% who have a radial ray abnormality [6]), the severity spectrum is vast as it can go from discrete flat thenar eminences to obvious oligodactyly or polydactyly [105], and may affect either a single or both upper limbs [106]. The pathophysiologic basis of radial ray abnormalities in FA remains unknown [104]), yet it is well recognized that genetic factors have an important role in the pathogenesis of radial ray deficiencies, for instance fibroblast growth factor (FGF) expression has been identified as necessary for appropriate radial development [107]. Although this has not been explored in the context of FA, it is possible that the ubiquitous FA/BRCA pathway may somehow interact with other developmental pathways adding to the stochastic factors contributing to the variability of upper limb phenotypes in patients with FA [35].

The relevance of stochastic factors in developmental phenotypes is also illustrated by renal malformations. The patterns of abnormalities found in these paired organs, point to disruption of

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migration patterns of embryonal organs to their final position occurring at an early developmental stage, suggesting that the FA pathway may have a role in this [108]. A literature review estimates a frequency of kidney malformations in nearly 30% of patients [6], but this seems to be an understatement since intentional assessment of renal anatomy has shown that nearly 50% of studied patients have alterations.

A more flagrant misestimation of occurrence is found for skin pigmentation changes, which have been described in almost 40% of published cases of patients with FA [6]. Yet, a recent study designed to delineate the cutaneous findings in FA found, after direct examination, that almost all patients with FA had at least one pigmentary alteration. The more frequent being café au lait macules, but also identifying hypopigmented macules of which the skin-fold freckle-like macules variety could be characteristic of FA [109]. It has long been established that anomalous pigmentation is associated to chromosomal alterations, and that they may only be found when skin tissue is analyzed [110,111]. Pigmentary changes in FA have not been thoroughly studied and are poorly understood, although an evident hypothesis is that they respond to accumulated genomic instability. The fact that pigmentary changes in FA appear to increase with age supports this possibility and that they occur in both exposed and non-exposed areas [109] would support that the chromosomal instability does not result from UVA exposure but other type of damage like the one arising from a defective FA/BRCA pathway (Figure 9).

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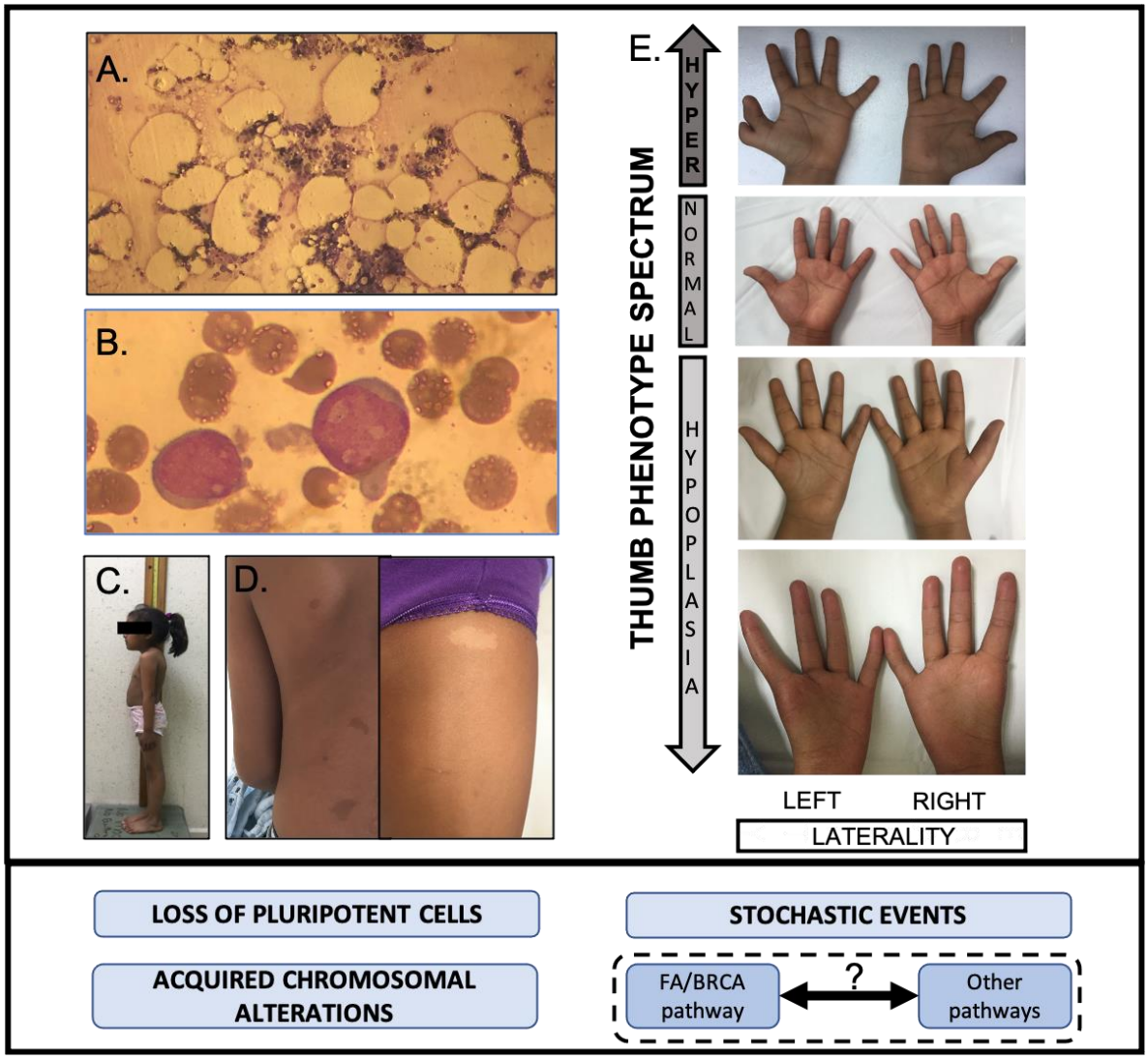

Figure 9. Deficit of the FA/BRCA pathway impacts the Fanconi anemia phenotype. A) Bone marrow failure results from bone marrow attrition due to loss of hematopoietic progenitors. B) Acute myeloblastic leukemia is the consequence of clonal evolution with acquired chromosomal aberrations. C) Somatometric features: short stature and microcephaly are thought to express an early loss of pluripotent cells during development. D) Pigmentation: a hypothesis is that café au lait and hypochromic macules could be subjacent to acquired chromosomal alterations in skin cells. E) Thumb abnormality spectrum. Stochastic events are credited for the high variability found in malformative phenotypes of patients with FA, the interaction of the FA/BRCA pathway with other developmental pathways remains to be investigated.

4.2 Hematological manifestations

Bone marrow failure (BMF) is the more characteristic feature of patients with FA, it has been estimated that the cumulative incidence of severe BMF goes to 70% by age 50 years, with a median of 7 years [71,112].

The primary genetic defect of FANC genes present in hematopoietic stem cells (HSC) hinders their ability to deal with replicative stress during prenatal HSC expansion by triggering an apoptotic p53/p21 mediated response that results in a prenatally reduced fraction of CD34+ cells in patients with FA [50]. This compromised HSC pool is further challenged by DNA damage accumulation

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during extrauterine life. Reactive aldehydes, by-products of normal cellular metabolism are important genotoxics neutralized by the FA/BRCA pathway, the exposure of FA deficient cells to aldehydes result in the accumulation of chromosomal aberrations [113]. The aldehyde dehydrogenases enzymes are known to be important for aldehyde detoxification [114]. Mice HSC have been found to heavily rely on the Aldh2 enzyme function to protect from aldehyde toxicity, double Fancd2-/- Aldh2-/- mutants have a severe defect in their HSC pool that correlates with an increase in the DNA damage marker γH2AX [95]. It is widely accepted that BMF in patients with FA is driven by endogenous-aldehyde induced toxicity of HSC cells, cytokine overproduction has also been hypothesized to contribute to the BMF phenotype in FA, but it is not yet clear if this sensitivity is related to the DNA repair deficiency or results from alternate roles of FA proteins [115]. Recent data illuminates another mechanism that contributes to BM attrition in FA: activation of the MYC oncogene in CD34+ FA cells inhibits the expression of the cell-adhesion CXCR4 molecule that keeps the CD34+ cells in the hematopoietic niche, resulting in CD34+ cells detachment from the niche and an increase of these cells in the peripheral blood compartment of patients with FA.

Every replication cycle is an occasion for chromosome instability in FA deficient cells. As we have seen, this DNA damage can result in a p53/p21 response that leads to bone marrow attrition. Nevertheless, the genomic instability inherent of FA cells can also bring clonal evolution that leads to neoplasia.

4.3 Oncologic

Patients with FA have a significant risk to develop cancer. The cumulative incidence of leukemia has been estimated to be under 5% by age 30, while the myelodysplatic syndrome (MDS) cumulative incidence was found to be 50% by age 50, regarding solid tumors, the cumulative incidence is about 20% by age 65, with a hazard rate that increases exponentially after the age of 30. This translates into a reduced median overall survival of patients with FA of 39 years [112].

4.3.1 Hematologic neoplasias

i. MDS

Non-transplanted patients with FA have an outstanding risk to develop MDS, that has been shown to be over 5,500-fold over that of the general population. The hazard rate of MDS reaches 1% by age 10 [116] and the cumulative incidence of reaches 50% by age 50 [112], starting at 1% at age 10. The more frequent subtype found in patients with FA is refractory cytopenia with multilineage dysplasia, this morphologic diagnosis is usually reached when the bone marrow shows dyserythropoiesis, a feature found in over 90% of patients, accompanied by over 10% dysplastic cells in 1 or 2 other myeloid cell lines [117]. Morphologic data of MDS is correlated to clonal evolution [Cioc 2010], the MDS progression to leukemia has been estimated at 9% in patients with FA [72].

ii. Leukemia

Leukemia has been found to occur in 3% of patients with FA [112]. In over 80% of cases it is acute myelogenous leukemia (AML) [116], although cases of acute lymphoblastic leukemia (ALL)

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have also been reported [66]. Hazard for AML rises steadily after age 10 and plateaus by the age of 20-30 years [116] so that by age 30 cumulative incidence of leukemia has been estimated to be under 5% [70]. To our knowledge, AML FAB subtypes in patients with FA haven seldom been reported [118]. But it is well known that patients with FA do not have recurring rearrangements like t(8:21); inv (16) and other aberrations usually found in de novo AML and that aid to classify according to FAB subtypes [119]. The signature recurring aberrations found in the bone marrow of FA patients bear witness on clonal evolution mediated by deficiency of the FA/BRCA pathway.

4.3.2 Solid tumors

Solid tumors were found in 12% of patients with FA, major cancer sites have consistently been reported as head and neck squamous cell carcinoma (HNSCC), vulva, esophagus and brain. Their cumulative incidence is about 20% by age 65, with a hazard rate that increases exponentially after the age of 30. This translates into a reduced median overall survival of patients with FA of 39 years Overall a 19 observed/expected ratio for all solid tumors in non-transplanted patients with FA was found, with a median age of presentation of 34 years. And an increased risk of solid tumors in transplanted patients was confirmed [112]. Molecular analysis of a subset of squamous cell carcinomas (SCC) from patients with FA showed that the allelic loss in these tumors is similar to sporadic SCC, suggesting that the same genes and chromosomal locations are targeted in SCC irrespective of their etiologic cause [121]. Genomic instability in epithelial cells from patients with FA has been evidenced by two recent studies, the first one evaluated the frequency of micronuclei (MN) in exfoliated buccal cells from patients with FA, in whom a higher frequency of MN was found when compared to a control group, and an even higher frequency was observed in patients who had had SCC. The MN frequency was found to be such a good biomarker of chromosomal fragility in epithelial cells that it is already being studied as an endpoint in clinical trials for chemoprevention interventions [122]. The second one studied brush biopsy specimens from oral lesions of patients with FA and showed that DNA aneuploidy is a good biomarker for oral epithelial dysplasia or SCC [123].

4.3.3 Skin cancer

Skin cancer is also extremely frequent in patients with FA. In the NCI cohort, the overall number of non-melanoma skin cancers in both non-transplanted and transplanted patients is larger than the number of solid tumors, and over 70% (8/11) of patients who had skin cancers had more than one [112]. The published information concerning skin cancer in patients with FA is scarce and vague, more precise data gathering could shed light into factors that may contribute to their development, like the anatomical region where they develop and if they initiate in skin with pigmentation changes. An interesting observation made by Kao et al. when analyzing DNA repair pathways in different skin cancers is that the FA/BRCA pathway may be contributing to melanomagenesis, since genes from this pathway were found to be upregulated in melanoma tumors [124]. It is interesting that melanoma was not reported in the NCI cohort, and to the best of our knowledge has not been reported in any patient with FA, a hypothesis may be that the deficiency in the FA/BRCA pathway may be protecting patients with FA from this kind of cancer.

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4.3.4 Childhood solid cancer

Besides the more frequent solid tumors like HNSCC, there are around 40 patients with FA in whom primary solid tumors have been found during childhood, the known genotypes of these patients are either *FANCD1 (BRCA2*) or *FANCN (PALB2*) in whom cancer usually occurs in the first decade of life [125,126]. The type of tumors associated with this presentation are brain tumors, nephroblastomas and neuroblastomas, and many patients with such genotypes develop multiple primary neoplasms. Microarray- based CGH analysis of tumors from these patients has shown a large number of segmental alterations, but they do not show a recurrent pattern as what has been described for MDS and leukemia in patients with FA. Although, the alterations found have been associated to aggressive phenotypes [125].

4.3.5 Increased risk for heterozygotes

Most patients with FA bear mutations in autosomic genes that require biallelic mutations to reveal the recessive character of the disease. In a classic paper from 2002, Howlett et al. linked in a same pathway, known *FANC* genes and the hereditary breast and ovarian cancer (HBOC) genes *BRCA1* and *BRCA2* [127], substantiating the observation that some family members from patients with FA could have an increased risk of cancer. Family members of patients with FA who are heterozygous carriers of pathogenic variants in *FANCD1 (BRCA2), FANCJ (BRIP1), FANCN (PALB2), FANCO (RAD51C) and FANCS (BRCA1*), are at increased risk to develop cancer. As a corollary, carriers of pathogenic variants in those genes are at increased risk of conceiving children with FA if their partners are also carriers [34].

4.3.6 Somatic mutations in FANC genes in sporadic cancer

The ability to perform DNA repair is a central mechanism for the protection of genome stability. It is of no surprise then that inappropriate genome maintenance conducts to cancer. Genes from the FA/BRCA pathway are of course susceptible of gaining mutations in the course of malignant transformation for a number of cancers. The sporadic forms of cancers typically found in patients with FA (breast, ovarian and HNSCC cancers and with a lesser frequency AML), have been reported to have an assortment of somatic mutations in genes from the FA/BRCA pathway. This somatic susceptibility for mutations in genes of the FA/BRCA pathway is not limited to such cancers, but can also be found in infrequent cancers in FA like melanoma, evidencing the importance of this pathway for genomic stability in a myriad of cell types [34].

4.4 Infertility

Fertility issues have been reported in both female and male patients with FA. Pregnancy in FA is a rare event, to our knowledge there are less than 50 pregnancies reported in the literature. Although both transplanted and non-transplanted patients have become spontaneously pregnant [78], their pregnancy rates are under 15% [69]. Most of these pregnancies occurred when the women were in their early twenties, which is not surprising since premature ovarian insufficiency (POI) has been found to be a feature of FA [69]. Moreover, heterozygous rare variants in FANC genes have been found in patients with non-syndromic POI [128], and male subjects have been identified as having FA after molecular diagnosis was ordered during causal investigation for azoospermia [129], substantiating the role of the FA/BRCA pathway in fertility.

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Mice models have proven essential to further understand the mechanisms that result in subfertility in FA. The FA/BRCA pathway is needed in the response to replication stress, it becomes essential in situations of rapid proliferation like expansion and maintenance of primordial germ cells (PGC)[130], mice studies have shown that non-functional Fanc proteins result in PGC attrition [130–133]. Although the FA/BRCA pathway is essential for ICL repair in somatic cells, the core complex does not seem essential for programmed DSB during meiosis, yet the role of FA proteins in mammalian meiosis has not been largely studied [45]. Studies in diverse non-mammalian species have shown that FA proteins have a role in meiotic recombination during DSB repair and crossing-over, but the attrition PGC phenotype seen in mammals is probably masking the meiotic phenotype in mice [45].

5. Conclusions

The FA/BRCA pathway coordinates the repair of ICLs through the error-free DNA repair mechanism known as HR, when constitutional PV in FANC genes render this pathway non-functional, the FA phenotype arises. Clinically it is characterized by a highly variable presentation including any or a combination of developmental alterations, bone marrow failure and an extremely high risk to develop cancer. In sharp contrast, the cellular phenotype is markedly constant, it is characterized by a prolonged G2 cell cycle phase, proclivity to apoptosis and most notably by chromosomal instability. This cellular phenotype is true irrespective of the role performed by the affected FANC protein in the intricated FA/BRCA pathway; it serves as a reminder that FANC proteins function comprehensively to protect the integrity of the genome.

A non-functional FA/BRCA pathway translates at the cellular level in the continuous generation of DSBs which promote the formation of SCA that are themselves susceptible to further generate other chromosomal alterations, bolstering a vicious cycle of chromosomal instability. The resulting load of DNA damage accumulated in FA cells leads to hyperactivation of cell cycle checkpoints that impede cellular division. Unsatisfied checkpoints can concurrently drive increased apoptotic rates or the activation of the senescence program. However, a proportion of FA cells manage to either ignore the cell cycle checkpoints or to override apoptosis giving rise initially to cells with non-clonal chromosomal aberrations that can eventually promote the formation of genomic rearrangements that propel the evolution of malignant clones.

In brief, the two alternatives for cells that cannot repair their damaged DNA are either cell death or survival despite genomic damage, these two cellular fates are at the center of the phenotypic presentation of patients with FA. On the one hand, loss of pluripotent stem cells, particularly when subjected to replicative stress during expansion, affects a myriad of tissues and partly accounts for features like short stature, microcephaly, infertility and the more extensively studied bone marrow failure, which is currently more thoroughly understood. In the bone marrow, HSPCs with genomic damage can activate the p53 dependent apoptotic program, stop proliferating in a TGFβ dependent manner or divide, mediated by the overexpression of the MYC oncogene. MYC overexpression is thought to preserve the population of HSPCs that allows the survival of patients with FA (Figure 8B). Nevertheless, most patients with FA eventually develop bone marrow failure, suggesting assistance of additional mechanisms that disrupt this precariously balanced state. Acute inflammation events may contribute to the decline of HSPCs, this may be triggered by excessive DNA damage, infection by diverse pathogens or even sterile inflammation, which is independent of pathogen infection but could well be contributing to the pro-inflammatory phenotype of FA patients. On the other hand, the pro-inflammatory bone marrow microenvironment in FA, partly mediated by TNFα and additional DAMPs (Figure 8A and B), could be sweeping fragile HSPCs out of the marrow while promoting the emergence of hematopoietic malignant clones resistant to apoptosis. Moreover, there is reason to hypothesize that a possible explanation for pigmentary features of FA lies in the development of chromosomal alterations in skin cells.

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The lack of animal models that fully recapitulate the human FA phenotype have particularly difficulted the study of mechanisms that give rise to the malformative phenotype of FA, today its pathophysiologic basis remains mainly unknown. Stochastic events, including aldehyde exposure at particular developmental stages appear to be important, however, mechanistic investigations into the physical phenotype of patients with FA is an area awaiting to be explored.

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