Fanconi anemia and the cell cycle: new perspectives on aneuploidy
Grzegorz Nalepa1-3* and D. Wade Clapp1,3-5*

Addresses: 1Department of Pediatrics, 2Division of Pediatric Hematology-Oncology, Indiana University School of Medicine, Riley Hospital for Children, 705 Riley Hospital Drive, Indianapolis, IN 46202, USA; 3Department of Medical and Molecular Genetics, 4Department of Microbiology and Immunology, 5Department of Biochemistry and Molecular Biology, Wells Center for Pediatric Research, 1044 W. Walnut Street, Indiana University School of Medicine, Indianapolis, IN 46202, USA

* Corresponding authors: Grzegorz Nalepa (gnalepa@iu.edu); D. Wade Clapp (dclapp@iu.edu)

Abstract
Fanconi anemia (FA) is a complex heterogenic disorder of genomic instability, bone marrow failure, cancer predisposition, and congenital malformations. The FA signaling network orchestrates the DNA damage recognition and repair in interphase as well as proper execution of mitosis. Loss of FA signaling causes chromosome instability by weakening the spindle assembly checkpoint, disrupting centrosome maintenance, disturbing resolution of ultrafine anaphase bridges, and dysregulating cytokinesis. Thus, the FA genes function as guardians of genome stability throughout the cell cycle. This review discusses recent advances in diagnosis and clinical management of Fanconi anemia and presents the new insights into the origins of genomic instability in FA. These new discoveries may facilitate the development of rational therapeutic strategies for FA and for FA-deficient malignancies in the general population.

Fanconi anemia: a disorder of genomic instability
FA is a group of genetic syndromes associated with bone marrow failure, congenital abnormalities, hypersensitivity to DNA-damaging agents, and a high cancer risk [1-5]. Few congenital disorders are as genetically and clinically complex as FA. We currently know at least 16 human FA genes (FANCA through FANCG), and more FA genes remain to be discovered [6-8].

Translational and basic research continues to shed new light on the mechanisms of carcinogenesis resulting from disruption of FA signaling. The genomic instability in FA results from impaired interphase DNA damage repair as well as from error-prone chromosome segregation during mitosis. Thus, we propose that FA signaling functions as a guardian of the genome throughout the cell cycle.

Here we provide a brief overview of clinical aspects of FA, followed by a discussion of new discoveries regarding the pathophysiology of FA. We hope these new findings will open new inroads to rational targeted therapies in FA.

Diagnostic and therapeutic challenges
The prevalence of FA is grossly estimated at 10 cases per 1 million individuals [9]. The median age of diagnosis is 7 years of age [10], but FA may remain unrecognized until adulthood due to the high clinical heterogeneity of this disease [11-13]. Developmental abnormalities seen in FA include short stature, musculoskeletal malformations, such as deformities of thumbs and radii, café-au-lait spots, structural kidney and genitourinary tract defects, and VACTERL (Vertebral anomalies, Anal atresia, Cardiac defects, Tracheoesophageal fistula and/or Esophageal atresia, Renal anomalies, and Limb defects) association [14]. Importantly, at least one in three FA patients does not have any visible malformations [15,16]. Thus, diagnosis of FA requires a high clinical index of suspicion [16].

Progressive bone marrow failure is the consistent clinical hallmark of FA. Gradual depletion of bone marrow stem cells impairs hematopoiesis. Decreased blood counts lead to symptoms that often appear in the first decade of life but may remain undetected for much longer because of their insidious onset. These symptoms include malaise
and pallor due to anemia, bruising and bleeding due to low platelet counts, and infections due to falling neutrophil counts. This clinical scenario requires a bone marrow aspirate to exclude leukemia. In most FA patients, bone marrow analysis reveals decreased hematopoiesis but no malignancy. If the blood counts do not recover within 1-2 months, a chromosome breakage test to exclude FA is indicated. This test is based on the fact that exposure to low-dose DNA-crosslinking chemotherapeutics such as diepoxybutane (DEB) [17] causes chromosome fragmentation in living FA patient cells but not in normal cells. A positive DEB test is followed by candidate FA gene sequencing, which allows family counseling and helps personalize patient care as the genotype-phenotype correlations in FA are beginning to emerge [15].

FA is associated with a high risk of cancer: at least 20% FA patients will suffer from a malignancy during their lifetime [1,4,18]. The most common cancers in FA patients are acute myeloid leukemias (AMLs) followed by squamous cell carcinomas as well as brain and soft tissue tumors. While more studies are needed, the affected FA gene and the type of mutation appear to correlate with disease severity. Biallelic loss-of-function mutations of the well-known breast cancer susceptibility gene (FANCD1/BRCA2 [19]) are associated with severe FA that includes predisposition to childhood brain tumors and other solid tumors in addition to early onset of bone marrow failure and hematopoietic malignancies [20-23]. This particular observation provides clinical evidence of the functional link between the FA signaling and the breast cancer susceptibility pathways (reviewed in [24]).

Clinical management of FA patients requires multidisciplinary expertise. For young FA children with asymptomatic pancytopenia, close observation may be appropriate. In some FA patients with worsening bone marrow failure, anabolic androgens can improve blood counts [25], although this strategy may be associated with significant morbidities [26]. Severe bone marrow failure requires a stem cell transplant. The choice of preparative regimen is a matter of ongoing debate [27,28]. For FA patients, modern stem cell transplant strategies based on low-dose preparative regimens are well tolerated and lead to promising outcomes.

The risk of AML in FA appears to steadily increase after 10 years of age in patients who have not undergone a stem cell transplant. Treatment of AML in FA requires the use of modified chemotherapy and stem cell transplant to maximize the chance of cure. Adolescent FA patients face a high risk of developing squamous cell carcinomas (SCCs) [29,30]. The etiology of increased risk of SCC in FA is unclear. In 2005, it was reported that stem cell transplant increases the risk of SCCs in FA [31], but it is not known whether this risk is associated with modern stem cell transplant regimens. The role of HPV (human papillomavirus) had been implicated, but the studies aimed to detect HPV in FA-associated SCCs have generated conflicting results [32,33]. Since the SCCs in FA patients are very difficult to treat, a close surveillance followed by prompt surgical resection of suspicious lesions is essential.

Most FA patients experience medical issues throughout life, including endocrine abnormalities [34,35] as well as therapy-related morbidities. As with other rare chronic illnesses, FA causes significant psychological stress to the family and the patient [36,37]. Therefore, the importance of psychological help cannot be underestimated.

Gene therapy for FA-associated bone marrow failure may become available in the future. Although early attempts at FA gene therapy were hampered by difficulties with engraftment of corrected cells [38], several groups have successfully used gene therapy in mouse models of FA [39,40], and human clinical trials are anticipated soon [41,42]. We hope that better understanding of the origins of genomic instability in FA will allow rational development of targeted therapies.

**Fanconi anemia: genome surveillance throughout the cell cycle**

*The canonical Fanconi anemia DNA damage response pathway in interphase*

All known FA proteins work in concert to control the DNA damage response (Fig. 1). Disrupted chromatin activates a network of kinases (including ATR and CHK1) to engage DNA damage pathways and arrest the cell cycle until the lesions are removed from the genome [43]. Some FA proteins are essential for this initial step in damage signal transduction: FANCM activates ATR [44] and CHK1 [45], and FANCJ promotes ATR-mediated cell cycle checkpoint signaling [46]. In return, ATR and CHK1 phosphorylate FANCA, FANCE, and FANCM [47-50]. This positive feedback loop connects the DNA damage kinases and FA proteins to amplify the DNA damage recognition signal. This in turn promotes the activation and assembly of the multiprotein FA core complex, which consists of eight FA proteins as well as several accessory proteins [51,52].

Next, assembly of the core complex promotes monoubiquitination and activation of a FANCD2/FANCl heterodimer by the FANCL ubiquitin ligase [53,54]. Notably, phosphorylation of both FANCI [47,55] and FANCD2 [56] by the DNA damage kinases is essential for the FANCD2/FANCJ monoubiquitination, again highlighting the complex interplay between the DNA damage kinases and Fanconi proteins.
Phosphorylation and monoubiquitination of the FANCD2/FANCI heterodimer activate the DNA repair pathways. A group of FA proteins responsible for this step is remarkable for several signal transducers that connect the breast cancer susceptibility pathways with the FA signaling. BRCA2, also known as FANCD1, is the most known gene that causes FA when both alleles are mutated and is associated with breast cancer risk when one allele is disrupted. At least three other genes (FANCN/PALB2, FANCI/BRIP1, and FANCO/RAD51C) appear to follow a similar pattern of inheritance [23,24,57-61]. The clinical heterogeneity of the Fanconi phenotypes seems at least partially due to the divergence of gene-specific downstream signaling pathways.

The FA signaling pathway selectively activates the high-fidelity homologous recombination-based damage repair, which is a default choice especially during the DNA replication [62]. FA-deficient cells attempt to repair the lesions through error-prone non-homologous end joining (NHEJ). At least three other genes (FANCN/PALB2, FANCI/BRIP1, and FANCO/RAD51C) appear to follow a similar pattern of inheritance [23,24,57-61]. The clinical heterogeneity of the Fanconi phenotypes seems at least partially due to the divergence of gene-specific downstream signaling pathways.

The FA signaling pathway selectively activates the high-fidelity homologous recombination-based damage repair, which is a default choice especially during the DNA replication [62]. FA-deficient cells attempt to repair the lesions through error-prone non-homologous end joining (NHEJ). Interestingly, the observation that silencing the NHEJ pathway alleviates DNA repair defects in some FA-deficient cells [63] suggests that increased mutagenesis in FA is at least partially due to the overuse of the NHEJ-based DNA repair.

It is not entirely clear how the FA signaling is turned off upon completion of the DNA damage repair, but recent work showed the critical role for the USP1 complex that deubiquitinates the FANDC2/FANCI heterodimer [64,65]. USP1 itself is downregulated by the DNA damage [65], providing another feedback loop to facilitate accumulation of the monoubiquitinated FANCD2/FANCI heterodimer in injured cells. Interestingly, Usp1-deficient mice display a phenotype similar to FA [66]. This observation implies that the ability to cycle the FA pathway on and off is essential to maintain the functionality of the FA signaling.

**The origins of DNA damage in Fanconi anemia-deficient cells**

The FA signaling pathway is essential to alleviate the mutagenic impact of a wide range of genotoxic insults, from chemotherapeutics [67] to ionizing radiation [68]. However, until recently, we did not know why FA patients develop genomic instability, even though most of them are not exposed to chemotherapy or radiation. New evidence indicates that genomic instability in FA is due to a combination of abnormal cell cycle progression and hypersensitivity to normal metabolic by-products such as aldehydes.

**Replication hazards: Fanconi anemia and the S-phase checkpoint**

DNA replication carries an intrinsic risk of DNA damage. Replication-associated DNA breaks are caused
by topological stress induced by unwinding of chromatin fibers tethered to the nuclear matrix and by collisions between the DNA polymerases and the RNA transcription machineries [69,70]. In the presence of DNA crosslinks, the double helix cannot be unwound, and the replication forks stall to repair the crosslink before the replication continues. The S-phase checkpoint ensures stability of replication forks and relieves the effect of replication stress on the genome [71,72]. Notably, the same kinases that control the FA signaling during DNA damage response (e.g. ATR, CHK1) are essential for the S-phase checkpoint [73]. Thus, it is not surprising that the FA signaling is essential for the intra-S-phase checkpoint triggered by DNA interstrand crosslinks [74,75].

The pain of living: Fanconi anemia signaling alleviates metabolic collateral damage to the genome

We are constantly bombarded by toxic molecules that are either required for our metabolism (oxygen) or generated as metabolic by-products (such as aldehydes). FA signaling protects our genomes from harmful effects of normal life processes.

The hypersensitivity of FA-deficient cells to oxidative stress is well known [76,77]. Recent studies revealed that the FA signaling provides a functionally important shield against the DNA damage induced by endogenous aldehydes. Since aldehyde metabolism may be amenable to small-molecule manipulation [78,79], this finding may provide future opportunities to decrease DNA damage and functional depletion of bone marrow cells in FA patients.

Aldehydes are highly reactive, carcinogenic molecules generated through metabolism of multiple chemicals, including alcohols [80]. FA-deficient chicken cells are hypersensitive to aldehyde-induced DNA damage [81]. To examine the potential role of endogenous aldehyde-induced DNA damage in FA-associated bone marrow failure and cancer, Langevin et al. disrupted the aldehyde detoxification pathway in Fancd2<sup>−/−</sup> mice by knocking out the Aldh2 aldehyde dehydrogenase. The Fancd2<sup>−/−</sup> mice are prone to developmental defects and epithelial cancers [82] but do not spontaneously develop leukemia, although their bone marrow stem cells have mild baseline proliferation defects [83]. However, the double-knockout Fancd2<sup>−/−</sup> Aldh2<sup>−/−</sup> mice accumulate hematopoietic stem cell mutations [84]. These mice tend to die of acute leukemias early in life [82], and a small fraction of surviving leukemia-free Fancd2<sup>−/−</sup> Aldh2<sup>−/−</sup> mice develop bone marrow aplasia at a later age [84]. Interestingly, this sequence of events is reversed compared with humans with FA, who develop bone marrow failure before the onset of leukemia. More work is needed to evaluate the contribution of endogenous aldehydes to genomic instability in FA patients and to evaluate activation of aldehyde detoxification as a therapeutic strategy. Nevertheless, it is prudent to advise FA patients to avoid alcohol as it is metabolized to aldehydes.

Division sickness: Fanconi anemia signaling prevents aneuploidy by regulating mitosis

Abnormal chromosome segregation during cell division leads to gross genomic instability and aneuploidy, which are both a cause and consequence of cancer [85]. Cell cycle checkpoints prevent chaotic chromosome segregation in mitosis. The spindle assembly checkpoint (SAC) arrests cell division until all chromosomes are captured by the mitotic spindle microtubules [86]. Weakened spindle checkpoint increases the risk of random chromosome segregation, aneuploidy, and cancer. Not surprisingly, many SAC regulators are tumor suppressors [86].

We recently showed that disruption of FA signaling weakens the SAC, and we visualized multiple FA proteins on the mitotic spindle and centrosomes [87]. This connection between the FA signaling and the SAC helps us understand the origins of gross chromosomal instability in FA and sheds new light on the previously reported biochemical interactions between FA proteins and the key SAC regulators, such as the CDC2 cyclin-dependent kinase [88,89]. Efforts are under way to identify FA-regulated branches of the highly complex SAC signaling network. Since multiple SAC regulators are amenable to small-molecule targeting, we expect that dissecting the links between the FA signaling and the spindle assembly checkpoint may reveal new therapeutic targets in FA-deficient cancers – both in FA patients and in the general population.

Recent work has shown that multiple FA proteins associate with centrosomes during mitosis to control centrosome function and physically interact with regulators of centrosome maintenance [90-92]. FA-deficient cells cannot maintain normal centrosome numbers under normal conditions [87,92-95] and upon exposure to DNA cross-linking agents [91]. While the essential role of FANCD1/BRCA2 in centrosome maintenance and mitosis is well established [21,94,95], these new findings reveal the essential role of the entire FA signaling network in ensuring genome stability during mitosis. The presence of supernumerary centrosomes further disrupts chromosome segregation through abnormal kinetochore-spindle interactions [96]; therefore, we hypothesize that centrosome dysfunction further worsens genomic instability in FA-deficient cells through abnormal chromosome segregation. The key FA-dependent signaling pathways responsible for centrosome maintenance and high-fidelity...
execution of mitosis remain unknown, although the polo-like kinase 1 (PLK1) was implicated in FA-dependent centrosome replication under genotoxic stress [91] and the never-in-mitosis-gene A (NIMA)-related kinase 2 (NEK2)-dependent phosphorylation of FANCA was proposed to regulate baseline centrosome replication [92]. Seamless execution of later stages of mitosis also depends on the functional FA pathway. Some FA proteins resolve entangled DNA during mitosis by associating with anaphase bridges in collaboration with the BLM signaling pathway [97,98]. Finally, FA-deficient cells undergo abnormal cytokinesis [99], which further impairs chromosome segregation at the exit from mitosis.

Together, these data reveal that the FA signaling prevents genome instability through ensuring DNA damage repair in interphase and normal progression through mitosis (Fig. 2).

**Fanconi anemia and p53: two guardians wrestling**

Improved understanding of genomic instability mechanisms in FA (Fig. 2) allows us to reconstruct the genome instability seen in FA cells. Another key tumor suppressor, p53, determines the fate of proliferating FA-deficient cells. Chronic DNA damage activates the p53 response and promotes the p53/CHK1-dependent G2/M cell cycle arrest [100-103], which decreases the pool of proliferating stem cells leading to bone marrow failure [100]. The proliferating cells develop progressive genomic instability as they undergo abnormal mitosis. The grossly aneuploid multinucleated cells generated through failed cell division are subject to further mutagenesis. This vicious cycle of genomic instability ultimately leads to either bone marrow failure (if the p53 cell cycle arrest prevails [100]) or cancer (if the p53 response is inactivated and genetically unstable cells are allowed to proliferate [104,105]).

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**Figure 2. Fanconi anemia (FA) signaling pathway guards the genome throughout the cell cycle**

FA signaling prevents accumulation of DNA damages in interphase and ensures high-fidelity chromosome segregation in mitosis. Abbreviations: SAC, spindle assembly checkpoint.
Cytokine storm in Fanconi anemia

FA-deficient cells express abnormally high levels of several pro-inflammatory cytokines, including tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) [106,107]. It was confirmed that these essential mediators of inflammation are overexpressed in bone marrow of FA patients [108]. This hyperactivation of the cytokine network appears particularly detrimental to the survival of FA-deficient hematopoietic cells because loss of the FA signaling pathway renders hematopoietic cells dramatically hypersensitive to the growth-inhibitory and death-promoting effects of the cytokine storm. Hematopoietic cells of the Fancc−/− mice are hypersensitive to the growth-inhibitory effect of IFNγ both in vitro [109,110] and in vivo when IFNγ is administered as continuous infusion [111,112]. This is at least partially mediated by increased activity of the Fas apoptotic programme, leading to the activation of caspases and death of hematopoietic cells [113-115].

Importantly, exposure to pro-inflammatory cytokines not only promotes the death of FA-deficient cells but also promotes the emergence of genomically unstable leukemic clones [116,117], highlighting the potential role of abnormal inflammatory response in leukemogenesis in FA. Since genetic correction of FANCC patient cells does not normalize expression of the essential p53 downstream gene (p21) [118], the inflammatory and p53-dependent pathways of hematopoietic cell loss and malignant transformation in FA are likely to be separate. Therefore, it is important to consider both signaling pathways as candidate therapeutic targets in future studies aiming to improve survival and decrease leukemogenic potential in FA patients.

Summary

Recent studies have provided exciting insights into the origins of genomic instability in FA. We now know that loss of FA signaling not only disrupts DNA damage recognition and repair in interphase [24] but also disrupts chromosome segregation in mitosis through weakening multiple cell division checkpoints [87,97-99]. These discoveries explain why loss of FA signaling promotes point mutations as well as gross chromosomal abnormalities. The signaling nodes connecting the FA signaling to other signal transduction pathways are being dissected through system biology studies. More detailed understanding of functional interactions between FA proteins, DNA damage kinases, and other signal transducers provides opportunities for a rational choice of therapeutic targets in FA-associated bone marrow failure and cancer. For example, FA-deficient cancer cells are hypersensitive to inhibition of the ataxia telangiectasia mutated (ATM) kinase [119], which is not surprising given the cross-talk between the DNA damage detection and the FA signaling pathway. Since cells deficient in homologous recombination-based DNA repair are hypersensitive to inhibition of the poly-(ADP-ribose) polymerase (PARP) pathway, small-molecule PARP inhibitors are being explored as targeted therapies of FA-deficient cancers [120]. However, the selected candidate targets should be carefully evaluated in preclinical studies to evaluate potential risks and benefits. For example, while silencing the p53 response may alleviate bone marrow failure in FA, it may also promote cancer [100,104,105]. Finally, better understanding of genotype-phenotype correlations in FA as well as optimizing clinical care of FA patients through clinical research will allow us to offer evidence-based interventions to this complex yet extremely rewarding group of patients.

Abbreviations

AML, acute myeloid leukemia; DEB, diepoxybutane; FA, Fanconi anemia; HPV, human papillomavirus; IFNγ, interferon gamma; NHEJ, non-homologous end joining; PARP, poly-(ADP-ribose) polymerase; SAC, spindle assembly checkpoint; SCC, squamous cell carcinoma.

Disclosures

The authors declare that they have no disclosures.

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