Spontaneous abrogation of the G2 DNA damage checkpoint has clinical benefits in Fanconi anemia patients

Raphael Ceccaldi,1,2,3 Delphine Briot,1,2,3 Jérôme Larghero,4 Nadia Vasquez,1,2,3 Catherine Dubois d’Enghien,5 Delphine Chamoussset,1,2,3 Maria-Elena Noguera,1,2,3 Quinten Waisfisz,6 Olivier Hermine,7 Corrine Pondarre,8 Thierry Leblanc,9 Eliane Gluckman,10,11 Hans Joenje,6 Dominique Stoppa-Lyonnet,5 Gérard Socié,11 and Jean Soulier1,2,3

DNA damage checkpoints in the cell cycle may be important barriers against cancer progression in human cells. Fanconi anemia (FA) is an inherited DNA instability disorder that is associated with bone marrow failure and a strong predisposition to cancer. Although FA cells experience constitutive chromosomal breaks, cell cycle arrest at the G2 DNA damage checkpoint, and an excess of cell death, some patients do become clinically stable, and the mechanisms underlying this, other than spontaneous reversion of the disease-causing mutation, are not well understood. Here we have defined a clonal phenotype, termed attenuation, in which FA patients acquire an abrogation of the G2 checkpoint arrest. Attenuated cells expressed lower levels of CHK1 (also known as CHEK1) and p53. The attenuation could be recapitulated by modulating the ATR/CHK1 pathway, and CHK1 inhibition protected FA cells from cell death. FA patients who expressed the attenuated phenotype had mild bone marrow deficiency and reached adulthood, but several of them eventually developed myelodysplasia or leukemia. Better understanding of attenuation might help predict a patient’s clinical course and guide choice of treatment. Our results also highlight the importance of evaluating the cellular DNA damage checkpoint and repair pathways in cancer therapies in general.

Introduction

Checkpoint pathways are complex biological pathways that regulate responses to DNA damage and other cellular events (1–3). The typical DNA damage checkpoint response triggers cell cycle arrest, allowing time for DNA repair, and impairments in checkpoints favor genomic instability and cancer (4). In humans, DNA damage checkpoint pathways include the post-translational activation of the transduction proteins CHK1 checkpoint homolog (Schizosaccharomyces pombe) (CHK1, also known as CHEK1) and CHK2 checkpoint homolog (S. pombe) (CHK2) by PI3-like kinase ataxia telangiectasia and Rad3 related (ATR) and ataxia telangiectasia mutated (ATM) (4, 5). If cells can not repair damage during cell cycle arrest, cell death is induced. Recently, DNA damage response pathways have also been implicated in cellular responses to oncogenic activation, and checkpoint pathways are believed to constitute a first-line anticancer barrier (6–8). Therefore, an important step of cancer progression is the inactivation of checkpoint pathways. Here we studied a series of patients with Fanconi anemia (FA), a genetic disease that is characterized by genomic instability, checkpoint arrest, and cancer predisposition and identified what we believe to be a new cellular phenotype that is associated with clonal evolution and leukemogenesis.

FA is an inherited condition that is caused by mutations in 1 out of the 13 FA-associated genes (9, 10). Products of these genes interact in the FANC/BRCA pathway, in which a pivotal event is the monoubiquitination of FA, complementation group D2 (FANCD2) (11). Affected patients can develop various congenital abnormalities, including short stature (12, 13). They usually experience bone marrow failure during childhood, and they have a considerable predisposition to cancer, especially myelodysplasia (MDS), acute myeloid leukemia (AML), and head and neck cancers (12–16). FA cells are hypersensitive to DNA interstrand crosslinking (ICL) agents and harbor high levels of spontaneous and induced chromosome breaks (9, 10, 12, 13). On exposure to ICL agents, such as mitomycin C (MMC), FA cells undergo massive G2 arrest, which is considered a normal checkpoint response to unresolved DNA damage during S phase (17–19). Consequently, FA cells experience slower progression through the cell cycle, inhibition of growth, increased p53 expression, and cell death (20–22). FA cells can undergo somatic mosaicism by spontaneous reverse mutation or mitotic recombination (23–25). This phenomenon of reversion allows genetically corrected cells to be selected to repopulate the bone

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Results

A new phenotype in FA patients — attenuation of the G2 checkpoint in response to DNA damage. FA cells are characteristically hypersensitive to DNA crosslinking agents. During diagnosis or follow-up evaluation of FA patients, we noted patients in whom the FA tests on peripheral blood lymphocytes (PBLs) were dissociated. In these patients, phytohemagglutinin-stimulated (PHA-stimulated) fresh PBLs did not arrest in G2 phase after MMC exposure, although they harbored chromosomal breaks and lacked FANCD2 monoubiquitination (Figure 1 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI43836DS1). The abrogation of the G2 checkpoint was previously reported in rare (non-FA) cancer cell lines, and it was defined as attenuation (28). Based on our observation, we hypothesized that the G2 checkpoint can be attenuated in FA cells, prompting us to evaluate this phenomenon in a large cohort of FA patients. We defined the attenuated phenotype as the combination of (a) a high level of chromosomal breaks after MMC exposure in the Fanconi range (“positive chromosome breakage test”); (b) a lack of FANCD2 monoubiquitination, characteristic of FA cells with an upstream defect in the FA/BRCA pathway (“positive FANCD2 test”); and (c) a paradoxical absence or significant decrease in MMC-induced FA cell G2 arrest (“negative cell cycle test”). These criteria allowed us to differentiate the attenuated phenotype from that of “classical” FA, in which all 3 tests are positive, and from that of reversion, in which the 3 tests are completely or partially normalized (somatic mosaicism).

Attenuation is found in older FA patients and is associated with milder bone marrow failure or MDS/leukemia. We evaluated a cohort of 97 FA patients (group FA-A, n = 80; FA-G, n = 9; FA-D2, n = 7; other, n = 1) for the presence of attenuation. This cohort included FA patients at Saint-Louis Hospital who underwent a complete analysis of fresh PBLs, particularly in the oldest patients, and excluded bone marrow transplantation patients. The 97 patients were classified as classical FA (i.e., patients with a standard FA phenotype), revertant, or attenuated. Sixty-eight out of ninety-seven patients had classical FA (70.1%), 12 patients had a revertant phenotype (12.4%), and 17 patients had the attenuated phenotype (17.5%). Patients with the attenuated phenotype were clearly not revertants, because they had a positive chromosome breakage test and lacked FANCD2 monoubiquitination in PHA-stimulated PBLs. In addition, reversion events were not observed after resequencing the FANC mutations in the PBLs of the attenuated patients (n = 10), whereas 3 revertant cases, tested as controls, harbored a point reversion in DNA from their PBLs (data not shown).

We analyzed the distribution of the FA phenotypes by patient age (Figure 1B). Notably, whereas reversion and attenuation were rare compared with classical FA in young patients, most patients with an attenuated or revertant phenotype were over 20 years of age. The median blood cell count in attenuated patients approximated normal levels (hemoglobin, 12.6 g/l; white blood cells, 3.9 × 10^9/l, neutrophil cells, 1.8 × 10^9/l, platelets 106 × 10^9/l), suggesting that attenuation, like reversion (26), was associated with a better clinical outcome with respect to bone marrow failure, allowing patients to reach adulthood (7 out of the 17 patients with the attenuated phenotype did not experience bone marrow failure; Supplemental Table 1). Notably, 5 FA patients (aged 16, 20, 26, 36, and 50 years) with the attenuated phenotype in PHA-stimulated PBLs developed MDS or AML (Supplemental Table 1).

Collectively, these data demonstrate that attenuation is frequent in older FA patients and is associated with better clinical outcomes but also that it can be found in patients with MDS or leukemia. The attenuated phenotype is acquired. Next, we determined whether the attenuated phenotype was acquired or constitutive. The FANC data from the 17 attenuated phenotype patients could not exclude in all cases the possibility of hypomorphic mutations (Supplemental Table 1 and ref. 29). Therefore, we performed FA tests on primary fibroblasts, as constitutive cells, which we could perform for 16 out of 17 patients. Two patients experienced intermediate G2 arrest and mild MMC sensitivity in their fibroblasts, and they were excluded from further analysis. In the remaining 14 patients, primary fibroblasts showed massive MMC-induced G2 arrest, typical of FA (Figure 1B and Supplemental Table 1). The patient difference between the PBL and fibroblast data demonstrated that attenuation, like reversion, was an acquired, not constitutive, phenotype. In addition, conversion from the classical to attenuated FA phenotype occurred in 2 patients at distant analyses (at 6- and 3-year intervals, respectively; Supplemental Figure 2).

Clonality of attenuated cells. To determine whether attenuation in PBLs was related to clonal evolution, we searched for somatic chromosomal abnormalities. Genomic DNA from PBLs of attenuated patients was subjected to high-resolution chromosomal profiling (array-CGH/SNP). In 8 of 13 cases, gross chromosomal copy number alterations were observed (Figure 2A and Supplemental Table 1). Notably, such alterations included several chromosomal abnormalities that are common in FA clonal evolution, such as gain of chromosome arm 1q or 3q and deletion of 11q.

In a separate approach, we measured clonality by X-linked inactivation analysis (Figure 2B). All 5 patients with the attenuated phenotype who could be analyzed, including 3 without detectable chromosomal abnormalities, showed skewed allelic expression that was consistent with clonal selection. In contrast, nearly all healthy controls had a balanced pattern. As expected, the revertant patient showed skewed allelic expression. Balanced allelic expression was observed in 3 out of 6 classical FA patients, consistent with polyclonal cells, and interestingly, a skewed expression was noted in the 3 others, suggesting that mechanisms other than attenuation also lead to clonal restriction in FA.

Collectively, these data are consistent with a model of natural selection of the attenuated cells in FA patients. Downregulation of checkpoint proteins in attenuated FA cells. We hypothesized that an acquired defect in the checkpoint pathways caused the attenuated phenotype. Expression of the prototypical DNA damage response proteins CHK1, CHK2, and p53 was measured in fresh blood samples from 9 attenuated phenotype
patients, 14 classical FA subjects, and 4 healthy samples (Figure 3). p53 expression was high in classical FA cells compared with that in healthy controls (Figure 3A), suggesting that p53 is induced in fresh FA cells in response to endogenous and induced DNA damage, consistent with previous reports of human FA EBV cell lines and murine fibroblasts (20–22). Notably, we observed low expression of CHK1 and p53 in the PHA-stimulated PBLs of 6 out of 9 attenuated phenotype patients compared with that in classical FA
subjects but not in the fibroblasts (Figure 3A and data not shown). Moreover, low levels of CHK1 but not CHK2 transcripts were detected by real-time quantitative PCR (RQ-PCR) in most attenuated patients compared with patients with classical FA (Figure 3B). By analyzing sequence and copy number of CHK1 and TP53 in low-expression level patients, no genomic mutation or focused deletion was detected, suggesting other mechanisms of deregulation. We therefore investigated epigenetic changes. Whereas we found no DNA methylation changes in attenuated cells (Supplemental Figure 3A), in silico analysis of CHK1 3′ UTR predicted numerous target sites for 2 microRNAs, namely miR15-a and miR16-1, which were previously reported to target CHK1 expression (30); interestingly, we found a higher expression of miR15-a, but not miR16-1, in attenuated cells compared with that in classical FA cells, suggesting a mechanism of CHK1 expression downregulation (Supplemental Figure 3B). In addition, unlike CHK2, which is a stable protein, CHK1 is a rather unstable protein due to proteasome degradation (31). We evaluated CHK1 turnover and found an acceleration in the attenuated cells, demonstrating that posttranslational mechanisms could also contribute to the low levels of CHK1, which were observed during attenuation (Figure 3C).

Based on these data, the transcriptional and/or posttranslational downregulation of CHK1 expression constituted a putative mechanism for attenuation of the G2 checkpoint in FA cells.

Suppression of the ATR/CHK1 checkpoint pathway reproduced the attenuated phenotype and protects FA cells from DNA damage-induced cell death in short-term culture. We determined whether attenuation could be recapitulated by checkpoint inhibition. We used an siRNA

Figure 2
Clonality of attenuated FA cells. (A) Array-CGH showed clonal chromosomal abnormalities in PHA-stimulated PBLs from an attenuated patient. MGG stains confirmed the activated lymphoblast morphology (original magnification, ×400). Arrows indicate copy number abnormalities. (B) X-linked–based analysis of clonality; female FA patients who were heterozygous for 1 or more of the 3 SNPs in the X chromosome were identified by direct sequencing of genomic DNA. Then, allelic X-linked inactivation was evaluated in a semiquantitative manner by sequencing PBL cDNA from these patients. The left panel shows balanced expression of the heterozygous MMP1 SNP in a healthy control and a classical FA patient, whereas skewed expression was detected in the attenuated and revertant patients (asterisks). The right panel summarizes the X-linked clonality data in heterozygous PBLs from healthy female controls (n = 11), attenuated patients (n = 5), revertants (n = 1), and classical FA subjects (n = 6). The percentage of skewing expression is quantified for each case, and median values are indicated by horizontal bars. The threshold for skewed unbalanced expression was arbitrarily fixed (dashed horizontal line). Each symbol represents a patient.
FANCD2-silenced HeLa cell line as an FA-like cell line for its convenience with cotransfection. G2 arrest was abrogated in CHK1- and ATR-depleted cells; in contrast, ATM, CHK2, and BRCA1 depletion did not affect G2 arrest (Figure 4A). We then evaluated the effect of checkpoint inhibitors on FA lymphoblastoid (EBV-immortalized) cells. An inhibitor of CHK1, but not CHK2, relieved the G2 arrest (Figure 4B). Similarly, fresh FA PBLs were converted to attenuated cells by the CHK1 inhibitor (Supplemental Figure 4).

We next examined whether CHK1 inhibition protected FA cells from MMC-induced cell death in short-term cultures. FA primary fibroblasts were grown with or without CHK1 inhibitor in increasing concentrations of MMC. Cell death was reflected by the nascent fraction of cellular fragments (sub-G1) by flow cytometry. Strikingly, FA cells that were incubated with the CHK1 inhibitor were protected from MMC-induced death (Figure 5 and Supplemental Figure 5). These data demonstrate that inhibition of CHK1 or ATR, but not CHK2, ATM, or BRCA1, mimics the attenuated phenotype in FA cells. The absence of CHK1 abrogates the G2 checkpoint and protects FA cells from death, consistent with our hypothesis that clonal attenuation in FA improves survival.

Attenuated phenotype in FA patients with MDS with excess blasts or acute leukemia. Five out of the ninety-seven FA patients had MDS with an excess of blasts or overt myeloid leukemia. In all of these patients, aged 16, 20, 26, 36 and 50 years, the PHA-stimulated PBLs had the attenuated phenotype (i.e., chromosome breaks and no FANCD2 monoubiquitination but no G2 arrest). This finding raises the possibility that the attenuated phenotype is involved in malignant transformation. We purified blast cells from fresh bone marrow aspirate in 4 out of the 5 patients and measured CHK1 and CHK2 expression. The clonal origin and purity of the CD34-sorted cells were confirmed by array-CGH (data not shown).

Importantly, the tumoral cells expressed minute levels of CHK1 but not of CHK2 (Figure 6), reminiscent of what was observed in attenuated PBLs, suggesting that leukemic cells would be unable to arrest through the ATR-CHK1 axis. In line with published data (32), very low levels of CHK1 were also found in a reference series of primary AML cells from non-FA patients (Figure 6), consistent with a model of suppression of the DNA damage response in AML as a late transformation event (Figure 7). Importantly, chemotherapy treatment (33) effected complete remission in these FA patients with MDS/AML, demonstrating that the leukemic cells were sensitive to chemotherapy.

Discussion

Based on our analysis of patients who were evaluated in a specialist center, we identified a type of spontaneous evolution in FA — attenuation, an acquired abrogation of the G2 checkpoint in FA cells (Figure 1). FA patients with the attenuated phenotype were older than classical FA subjects and had near-normal blood cell counts, suggesting that this phenotype is beneficial for hematopoiesis. Therefore, our working hypothesis has been that checkpoint attenuation allows FA cells to survive despite...
spontaneous DNA damage, whereas in classical (not attenuated) FA cells, the DNA damage response pathway is deleterious by triggering unresolved checkpoint arrest. The distribution of the various phenotypes (classical, attenuated, and revertant) throughout different age groups suggests that a model exists in which hematopoiesis evolves clonally in most FA patients during adolescence or early adulthood as a response to stem cell exhaustion and selective pressure. Such evolution can arise spontaneously through attenuation or reversion, or it can be driven by allogeneic bone marrow transplantation. Attenuation

Figure 4
Inhibition of CHK1 and ATR, but not CHK2, ATM, or BRCA1, mimics the attenuated phenotype in FA cells. (A) Cell cycle analysis of MMC-induced G2 arrest in HeLa cells transfected with combinations of siRNAs (si) against luciferase (control), FANCD2, and the checkpoint genes CHK1, CHK2, ATR, ATM, and BRCA1, as indicated. FANCD2-silenced HeLa cells displayed a strong MMC-induced G2 arrest and were used as FA-like cells for convenience in cotransfection experiments. The immunoblot shows the knockdown of different proteins for each siRNA. Arrows and asterisks indicate typical FA G2 arrest and its abrogation, respectively. All data were obtained in at least 3 independent experiments for each targeted gene with consistent results. The arrows indicate the MMC-induced G2 arrest, and the asterisk designates the G2 checkpoint abrogation. Horizontal bars in A and B indicate the G1 and G2 cell cycle phases (M1 and M2, respectively).
could explain the pattern in families in which FA siblings have patent phenotypic differences despite similar genotypes, reminiscent of what has been observed for reversion. Notably, neither attenuation (this work), nor reversion (27) appears to protect against transformation into MDS and leukemia, and we believe that all FA patients have to be monitored for cancer detection for life, including, if possible, by a yearly bone marrow aspirate.

We investigated the biological mechanisms of attenuation by analyzing the prototypical checkpoint pathways in FA cells. CHK1 levels, but not CHK2 levels, were frequently very low in attenuated blood cells. CHK1 downregulation was related to transcriptional and/or posttranslational mechanisms, and interestingly, miR15a, previously reported to silent CHK1 (30), could contribute to the low CHK1 levels. Downregulation of the ATR-CHK1 axis, but not ATM-CHK2 or BRCA1, by siRNA or chemical inhibitors abrogated the G2 arrest, mimicking the attenuated phenotype and confirming that CHK1 function is pivotal for G2 accumulation in FA cells (34–36). Moreover, CHK1 inhibition protected FA cells from DNA damage-induced immediate cell death, consistent with our model, in which G2 checkpoint attenuation allows FA cells to grow despite spontaneous DNA damage, in contrast to deleterious checkpoint responses in classical FA cells. In addition, CHK1 downregulation is likely to contribute to the genetic instability through deregulation of the normal cell cycle transitions, especially G1/S and G2/M (refs. 37 and 38 and Supplemental Figure 6). In genomic analyses of attenuated PBLs, we did not detect any mutation or focused deletion in CHK1 or TP53 but observed that these cells experienced frequent gross somatic chromosomal alterations, such as duplication of chromosome 1q (Supplemental Table 1). Notably, this chromosomal abnormality is commonly associated with clonal evolution in FA (39, 40), and it might be involved in the attenuation mechanism. It is noteworthy that the molecular target and related downstream pathways of gross chromosomal abnormalities in neoplasias remain largely to be identified, see for instance trisomy 8 or monosomy 7 in clonal evolution of aplastic anemia or MDS/AML in non-FA patients (41–44).

Finally, it is likely that other changes in pathways that regulate cellular stress, apoptosis, and senescence can also contribute to cell survival and transformation throughout the natural history of FA patients, as occurs in mice (45).

Genomic instability is a common feature of cancer, wherein it promotes the accumulation of genomic alterations, leading to the deregulation of oncogenes and tumor suppressor genes (46). In very rare genetic conditions, such as FA, the instability is constitutive, and patients with this condition have a strong predisposition to cancer (12–16). Recently, the DNA damage response has emerged as a major anticancer barrier, and DNA damage response inactivation is an important step for cancer progression (6–8). In our FA cohort, 5 patients with attenuated PBLs experienced MDS or AML. Strikingly, the leukemic cells expressed CHK2 but very low levels of CHK1, suggesting that these cells would fail to elicit CHK1-dependent checkpoint arrest. Therefore, the attenuation

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**Figure 5**

CHK1 inhibitor protects FA cells from DNA damage-induced cell death in short-term cultures. (A) Representative flow cytometry–based analysis of cell death of FA primary fibroblasts, with or without CHK1i. The fraction of cellular fragments (sub-G1, bars) was measured in each condition. Consistent data were obtained in at least 2 experiments for each of 4 unrelated FA patients. (B) Graphic representation of the percentage of cellular fragments measured in A. Annexin V staining is shown in Supplemental Figure 5. (C) MGG staining of FA primary fibroblasts with or without CHK1i treatment with 25 ng/ml MMC (original magnification, x160).
phenomenon might contribute to leukemogenesis by promoting survival in an FA background of constitutive genomic instability (Figure 7). In patients who do not have a genetic predisposition (non-FA patients), the common stepwise order of oncogenic events likely differs slightly (Figure 7). The genomic instability and induction of the DNA damage checkpoint are not constitutive but are likely to be induced as a consequence of the initial oncogenic events (6–8), as in primary human MDS and AML (32, 44). As a result, the inactivation of DNA damage response pathways, often by TP53 inactivation, would allow cells to survive despite the persistent genomic instability, accelerating the progression to late stages (6–8, 32, 44, 47).

These findings and concepts have important implications, not only for gaining a better understanding of the clinical course of FA, but also for developing cancer treatments for FA and non-FA patients. Based on our data, the acquired attenuation of checkpoints gives a survival advantage to FA cells in a background of endogenous DNA damage. In contrast, one can predict that attenuated cancer cells will be highly sensitive to chemotherapy, unlike revertant cancer cells (27). In an environment of high and sustained DNA damage, such as that during chemotherapy, attenuated cells will die due to checkpoint deficiencies and mitotic dysfunction. Accordingly, the 5 FA patients with MDS/AML and the attenuated phenotype who we evaluated responded very well to usual FA chemotherapy (33), and only 1 experienced a relapse 7–42 months after treatment. Interestingly, these results and concepts in patients are consistent with a recent preclinical model in mice, in which concomitant abnormal levels of Atm, Chk2, and p53 are more sensitive to chemotherapy (48).

Figure 6
Low expression of CHK1, but not CHK2, in the leukemic cells from MDS/AML FA patients (n = 4). Leukemic cells were purified by CD34+ selection; the clonality and high purity of the blast cell fraction was demonstrated in all of the 4 MDS/AML FA patients by array-CGH profiling, which detected chromosomal alterations (data not shown). Data from classical FA patient PHA-stimulated PBLs are indicated as reference (several samples were reanalyzed with consistent results). Leukemic cells from a series of non-FA patients (n = 15) with AML were analyzed as malignant reference. RQ-PCR copy number values are expressed using the ΔCT method relative to a housekeeping gene (reverse log2 scale). Mean values are indicated by horizontal bars. Statistical analyses were performed using the non-parametric test Wilcoxon test (**P < 0.001). As expected from published data in MDS and AML in non-FA patients (32), very low levels of CHK1 transcripts were also found in the non-FA AML cells. Each symbol represents a patient.

In conclusion, we believe that a careful evaluation of hematopoietic attenuation and reversion in FA patients can help understand and predict the clinical course of the disease. Further, in non-FA patients, the evaluation of genomic instability and checkpoint and repair pathways in cancer cells should also help clinicians tailor the chemotherapy and specifically target the relevant checkpoint/repair pathways (16, 49–53).

Methods
Patients and biological material collection and cell lines. This study was based on a cohort of FA patients, followed for 6 years at Saint-Louis Hospital, the French national reference center for FA. Written informed consent was obtained from the patients and/or their relatives. The study was approved by the Institutional Review Board at Hôpital Saint-Louis. Patients were aged 1 to 56 years (median age, 12 years). All patients had an FA diagnosis based on classical FA tests, including the chromosomal breakage test (12, 26). The FA-associated gene mutation data have been entered into the international IFAR registry (www.rockefeller.edu/fanconi/mutate); mutations data in the patients with the attenuated phenotype are described in Supplemental Table 1. The 97 patients were distributed as follows: FA-A, n = 80; FA-G, n = 9; FA-D2, n = 7; other, n = 1, which is representative of all French FA patients. Patients were referred to using a unique identification number (EGF), following our nomenclature. Blood cells and primary fibroblasts were collected. CD34-positive cells from bone marrow of MDS/AML FA patients were purified using the CD34 MicroBead Kit (Miltenyi).

Several cell lines were EBV-derived from FA patients at our center and used for this study. The HeLa cell line that was used for functional studies was obtained from ATCC.

Cell culture and FA tests. Chromosomal breakage tests and FANCD2 monoubiquitination analysis by immunoblot were performed on PBLs and fibroblasts using conventional methods (12, 26, 54).

For cell cycle analysis, MMC was used at 0, 30, and 75 ng/ml for PHA-stimulated PBLs and at 0, 10, and 25 ng/ml for primary fibroblasts. In all experiments, growing cells (fresh PHA-stimulated PBLs or cultured primary fibroblasts) were treated with MMC, fixed in chilled 70% ethanol, washed with PBS after overnight storage at ~20°C, and resuspended in propidium iodide in PBS with 0.1% Triton X-100 and 100 μg/ml DNase-free RNase A. The stained cells were sorted on a Becton Dickinson FACSCalibur and analyzed using CellQuest (Becton Dickinson). Approximately 10,000 cells per sample were analyzed. EBV and HeLa cells were treated with 0 and 75 ng/ml or 100 ng/ml MMC, respectively.

FA cell death after a 96-hour incubation with MMC, with or without CHK1 inhibitor, was calculated by measuring the percentage of cellular fragments (sub-G1), derived from a flow-based MMC-hypersensitivity test in primary fibroblasts (55). In parallel experiments, FA primary fibroblasts were grown in a Lab-tekII Chamber Slide (Nalge Nunc International) in increasing dilutions of MMC, with or without CHK1 inhibitor, and analyzed by in situ staining of adherent cells with May Grunwald Giemsa (MGG). Apoptosis was further analyzed by Annexin V staining.
Figure 7
Genomic instability, DNA damage response, and stepwise oncogenesis — a model of stepwise progression to MDS/AML in patients with or without constitutional genetic instability. In FA patients, the first step is constitutive and leads to excess cell death related to the G2 checkpoint response; the attenuation phenomenon described here rescues cell survival and allows for the accumulation of additional oncogenic events that are favored by the genetic instability. In patients that are not genetically predisposed (non-FA patients), activation of oncogenes is associated with acquired genetic instability and induction of anticancer DNA damage response (6–8); further inactivation of this response, often by TP53 inactivation, allows cancer progression to late stages (6–8, 32, 44, 47). Notably, no TP53 mutation or deletion was observed in the 5 MDS/AML leukemia cases that developed in attenuated FA patients in our series (data not shown).

Chemical inhibitors. Chemical inhibitors against CHK1 (CHK1i; SB-218078 [Calbiochem]) and CHK2 (CHK2i; C3742 [Sigma-Aldrich]), were dissolved in DMSO and used at a final concentration of 1 μM and 100 nM, respectively. The diluted solution of DMSO was used as control. The translational inhibitor, cycloheximide (CHX; Calbiochem) and the proteasome inhibitor MG132 (Calbiochem) were dissolved in DMSO and used at a final concentration of 25 μg/ml and 20 μM, respectively. The diluted solution of DMSO was used as control.

Transfection. Targeted genes were knocked down by transient expression of siRNA against FANCD2 (5′-GGAGAUUGAUGGUCUCdAdTdT-3′), LUC (5′-CGUACCGGAUACUUGAdTdT-3′), CHK1 (5′-GGACUUCUCCAUGUAACdAdTdT-3′), CHK2 (5′-GACCCUGAGGCGUCCUACCdAdTdT-3′), ATR (5′-AACCCGUUGAUUGCGUUGAdTdT-3′), ATM (5′-AAGCGCCUGAUUCGAGAUCdAdTdT-3′), and BRCA1 (5′-CUAGAAAUUCUGUUGCUAGdAdTdT-3′). All siRNA duplexes were purchased from Eurogentec, except CHK1, which was purchased from Dharmacon. siRNA or the combination of 2 different siRNAs were used at a final concentration of 100 nM. siRNA duplexes were transfected using the Lipofectamine Reagent (Invitrogen) according to the manufacturer’s recommendations. Luciferase-specific siRNA served as the negative control. In all experiments, a fraction of transfected cells was analyzed by immunoblot to assess knockdown efficiency. For cell cycle experiments using transfected cells, HeLa cells were plated 24 hours before transfection, MMC was added 72 hours after transfection, and cells were harvested at 96 hours and immediately fixed.

Evaluation of clonality. Array-CGH (105K, Agilent Technologies) or SNP-array (500K, Affymetrix) were used for genomic PBL DNA according to the manufacturer’s protocols. For SNP analysis, paired fibroblast DNA was used as a reference. Data were analyzed using DNA Analytics (Agilent Technologies) or Genomic Suite (Partek).

For X-linked inactivation analysis (56), 3 SNPs in X chromosome genes, subjected to X-linked inactivation, were studied: dbSNP 1126762 (MPP1), dbSNP 9018 (FHL1), and dbSNP 1135363 (BTK). Primer sequences are shown in Supplemental Figure 7A. Briefly, SNPs were sequenced from genomic DNA of female FA patients to determine heterozygosity. The relative expression of each allele was determined by direct sequencing of PBL cDNA. Balanced or skewed ratios were determined by direct evaluation of the chromatograms. Healthy females were evaluated as control.

Immunoblotting. Cell lysates were prepared in 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, and 1% NP-40 buffer. Antibodies were purchased against CHK1, CHK2, p53, ATM, CDC25A, actin (all from Santa Cruz Biotechnology Inc.), FANCD2 (Novus Biologicals), ATR (Cell Signaling), BRCA1 (Calbiochem), and vinculin (Abcam). Signals were detected using an ECL kit (Pierce) and visualized with a Fuji LAS-3000 luminescent image analyzer system.

CHK1 and CHK2 gene expression, miRNA expression. RNA samples, extracted from PHA-stimulated PBLs were reverse transcribed, and the cDNA was analyzed for CHK1 and CHK2 expression by RQ-PCR using TaqMan methods (Applied Biosystems). Expression values were normalized to expression of the housekeeping gene GUS, using the ΔCT method, and are shown on a log2 scale.

After extraction of the dry pellet with the miRNeasy Kit, RT and RQ-PCR were performed with the miScript PCR System Kit (Qiagen), using specific primers to miR15-a and miR16-1. Data were normalized to RNU6b expression and represented in an arbitrary units, with a log2 scale representation.

Genome-wide analysis of DNA methylation was performed by genomic methylated DNA immunoprecipitation, capture, and hybridization on Agilent Human CpG Island Microarrays as recommended by the supplier (Agilent Technologies).

Sequence analysis. The entire CHK1 open reading frame was sequenced on both strands. PCR was performed on 50 ng genomic DNA with Taq polymerase (Sigma-Aldrich), and the products were sequenced using the
BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). PCR primer sequences are listed in Supplemental Figure 7B. TP53 status was analyzed by direct DNA sequencing and functional analysis of separated alleles in yeast using the FASAY method as described previously (57).

Statistics. Statistical analyses were performed using the 2-tailed non-parametric Wilcoxon test. P values of less than 0.05 and less than 0.001 were considered as significant.

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Address correspondence to: Jean Soulier, Genome and Cancer, INSERM U944 and CNRS UMR7212, Institute of Hematology, Saint-Louis Hospital, 1 Av Claude, Vellefaux 75010, Paris, France. Phone: 33.1.42-49.9891; Fax: 33.1.42-49.4027; E-mail: jean.soulier@sls.aphp.fr.