TNF-α induces leukemic clonal evolution ex vivo in Fanconi anemia group C murine stem cells

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The molecular pathogenesis of the myeloid leukemias that frequently occur in patients with Fanconi anemia (FA) is not well defined. Hematopoietic stem cells bearing inactivating mutations of FA complementation group C (FANCC) are genetically unstable and hypersensitive to apoptotic cytokine cues including IFN-γ and TNF-α, but neoplastic stem cell clones that arise frequently in vivo are resistant to these cytokines. Reasoning that the combination of genetic instability and cytokine hypersensitivity might create an environment supporting the emergence of leukemic stem cells, we tested the leukemia-promoting effects of TNF-α in murine stem cells. TNF-α exposure initially profoundly inhibited the growth of *Fancc*$^{-/-}$ stem cells. However, longer-term exposure of these cells promoted the outgrowth of cytogenetically abnormal clones that, upon transplantation into congenic WT mice, led to acute myelogenous leukemia. TNF-α induced ROS-dependent genetic instability in *Fancc*$^{-/-}$ but not in WT cells. The leukemic clones were TNF-α resistant but retained their characteristic hypersensitivity to mitomycin C and exhibited high levels of chromosomal instability. Expression of *FANCC* cDNA in *Fancc*$^{-/-}$ stem cells protected them from TNF-α–induced clonal evolution. We conclude that TNF-α exposure creates an environment in which somatically mutated preleukemic stem cell clones are selected and from which unaltered TNF-α–hypersensitive *Fancc*$^{-/-}$ stem cells are purged.

Introduction

Biallelic inactivation of any of 13 known FA genes causes Fanconi anemia (FA), a disorder characterized by early-onset BM failure often followed by evolution to acute myelogenous leukemia and myelodysplasia. (1–3) These genes, *FANCA*, *-B*, -C, *-D1* (*BRCA2*), *-D2*, *-E*, *-F*, *-G* (*XRCC9*), *-J* (*BACH1/BRIP1*), *-L* (*PHF9*), *-M* (*Hef*), *-N* (*PALB2*), and *-I* (*KIAA1794*), and the proteins they encode have no strong homologies to one another or to any other known proteins (except BRCA2, for which some truncating mutations appear to account for FA-D1) (4–18). Studies on the function of the FA proteins, particularly FA complementation group C (FANCC), indicate (a) that they function to protect against genotoxic stress by forming complexes with each other (19–22) and (b) that they protect hematopoietic stem cells from apoptotic cues by both suppressing apoptotic signaling pathways and enhancing survival signaling pathways (23–27).

Strong evidence indicates that progressive BM failure in children with FA results from excessive apoptosis and subsequent failure of the hematopoietic stem cell compartment (1, 3). Significantly, FA patients show altered expression of certain growth factors and cytokines (3). Particularly, several groups have reported abnormally elevated levels of serum, plasma, and intracellular TNF-α in FA patients (28–30). Cytokine hypersensitivity of FA cells to apoptotic cues is a major factor in the pathogenesis of BM failure in 3 FA mouse models (*Fanca*$^{-/-}$, *Fancc*$^{-/-}$, and *Fancg*$^{-/-}$) (31–33), and at least some myeloid leukemic clones evolving in a context of BM failure are cytokine resistant (34). We sought to validate the notion that cytokine hypersensitivity is an important selective force for the emergence of leukemic clones. Choosing to focus on the role of Fancc, the protein for which abundant biochemical evidence exists supporting its direct involvement in cytokine signaling (1, 23, 24, 26, 27, 30, 31), we tested the effect of TNF-α on murine *Fancc*$^{-/-}$ cells. We exposed murine hematopoietic stem cells ex vivo to TNF-α for periods of up to 30 days. In *Fancc*$^{-/-}$ cells but not in WT cells, we noted the late in vitro emergence of TNF-α–resistant hematopoietic clones, the progeny of which had an unambiguous and transplantable myeloid leukemic phenotype. No such clonal evolution was noted in *Fancc*$^{-/-}$ stem cells cultured in the absence of TNF-α, in WT cells cultured with TNF-α, or in *FANCC*-corrected *Fancc*$^{-/-}$ stem cells. Our results provide direct confirmation of the importance of selective pressure in the evolution of leukemic clones in FA of the C complementation group.

Results

Long-term TNF-α exposure promotes clonal proliferation of Fancc$^{-/-}$ *BM stem/progenitors*. We treated BM cells enriched for hematopoietic stem and progenitor cells isolated from WT or *Fancc*$^{-/-}$ mice with TNF-α for 30 days. WT cells were relatively resistant to TNF-α within the first 10 days but markedly reduced in number after 3 weeks (Figure 1A). *Fancc*$^{-/-}$ cells were sensitive to TNF-α, as expected (23, 31), within the first 3 weeks. After that time, however, from a small number of surviving cells a rapidly expanding population ultimately appeared. These outgrown cells, to which we refer herein as "preleukemic" *Fancc*$^{-/-}$ cells (see below), could be thereafter main-

Nonstandard abbreviations used: FA, Fanconi anemia; FANCC, FA complementation group C; γH2AX, phosphorylated histone H2AX; LM-PCR, ligation-mediated PCR; NAC, *N*-acetyl-L-cysteine.

Conflict of interest: The authors have declared that no conflict of interest exists.

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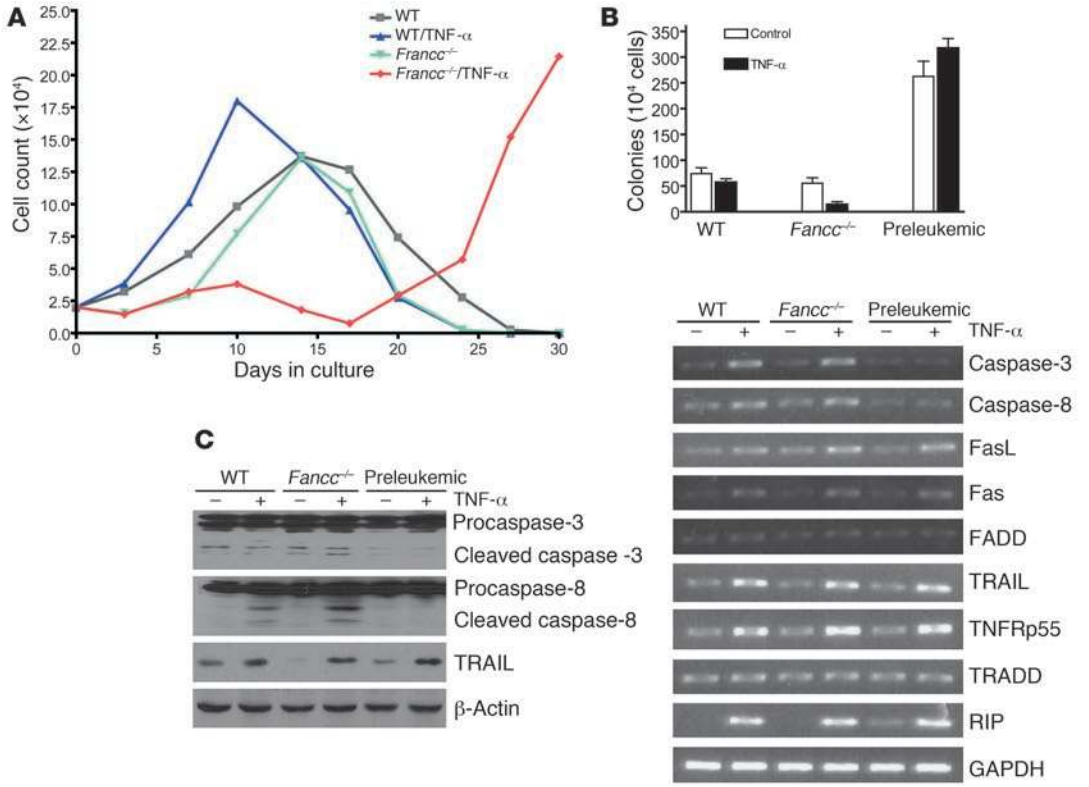
A
Cell count (×10⁴)
Days in culture
WT
WT/TNF-α
Fancc−/−
Fancc−/−/TNF-α
B
Colonies (10⁴ cells)
Control
TNF-α
WT
Fancc−/−
Preleukemic
C
WT
Fancc−/−
Preleukemic
TNF-α
Procaspase-3
Cleaved caspase -3
Procaspase-8
Cleaved caspase-8
TRAIL
β-Actin
Caspase-3
Caspase-8
FasL
Fas
FADD
TRAIL
TNFRp55
TRADD
RIP
GAPDH
D
t(10;11)
t(4;9)

Figure 1
TNF-α promotes clonal proliferation of TNF-α–resistant *Fancc*$^{-/-}$ BM progenitors. (**A**) TNF-α–mediated selection for resistant *Fancc*$^{-/-}$ BM cells. Lin-Sca-1^{+} BM cells from WT and *Fancc*$^{-/-}$ mice were cultured in complete medium containing IL-11, IL-6, Steel factor, and Flt-3 ligand and in the presence or absence of 10 ng/ml TNF-α. The total number of viable cells was counted by the trypan blue exclusion method at the times indicated. Result shown are representative of 5 separate experiments with similar results and expressed as mean of duplicates. (**B**) Colony growth assays demonstrated that while *Fancc*$^{-/-}$ BM progenitor cells were hypersensitive to TNF-α, the TNF-α–resistant *Fancc*$^{-/-}$ clonal progenitors were resistant in clonal assays. Data represent total number of colonies (mean ± SD) from 3 independent experiments. (**C**) Expression and activation of the TNF family signaling molecules in WT, freshly isolated *Fancc*$^{-/-}$, and outgrown *Fancc*$^{-/-}$ BM progenitor cells. Cells were cultured in cytokine-supplemented medium in the absence or presence of 10 ng/ml TNF-α for 12 hours. RNA and protein extracts were prepared, and gene expression (right) and activation of caspase-3 and -8 were analyzed by RT-PCR and immunoblotting, respectively. (**D**) Spectral karyotype (SKY) analysis of the TNF-resistant cells demonstrated clonal cytogenetic abnormalities, including a clone with t(10;11) and another with t(4;9). The images shown were obtained from 1 culture. We conducted cytogenetic analysis on 3 different cultures, which gave some shared (e.g., the 10;11 translocations and monosomy 14) as well as different aberrations.

tained in liquid medium supplemented with IL-3 for an additional 3 weeks and were no longer hypersensitive to TNF-α (Figure 1B) but retained their characteristic hypersensitivity to DNA cross-linking agent mitomycin C (data not shown). Cells from these TNF-α–resistant clones were highly replicative, as they formed large colonies (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI31772DS1) and could be sequentially replated in clonal assays up to 4 times (Supplemental Figure 1B). Wright-Giemsa–stained cytospin preparations of these outgrown preleukemic *Fancc*$^{-/-}$ cells revealed the presence of more immature myeloid cells compared with the freshly isolated WT and *Fancc*$^{-/-}$ controls (Supplemental Figure 1C).

The observation that the preleukemic *Fancc*$^{-/-}$ cells had acquired resistance to TNF-α prompted us to search for components in the TNF-α signaling pathway that might have been lost in the preleukemic *Fancc*$^{-/-}$ cells. We did not observe significant changes in the expression of several TNF family members, their receptors, and effector molecules in WT, freshly isolated *Fancc*$^{-/-}$, and outgrown preleukemic *Fancc*$^{-/-}$ BM progenitor cells (Figure 1C). However, we did observe that both caspase-3 and -8 mRNAs were strongly induced in WT and freshly isolated *Fancc*$^{-/-}$ BM progenitor cells but were not in preleukemic *Fancc*$^{-/-}$ cells after TNF-α treatment. Furthermore, in the preleukemic *Fancc*$^{-/-}$ cells, TNF-α–induced caspase-3 and -8 activation was suppressed (Figure 1C). This would suggest that the preleukemic *Fancc*$^{-/-}$ cells resist TNF-α killing through a mechanism involving suppression of apoptosis.

These preleukemic *Fancc*$^{-/-}$ cells were clones. While we observed no significant deviations from the normal number of 40 chromosomes in the preleukemic *Fancc*$^{-/-}$ cells (40.7 ± 1.4 compared with 40.1 ± 0.8 and 40.2 ± 0.5 chromosomes in freshly isolated WT and *Fancc*$^{-/-}$ BM progenitors, respectively), occasional chromosomal or chromatid breaks or gaps could be found in these preleukemic *Fancc*$^{-/-}$ cells but were absent in freshly isolated controls (Table 1). Spectral karyotyping revealed shared clonal cytogenetic defects in the preleukemic *Fancc*$^{-/-}$ cells that included loss of chromosome 14 as well as chromosome t(10;11) and t(4;9) translocations (Figure 1D).

The TNF-α–induced clones contain preleukemic stem cells. To determine whether these clonally evolved *Fancc*$^{-/-}$ cells could give rise to myeloid leukemia, we transplanted these cells into lethally irradiated congenic mice. The recipients of WT cells survived without signs of leukemia for 150 days following transplantation. However, all recipients (n = 12) of TNF-α–exposed *Fancc*$^{-/-}$ cells developed acute nonlymphocytic leukemia within 150 days (Figure 2A). The mice exhibited leukocytosis, myeloblastosis, and massive splenomegaly (Figure 2B). While TNF-α–treated WT cells failed to engraft, the TNF-α–exposed *Fancc*$^{-/-}$ cells constituted more than 90% of recipient blood cells, which were mostly of myeloid origin (Figure 2C).

Leukemic clones that developed in recipients of preleukemic *Fancc*$^{-/-}$ cells were readily transplantable to secondary recipients. The time required for leukemia to develop in recipients of leukemic marrow was shorter (Table 2) than it was in primary recipients of preleukemic clones (Figure 2A). All leukemic recipients had infiltrated BM, spleen, liver, and intestine at the time they were sacrificed, as determined histologically (Figure 2D) and by PCR genotyping (Supplemental Figure 2).

That cytogenetically marked preleukemic clones were the progenitors of overt leukemia was confirmed by the retention of the same cytogenetic defects (along with additional ones) in the leukemic cells that evolved in transplanted mice (Table 3). To confirm the clonal nature of the preleukemic cells, we analyzed insertion sites, using ligation-mediated PCR (LM-PCR), in leukemic cells from recipients of GFP vector–transduced *Fancc*$^{-/-}$ cells that had been exposed to TNF-α. If the initial transforming event resulted in the outgrowth of a single preleukemic clone, then all progeny from this clone must bear identical retroviral integration sites, independent of the recipient. Figure 2E demonstrates that the preleukemic cells that repopulated mice 1–6 were clonal in nature, as they exhibited an identical retroviral integration pattern in all BM cells. We thus conclude that the initial preleukemic transformation occurs at the single-cell level.

Correction of FA deficiency prevents clonal evolution and leukemogenesis. We determined whether correction of the FA defect in *Fancc*$^{-/-}$ hematopoietic cells would prevent clonal progenitor proliferation and leukemic development. BM progenitors from WT and *Fancc*$^{-/-}$ mice were transduced with retroviruses expressing either enhanced GFP (eGFP) alone or FANCC and eGFP. Infected BM cells (containing both GFP^{+} and GFP^{-} cells) were cultured in cytokine-supplemented medium in the presence of TNF-α for 30 days (Supplemental Figure 3A). During the 30-day period, the content of GFP^{+} cells in the vector group and WT cells infected with FANCC retroviral vectors remained steady at approximately 40% regardless of genotype (Supplemental Figure 3B), which was close to the initial transduction efficiency. In contrast, in *Fancc*$^{-/-}$ cells infected with FANCC viruses (37% transduction efficiency), expression of FANCC led to a significant increase in the number of eGFP-expressing cells in the first 20 days. Specifically, more than 70% of the cells were GFP^{+} at day 20 (Supplemental Figure 3B). However, the percentage of GFP^{+} cells decreased rapidly thereafter (Supplemental Figure 3, A and B). Interestingly, while both *Fancc*$^{-/-}$/vector and *Fancc*$^{-/-}$/FANCC groups contained cells that began to proliferate after 4 weeks in the presence of TNF-α, few of the cells present at that point in time expressed FANCC (Supplemental Figure 3, B and C). We conclude that ectopic expression of FANCC in *Fancc*$^{-/-}$ stem cells prevents clonal proliferation promoted by TNF-α.

To determine whether reexpression of FANCC prevents leukemogenesis, *Fancc*$^{-/-}$/vector or *Fancc*$^{-/-}$/FANCC cells cultured in the

Table 1
Clonal chromosome aberrations in outgrown *Fancc*$^{-/-}$ BM cells

Cell line	Treatment	Metaphase examined	Range (average)	Clonal aberrations	Frequency[A]
WT	–	30	38–42 (40.1)	–	–
	TNF-α	35	37–41(40.3)	–	–
Fancc$^{-/-}$	–	30	38–43(40.2)	–	–
	TNF-α	33	38–41(40.7)	t(4;9)	13/33
				t(10;11)	11/33
				–14	7/33

[A]The frequency is denoted by the incidence of the aberration in the indicated metaphases analyzed. Only recurrent aberrations that occurred in at least 15% of the cells examined are listed. Data shown were obtained from 1 culture. We conducted cytogenetic analysis on 3 different cultures, which gave some shared (e.g., the 10;11 translocations and monosomy 14) as well as different aberrations.

presence of TNF-α for 5 and 30 days were transplanted into irradiated recipients. Leukemia was not detected in recipients transplanted with 5-day cultured cells regardless of genotype within 6 months, whereas all mice receiving 30-day cultured cells died of leukemia within 140 days (Figure 3A). Engraftment analysis of recipients transplanted with 5-day and 30-day *Fancc*$^{-/-}$/FANCC cells showed that the majority of the donor-derived cells in recipients with 5-day *Fancc*$^{-/-}$/FANCC cells were GFP$^+$, while all of the engrafted cells in mice with 30-day *Fancc*$^{-/-}$/FANCC cells were GFP$^-$ (that is, these cells did not express FANCC) (Figure 3B). These results confirm that expression of FANCC prevents TNF-α–induced clonal proliferation and leukemogenesis.

TNF-α induces ROS-dependent genetic instability specifically in Fancc$^{-/-}$ *cells*. TNF-α is known to induce production of ROS (35, 36), which may represent one mechanism by which TNF-α mediates its leukemogenic effect. We thus investigated the role of ROS in TNF-α–induced leukemia development in *Fancc*$^{-/-}$ cells. Recipients transplanted with preleukemic *Fancc*$^{-/-}$ cells were challenged with TNF-α followed by administration of the ROS scavenger *N*-acetyl-L-cysteine (NAC). Mice transplanted with preleukemic *Fancc*$^{-/-}$ BM cells accumulated high levels of ROS, and administration of NAC significantly reduced ROS (Figure 4A). The time required to develop leukemia was significantly increased in recipients treated with NAC (Table 4). NAC also substantially reduced the amounts of secreted proinflammatory cytokines IL-1β, IL-6, and TNF-α in the serum of recipients (Table 4), indicating that the TNF-α–induced responses were mediated, at least in part, by ROS. These results are compatible with the view that TNF-α–induced ROS contribute to leukemia progression in recipients of *Fancc*$^{-/-}$ preleukemic cells. In light of the capacity of ROS to induce DNA damage (reviewed in ref. 37), we next sought to quantify the genotoxic effects of TNF-α–induced ROS in preleukemic cells.

Using the comet assay (38), we found no significant accumulation of DNA damage in BM cells from TNF-α–treated recipient mice transplanted with WT cells, irrespective of NAC treatment (Figure 5A). In contrast, BM cells from TNF-α–treated mice receiving *Fancc*$^{-/-}$ preleukemic cells showed excessive DNA strand breakage, which was effectively eliminated by NAC administration. A similar increase in 8-oxo-deoxyguanosine (8-oxodG), an established marker of oxidative DNA damage, was also demonstrated in BM cells from TNF-α–treated mice receiving preleukemic *Fancc*$^{-/-}$ cells compared with those with WT cells (Figure 5B), which was, again, eliminated by the administration of the antioxidant NAC.

Because high levels of oxidative DNA damage were retained in *Fancc*$^{-/-}$ leukemic cells, we asked whether the DNA damage response was persistently activated in these cells. To this end, we examined 2 well-established DNA damage response markers, phosphorylated histone H2AX (γH2AX), which forms foci in response to DNA double-strand breaks (DSBs) (39), and phosphorylated p53 at Ser20 (p53^{Ser20}), a specific indicator of oxidative DNA damage (40). In WT BM cells, we found that low levels of p53^{Ser20} and γH2AX could be detected within the first 2 hours of TNF-α treatment, but levels declined by 4 hours (Figure 5C). In contrast, TNF-α induced substantial levels of p53^{Ser20} or γH2AX in *Fancc*$^{-/-}$ preleukemic cells. Moreover, the expression of p53^{Ser20} and γH2AX remained high in these preleukemic cells even 2 hours after withdrawal of TNF-α from the culture medium (Figure 5C). In addition, high levels of p53^{Ser20} and γH2AX expression were observed in recipient mice transplanted with the *Fancc*$^{-/-}$ leukemic cells, which were almost completely abolished by NAC (Figure 5D). These markers were attended by clear structural evidence of genomic instability by karyotype analysis that demonstrated an increase in chromosomal aberrations in transplanted *Fancc*$^{-/-}$ cells compared with transplanted WT cells (Figure 5E and Table 5). Specifically, while most cells from recipients transplanted with WT cells remained diploid, the leukemic *Fancc*$^{-/-}$ cells showed a high degree of aneuploidy, with chromosomal numbers ranging from 37 to 159 (Table 6). In addition, the aberrations in these leukemic *Fancc*$^{-/-}$ cells consisted of breaks, gaps, dicentric chromosomes, double minutes, translocations, chromosome fragments, and fusions (Table 6). These data point to a dramatic increase in genomic instability during leukemic progression.

Fancc$^{-/-}$ *malignant transformation requires both oxidative DNA damage and suppression of apoptosis*. Because our results show that TNF-α induced overproduction of ROS and high levels of oxidative DNA damage in *Fancc*$^{-/-}$ leukemic cells (Figure 5), we sought to determine the contribution of oxidative DNA damage to *Fancc*$^{-/-}$ malignant transformation. We treated the *Fancc*$^{-/-}$ BM stem/progenitor cells with a low dose (10 μM) of H_2O_2 for 30 days. Interestingly, H_2O_2 alone failed to transform *Fancc*$^{-/-}$ cells (Figure 6A). However,

Table 2
Secondary recipients of WT versus *Fancc*$^{-/-}$ primary donor-derived leukemic cells

Primary donor cells	Secondary recipients[A]	Time to leukemia (d)	Spleen weight (g)	wbc × 10^3/μl	% Blast in PBL
WT	36.1–36.6	Sac'd	0.09 ± 0.02	10.4 ± 4	12 ± 4
	39.1–36.6	Sac'd	0.1 ± 0.04	12.3 ± 5	15 ± 8
Fancc$^{-/-}$	45.1–45.6	53 ± 4	0.29 ± 0.1	74.4 ± 15	82 ± 12
	46.1–46.6	51 ± 3	0.34 ± 0.1	66.7 ± 11	79 ± 8

[A]Secondary recipients (n = 6) were transplanted with BM cells from individual primary recipients that had received TNF-α–treated WT or preleukemic *Fancc*$^{-/-}$ BM cells. Sac'd, sacrificed; PBL, peripheral blood.

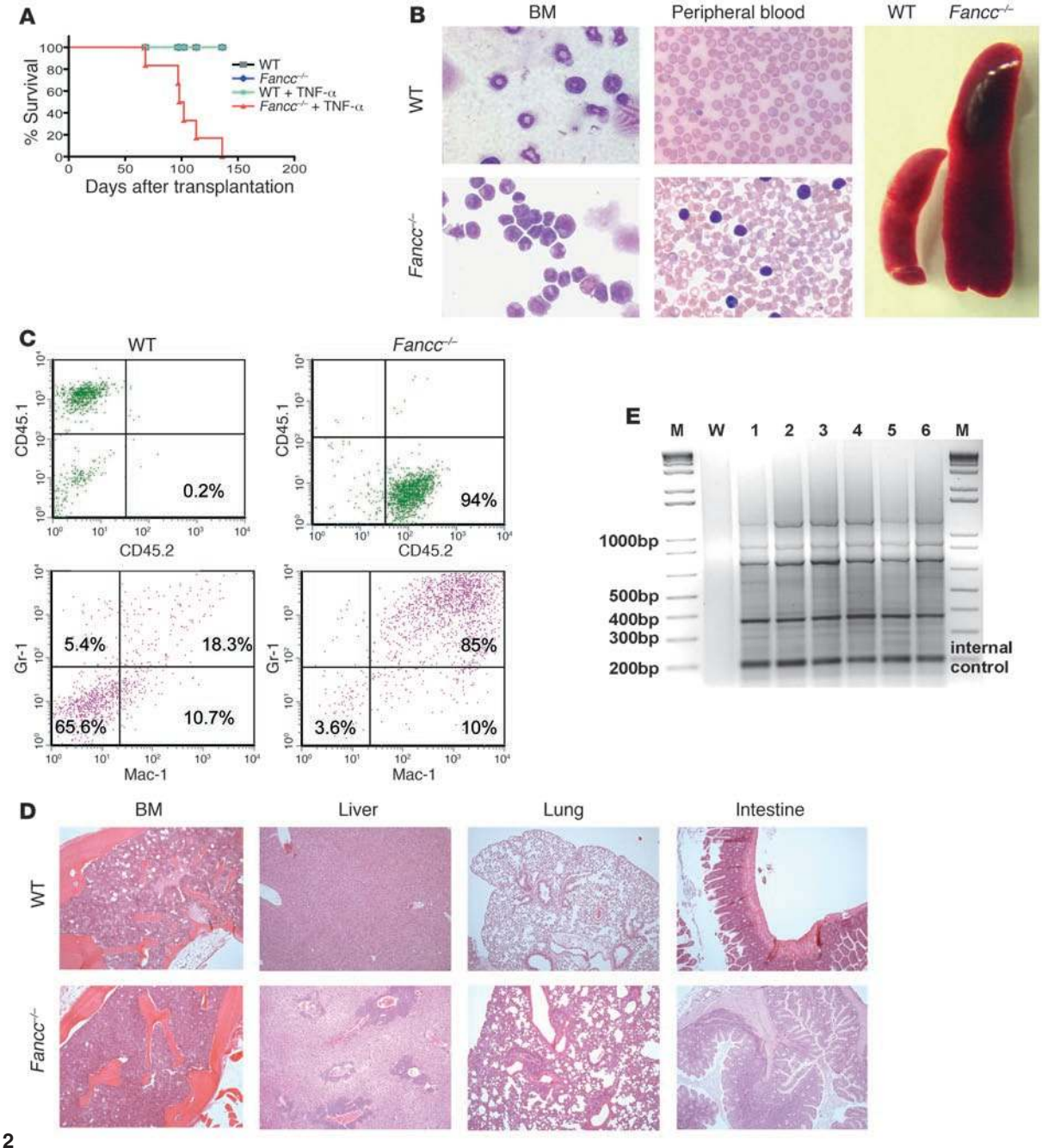

Figure 2

TNF-α–adapted *Fancc*$^{-/-}$ stem cells are preleukemic. (**A**) 1 × 10^6 untreated WT, *Fancc*$^{-/-}$, TNF-α–treated (10 days) WT or outgrown (30 days) *Fancc*$^{-/-}$ BM cells (along with 1 × 10^6 competitive cells) were injected i.v. into lethally irradiated recipients. Survival of recipient mice was quantified by Kaplan-Meier analysis. Experiments were performed 3 times, each with 4 recipient mice (total 12 mice per group). Data were obtained from recipient mice transplanted with cells from 1 culture. We performed BM transplantation with 5 separate cultures, which gave similar results. (**B**) Increased blast cells in BM and peripheral blood and massive splenomegaly in *Fancc*$^{-/-}$ leukemic mice. (**C**) Elevated expression of myeloid markers in *Fancc*$^{-/-}$ leukemic mice. (**D**) H&E staining of tissue sections of recipient mice transplanted with TNF-α–treated WT or *Fancc*$^{-/-}$ BM cells. Original magnification, ×10. (**E**) Clonal origin of a preleukemic clone. LM-PCR was performed to determine the retroviral integration pattern in animals transplanted with outgrown *Fancc*$^{-/-}$ cells transduced with control (enhanced GFP) vector. BM cells from animals 1–6 presented with an identical retroviral integration pattern, indicating that the repopulating cells were clonal in origin. The internal vector control was detectable at 200 bp in animals 1–6 and confirmed a functional LM-PCR reaction. M, marker; W, water.

treatment of the *Fancc*$^{-/-}$ stem/progenitors with a combination of H_2O_2 and the pan-caspase inhibitor Z-VAD-FMK (100 μM) supported the emergence of *Fancc*$^{-/-}$ preleukemic stem cells (Figure 6A). Notably, this sublethal dose of H_2O_2 caused a high level of oxidative DNA damage accumulation in *Fancc*$^{-/-}$ cells compared with WT cells, and Z-VAD-FMK treatment did not affect the accu-

Table 3
Recurring chromosome aberrations in *Fancc*$^{-/-}$ preleukemic and leukemic cells[A]

Preleukemic cells	Leukemic cells
t(4;9)	t(4;9)
t(10;11)	t(10;11)
–14	t(4;12)
	t(5;10)
	t(1;10)
	t(17;18)
	–14

[A]Data shown were obtained from 3 recipient mice transplanted with cells described in Table 1 and Figure 1D. We also analyzed recipients transplanted with cells from another culture containing different aberrations. In the latter case, the shared cytogenetic aberrations detected in the preleukemic clones were retained in the leukemic cells that evolved in transplanted mice.

mulation of DNA damage in either WT or *Fancc*$^{-/-}$ cells (Figure 6B). This result suggests that oxidative DNA damage may be necessary but not sufficient for oncogenic transformation and needs to collaborate with other (such as antiapoptotic) events in order to induce malignant transformation.

Prolonged TNF-α–induced JNK activation is required for Fancc$^{-/-}$ *malignant transformation*. We recently reported that prolonged activation of the MAPK kinase JNK contributes to the overproduction of ROS and exacerbated inflammation in *Fancc*$^{-/-}$ mice challenged with bacterial lipopolysaccharide, a potent inducer of TNF-α gene expression (41). We therefore assessed the role of JNK kinase in TNF-α–induced malignant transformation in *Fancc*$^{-/-}$ BM stem/progenitor cells. We first examined JNK activation in WT, freshly isolated *Fancc*$^{-/-}$, and preleukemic *Fancc*$^{-/-}$ cells. We found that JNK remained persistently activated in the preleukemic cells even in the absence of TNF-α (Figure 7A). Importantly, treatment of BM stem/progenitor cells with a pharmaceutical JNK inhibitor (SP600125) prevented TNF-α–induced outgrowth of *Fancc*$^{-/-}$ cells (Figure 7B). We then conducted experiments in which we inhibited JNK in the outgrown *Fancc*$^{-/-}$ cells using either a dominant mutant of an upstream kinase (MKK7-KM) or the pharmaceutical JNK inhibitor (SP600125). We found that both inhibitors reduced the activity of the endogenous JNK kinase (Figure 7C) and reduced TNF-α–induced ROS production (Figure 7D) in the preleukemic *Fancc*$^{-/-}$ progenitor cells.

Survival of leukemic clones is independent of NF-κB activation. The activation of the redox transcription factor NF-κB induced by TNF-α is known to enhance cell survival and proliferation (35, 36). To determine the role of NF-κB in progression of preleukemic clones to frank leukemia, we transduced freshly isolated and *Fancc*$^{-/-}$ preleukemic cells with a retroviral vector expressing an IκBα "super-repressor" mutant (IκBαAA; Figure 8A), which, because it is resistant to degradation (Figure 8B), suppresses NF-κB activation (42) (Figure 8C). IκBαAA expression clearly suppressed nuclear translocation of NF-κB (Supplemental Figure 4) and significantly suppressed clonogenic progenitor growth of WT and *Fancc*$^{-/-}$ cells as well as preleukemic cells. However this mutant had no effect on clonal growth of *Fancc*$^{-/-}$ leukemia cells (Figure 8D) and failed to prevent progression of leukemia in secondary recipients and progression to leukemia in recipients of *Fancc*$^{-/-}$ preleukemic stem cells (Figure 8E). Collectively, these results demonstrate that NF-κB activation is not required for survival and expansion of the *Fancc*$^{-/-}$ leukemic cells and that inhibition of NF-κB activation does not prevent TNF-α–promoted leukemogenesis in *Fancc*$^{-/-}$ hematopoietic progenitors.

Discussion

Although FA is known as a disorder of cytogenetic instability in which somatic cells are hypersensitive to bifunctional alkylating agents (43), the major life-threatening complications are commonly confined to hematopoietic tissues and include aplastic anemia and conditions resulting from clonal expansion of cytogenetically unstable stem cells, myelodysplasia, and acute myelogenous leukemia (44). The unique vulnerability of hematopoietic cells is likely reflective of key prosurvival roles of the FA proteins in hematopoietic cells. For example, while it is known that the FA proteins function to protect against genotoxic stress by forming complexes with each other (19–22), some are known to also protect hematopoietic cells, in part by suppressing apoptotic responses to extracellular

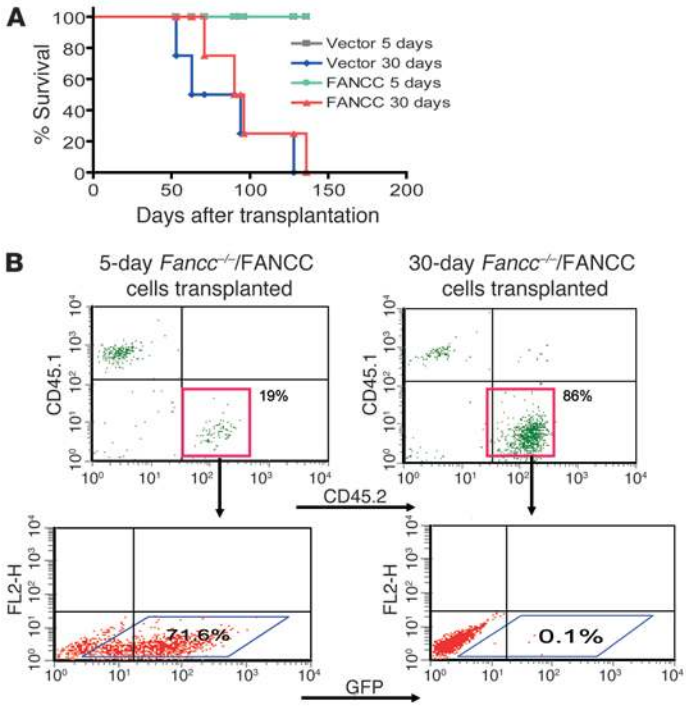

Figure 3
FANCC complementation of *Fancc*$^{-/-}$ stem cells prevents initiation of leukemia. 2 × 10^5 *Fancc*$^{-/-}$ Lin- BM cells transduced with bicistronic (GFP) control (Vector) or *FANCC* retroviral vectors were cultured in the presence of 10 ng/ml TNF-α for the indicated time periods and injected i.v. (along with 1 × 10^6 competitive cells) into lethally irradiated recipients. Because the transduction efficiency averaged 40% in each experiment, the exposed cells represented mixtures of transduced (GFP$^+$) and untransduced (GFP$^-$) cells. (**A**) Survival of recipient mice is shown using Kaplan-Meier analysis. Experiments were performed 2 times, each with 3 recipient mice (total 6 mice per group). (**B**) Peripheral blood cells from transplanted mice were stained with antibodies CD45.1-PE and CD45.2-APC, and donor-derived CD45.2$^+$ cells were gated and analyzed by flow cytometry for GFP$^+$ and GFP$^-$ cell populations. Note that the leukemic *Fancc*$^{-/-}$ cells were entirely GFP$^-$.

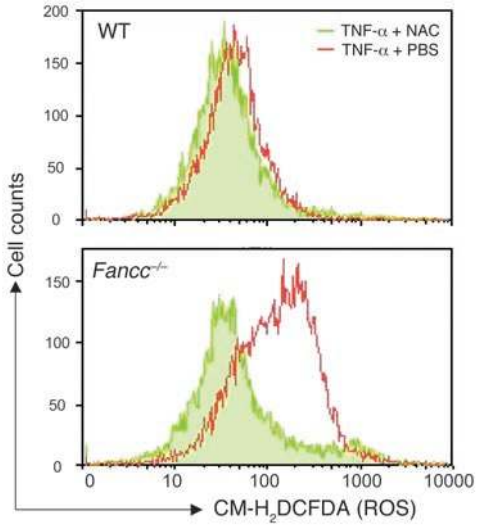

Figure 4
TNF-α induces overprotection of ROS in *Fancc*$^{-/-}$ leukemic mice. ROS production in transplanted recipient mice. 1 × 10^6 TNF-α–treated WT cells or *Fancc*$^{-/-}$ preleukemic cells were injected i.v. into lethally irradiated recipients, which after 10 days were injected i.p. with 1 dose (100 μg/kg) of TNF-α followed by NAC (100 mg/kg/d; TNF + NAC) or PBS (TNF + PBS) for 10 days. When recipients of *Fancc*$^{-/-}$ preleukemic cells became moribund, 12 (6 each in TNF + NAC or TNF + PBS) were analyzed in parallel with 12 recipients of WT cells (6 each in TNF + NAC or TNF + PBS). BM cells were stained with fluorescent probe CM-H_2DCFDA, and ROS were detected by flow cytometry. Shown is a representative flow cytometric analysis of ROS production in BM cells from the indicated recipient mice.

apoptotic cues, particularly TNF-α (23–28). The strongest evidence that FA proteins are likely multifunctional comes from studies on the role of FANCC in suppressing apoptotic responses. In fact, the antiapoptotic functions of FANCC, including mediating resistance to TNF-α, require structural features that differ from those that permit the formation and function of the canonical nuclear core complex (45), confirming structurally the multifunctionality of FANCC. Because aplastic anemia is nearly universal in FA patients, we suspect that most of the other 12 FA proteins will ultimately prove to be multifunctional. Although most of the FA proteins collaborate in the nuclear pathway to protect the genome (1–3), there is no evidence that the mechanisms by which they may promote survival of hematopoietic stem cells are precisely the same. For example, FANCA mutant cells are hypersensitive to TNF-related apoptosis-inducing ligand (TRAIL) (46), but FANCC mutant cells are not (47). Therefore, it is quite possible that specific molecular leukemogenic events in FA cells will differ from one complementation group to another because the adaptive responses required may differ. Alternatively, the leukemogenic events might function to suppress a signaling bottleneck for more than 1 apoptotic pathway, in which case the adaptive adjustment may be in a common effector pathway. That is, the leukemogenic events might be the same across the groups. These questions are not answered by this study, because we focused specifically on 1 well-defined complementation group, FA-C.

We argue that the multifunctionality of FANCC explains the high incidence of clonal evolution in FA stem cells of the C complementation group. First, FA stem cells are genetically unstable. Second, owing to their proapoptotic phenotype, the FA stem cells are less fit than normal stem cells (48). Third, the neoplastic FA clones seem to be resistant to precisely the same cytokines to which their non-neoplastic progenitors were hypersensitive (34, 49). Therefore, we have proposed that repeated cytokine release responses throughout the life of FA patients provides recrudescent selective sweeps that purge hematopoietic tissues of all but the selected or adapted neoplastic stem cells. Rarely, new stem cell clones can emerge through mutations that specifically correct the inherited mutation, a process leading to mosaicism that can be so complete that hematopoiesis is normalized (50). More often, emerging clones have not corrected the mutant FA gene. Encouraged by a preliminary report that suggested that certain conditions of ex vivo culture might enhance clonal evolution of *Fancc*$^{-/-}$ cells (49), we tested the hypothesis that TNF-α exposure in vitro can provide a selective sweep in *Fancc*$^{-/-}$ stem cells, resulting in the outgrowth of TNF-α–resistant leukemogenic clones. We specifically chose TNF-α and *Fancc*$^{-/-}$ mutant cells as the instruments of our studies because we and others have shown that FA-C cells are hypersensitive to TNF-α (23, 24, 26, 27, 31), because some of the roles of FANCC in the TNF-α signaling pathway have been defined biochemically (26, 27, 45), and because we have reported TNF-α resistance in clonally evolved human FA progenitor cells (34).

We found, as expected, that TNF-α exposure initially inhibited profoundly the expansion of *Fancc*$^{-/-}$ stem progeny but noted that longer-term exposure of these cells promoted the outgrowth of TNF-α–resistant, cytogenetically abnormal clones that upon transplantation into syngeneic WT mice led to acute myelogenous leukemia (Figures 1 and 2). That the recipient animals survived transplantation for more than 100 days indicated either that nonclonal stem cells repopulated the animals early or that the clonally derived cells had the potential for multilineage repopulation of the recipient animals and were, therefore, preleukemic. Once leukemia developed, the TNF-α–resistant leukemic cells retained their char-

Table 4
NAC delays leukemia development

Donor	Treatment	Time to leukemia (d)	Serum cytokines (pg/ml)		
			TNF-α	IL-1β	IL-6
WT	PBS	Sac'd	35.9 ± 3.5	8.7 ± 1.2	41.6 ± 6.3
	NAC	Sac'd	42.3 ± 7.6	11.3 ± 3.8	36.3 ± 5.6
Fancc$^{-/-}$	PBS	83.8 ± 7.7	98.4 ± 12.4	26.6 ± 2.6	118.5 ± 8.5
	NAC	184 ± 11.5	58.2 ± 9.2	10.8 ± 2.7	51.7 ± 6.6

Recipient mice described in Figure 4 were analyzed for leukemia development and proinflammatory cytokines. Six mice were examined in each group. Serum levels of the indicated cytokines were measured using commercial ELISA kits. Data are expressed as mean ± SD of 6 recipient mice per group.

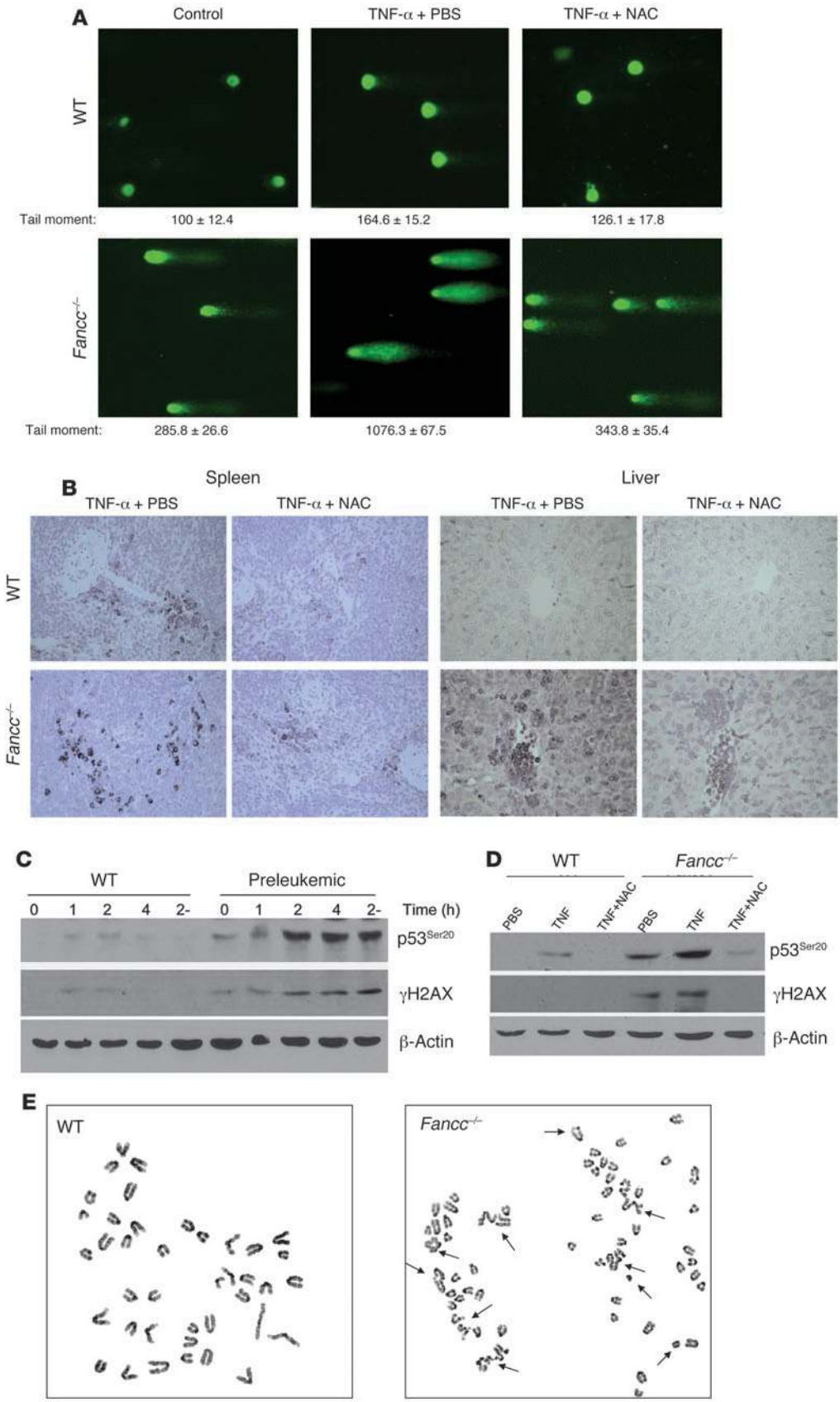

Figure 5

The role of TNF-α–induced ROS in genetic instability. (**A**) 1 × 10^6 TNF-α–treated WT cells or *Fancc*$^{-/-}$ preleukemic cells were injected i.v. into lethally irradiated recipients, which after 10 days were injected i.p. with 1 dose of TNF-α (100 μg/kg) followed by NAC (100 mg/kg/d) administration for 10 days. Control mice were injected with PBS only. The mice were sacrificed 24 hours after the last NAC injection, and BM cells were analyzed for DNA strand breaks by the comet assay. The mean tail moment of WT control sample is expressed as 100%. Larger tail moments represent higher levels of DNA damage. Three recipient mice from each group were analyzed, and 50 cells per mouse were scored from random sampling. (**B**) Tissue sections of liver and lung from recipient mice described in **A** were stained with anti–8-oxodG antibody and counterstained with H&E. Original magnification, ×40. (**C**) WT and *Fancc*$^{-/-}$ preleukemic cells were cultured in the presence of TNF-α (10 ng/ml), and protein extracts were prepared 0, 1, 2, and 4 hours after TNF-α treatment and analyzed by immunoblotting with anti–phospho-p53^{Ser20}, anti-γH2AX, and anti-actin antibodies. Extracts were also prepared from cells 2 hours (2–) after TNF-α withdrawal after the cells had been treated with TNF-α for 4 hours. (**D**) BM cells from recipient mice described in **A** were analyzed for p53^{Ser20} and γH2AX expression. (**E**) Examples of metaphase chromosomes prepared from donor-derived WT and *Fancc*$^{-/-}$ BM cells. Chromosomal aberrations (arrows) were noted in *Fancc*$^{-/-}$ cells but rarely in WT cells.

Table 5
Chromosome aberrations in recipient mice[A]

Donor	Metaphase examined	Aberrations	Frequency (%)
WT	93	At least 1 aberration	14
		At least 2 aberrations	3
		3 or more aberrations	1
		<39 chromosomes	4
		>40 chromosomes	1
Fancc$^{-/-}$	96	At least 1 aberration	89
		At least 2 aberrations	47
		3 or more aberrations	28
		<39 chromosomes	13
		>40 chromosomes	15

[A]Recipients of WT cells were sacrificed on the same day that mice transplanted with *Fancc*$^{-/-}$ preleukemic cells were found to be overtly leukemic.

acteristic hypersensitivity to mitomycin C and exhibited high-level constitutional chromosomal instability but did not require TNF-α–induced NF-κB activation for their survival and proliferation (Figure 8). We also noted that TNF-α enhanced ROS-dependent genetic instability in *Fancc*$^{-/-}$ but did not induce genetic instability in WT cells (Figures 4 and 5 and Table 4). These findings are consistent with prior indications that TNF-α's effects on other cell types are ROS dependent (35, 36) and with reports that FA cells are hypersensitive to oxidative stress (51–56).

Expression of *FANCC* cDNA in *Fancc*$^{-/-}$ stem cells protected them from TNF-α–induced clonal evolution (Figure 3). Of potential importance for prevention of clinical leukemogenesis, we also found that inhibition of ROS accumulation in vivo delayed leukemia development in recipients of preleukemic clones (Figure 4 and Table 4). We recently reported that prolonged activation of the MAPK kinase JNK contributes to the overproduction of ROS and exacerbated inflammation in *Fancc*$^{-/-}$ mice challenged with bacterial lipopolysaccharide (41). We have demonstrated herein that JNK is persistently activated in the preleukemic *Fancc*$^{-/-}$ cells and that inhibition of JNK kinase using either dominant mutant of an upstream kinase or a pharmaceutical JNK inhibitor could: (a) inhibit the activity of the endogenous kinases; (b) reduce TNF-α–induced ROS production; and (c) prevent the outgrowth of *Fancc*$^{-/-}$ progenitor cells (Figure 7). Mice transplanted with the TNF-α–promoted preleukemic *Fancc*$^{-/-}$ BM cells accumulated high levels of ROS, but NAC administration suppressed ROS as well as the levels of secreted proinflammatory cytokines in the recipients (Figure 4 and Table 4).

Intriguingly, oxidative DNA damage alone appears to be insufficient for *Fancc*$^{-/-}$ malignant transformation. We provide evidence that prolonged treatment of the *Fancc*$^{-/-}$ BM stem/progenitor cells with a sublethal dose of H_2O_2 failed to transform *Fancc*$^{-/-}$ cells, whereas combined treatment of the cells with the pan-caspase inhibitor Z-VAD-FMK and H_2O_2 supported the emergence of *Fancc*$^{-/-}$ preleukemic stem cells (Figure 6).

We reason that exposure of *Fancc*$^{-/-}$ stem cells to agents that damage DNA would not be enough to account for evolution of malignant clones. If this were true, one would expect that all somatic cells (all of which are hypersensitive to alkylating agents) would be potential targets for neoplastic transformation. In fact, the vulnerable tissues are limited. One of the key nonstochastic targets is the hematopoietic organ system that in *Fancc*$^{-/-}$ cells is inherently hypersensitive to the apoptotic effects of TNF-α. To gain a competitive advantage, clonal neoplasms must overcome any apoptotic hypersensitivities. Indeed, we have found (34) that TNF-α resistance occurs in clonal FA cells. That these TNF-resistant preleukemic and leukemic cells retain their characteristic DNA cross-linker hypersensitivity confirms that the key adaptive response in leukemogenesis involves TNF resistance, not resistance to cross-linking agents. That the leukemic cells retain genetic instability on the one hand and have a suppressed cytokine-dependent apoptotic response on the other favors rapid clonal evolution.

We conclude that TNF-α exposure creates an environment in which somatically mutated preleukemic stem cell clones are selected and from which unaltered TNF-α–hypersensitive *Fancc*$^{-/-}$ stem cells are purged. Moreover, TNF-α provides 2 functions of relevance to clonal evolution in vitro. First, it inhibits survival of unadapted (constitutionally unfit) cells, and second, the cytokine generates ROS-dependent genetic instability that may enhance the likelihood of adaptive mutations in vitro. The ability to reliably induce clonal evolution in vitro in such a short period of time

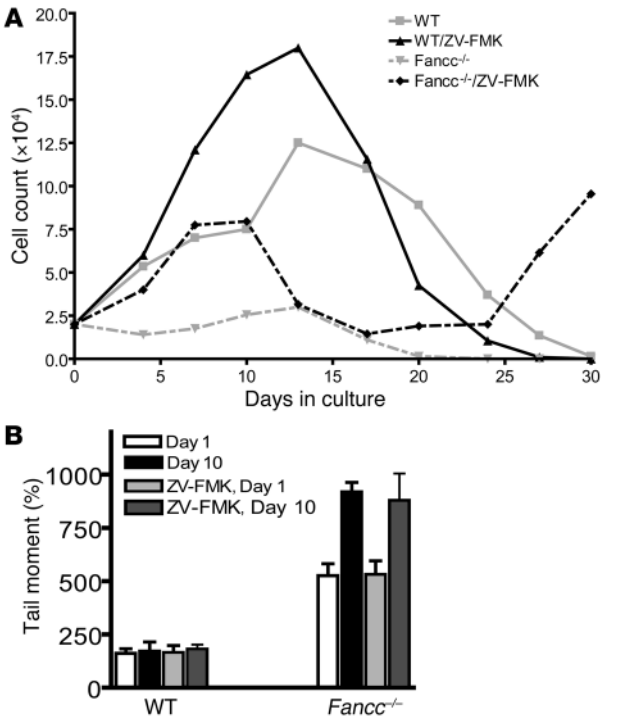

Figure 6
H_2O_2-mediated *Fancc*$^{-/-}$ malignant transformation requires inhibition of apoptosis. (**A**) BM progenitor cells isolated from WT or *Fancc*$^{-/-}$ mice were cultured in cytokine-supplemented medium in the absence or presence of the pan-caspase inhibitor Z-VAD-FMK (ZV-FMK; 100 μM) for 2 hours before H_2O_2 (10 μM) was added to the culture in each medium change. The cells were cultured for 30 days. The total number of viable cells was counted by the trypan blue exclusion method at the times indicated. Results were expressed as mean of duplicates. (**B**) Cells described in **A** were harvested on days 1 and 10 and analyzed for DNA strand breaks by the comet assay. The mean tail moment of WT control sample is expressed as 100%. Larger tail moments represent higher levels of DNA damage. Two cultures from each group were analyzed, and 50 cells per culture were scored from random sampling.

Table 6
Increased constitutional chromosome instability in *Fancc*$^{-/-}$ leukemia

Aberration	WT (%)	*Fancc*$^{-/-}$ (%)
Chromosome breaks	1	19
Chromosome fragment	0	5
Chromatid break	3	21
Chromatid gap	4	9
Complex rearrangement	0	6
Deletions	2	11
Dicentric chromosome	0	7
Double minute	0	3
Translocation	0	76

without expressing oncogenes ectopically provides an important opportunity for the general field of leukemogenesis.

Methods

Mice and treatments. WT and *Fancc*$^{-/-}$ mice were generated by interbreeding heterozygous *Fancc*$^{+/-}$ mice (a generous gift from Manuel Buchwald, University of Toronto, Ontario, Canada; ref. 57). The genetic background of the mice was C57BL/6 (CD45.2^{+}). The primers for genotyping were as follows: common, 5′-GAGCAACACAAATGGTAAGG-3′; WT, 5′-CCTGCCATCTTCAGAATTGT-3′; knockout, 5′-TTGAATGGAAGGATTGGAGC-3′. All of the mice were used at approximately 8–10 weeks of age. In mice that received TNF-α after stem cell transplantation, 1 injection (100 μg/kg) of mouse recombinant TNF-α (Peprotech) was given i.p. 10 days after transplantation. NAC (Sigma-Aldrich) was administered by subcutaneous injection daily at a dose of 100 mg/kg/d for 10 days following TNF-α injection. All experimental procedures conducted in this study were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

Isolation and long-term TNF-α treatment of BM hematopoietic stem/progenitor cells in vitro. Low-density BM mononuclear cells were depleted of lineage-positive cells using MACS columns (Miltenyi Biotec) in accordance with manufacturer's instruction. Lin-Sca1^{+} cells were then purified by staining the Lin^{-} cells with Sca-1-PE antibodies (BD Biosciences — Pharmingen) followed by cell sorting using a FACSCalibur (BD Biosciences). Cells were cultured in IMDM medium containing 100 ng/ml of SCF, 20 ng/ml of IL-6, 10 ng/ml of IL-11, and 50 ng/ml of Flt-3 ligand (Flt-3L) (Peprotech) with or without 10 ng/ml of TNF-α or 30 μM of the pharmaceutical JNK inhibitor (SP600125; Calbiochem) for 30 days. For H_2O_2 treatment, cells

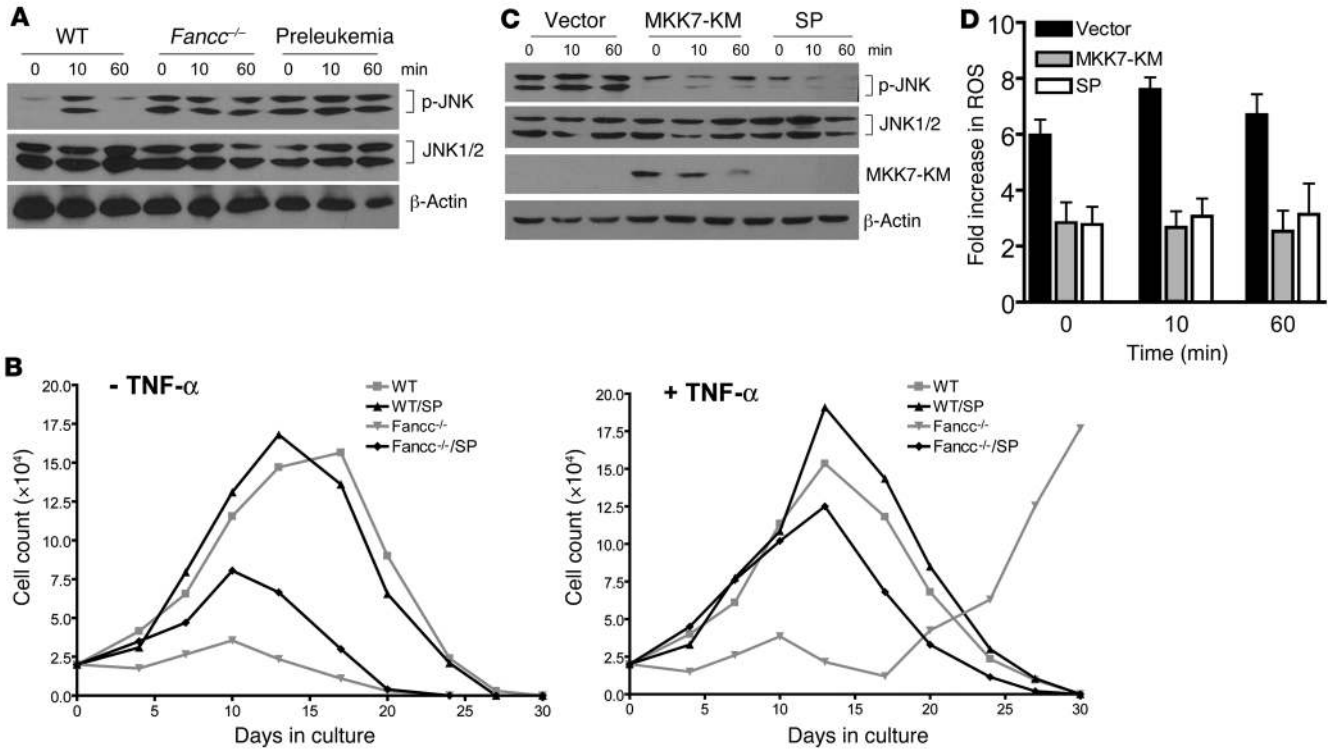

Figure 7
Role of JNK kinase in TNF-α–induced malignant transformation in *Fancc*$^{-/-}$ BM stem/progenitor cells. (**A**) Prolonged TNF-α–induced JNK activation in *Fancc*$^{-/-}$ cells. Low-density BM mononuclear cells freshly isolated from WT or *Fancc*$^{-/-}$ mice or *Fancc*$^{-/-}$ preleukemic cells were cultured in the presence of TNF-α (10 ng/ml) for the indicated time periods, and protein extracts were prepared and analyzed by immunoblotting with anti–phospho-JNK, anti-JNK, and anti-actin antibodies. (**B**) BM progenitor cells isolated from WT or *Fancc*$^{-/-}$ mice were treated with or without the pharmaceutical JNK inhibitor (SP600125 [SP]; 30 μM) in the absence (left panel) or presence (right panel) of TNF-α (10 ng/ml) for 30 days. The total number of viable cells was counted by the trypan blue exclusion method at the times indicated. Results are expressed as mean of duplicates. (**C**) Inhibition of JNK activation by overexpression of a dominant-negative MKK7 and SP600125 in *Fancc*$^{-/-}$ preleukemic cells. Cells were transduced with retroviruses carrying vector alone or MKK7-KM or treated with SP600125 (30 μM) and cultured in the presence of TNF-α (10 ng/ml) for the indicated time periods; and protein extracts were prepared and analyzed by immunoblotting with anti–phospho-JNK, anti-JNK, anti-Flag (for MKK7-KM), and anti-actin antibodies. (**D**) Inhibition of the JNK kinase reduces ROS production in *Fancc*$^{-/-}$ preleukemic cells. Cells described in **C** were cultured in the presence of TNF-α (10 ng/ml) for the indicated time periods, labeled with CM-H_2DCFDA, and subjected to flow cytometry. Data represent mean ± SD of 3 independent experiments.

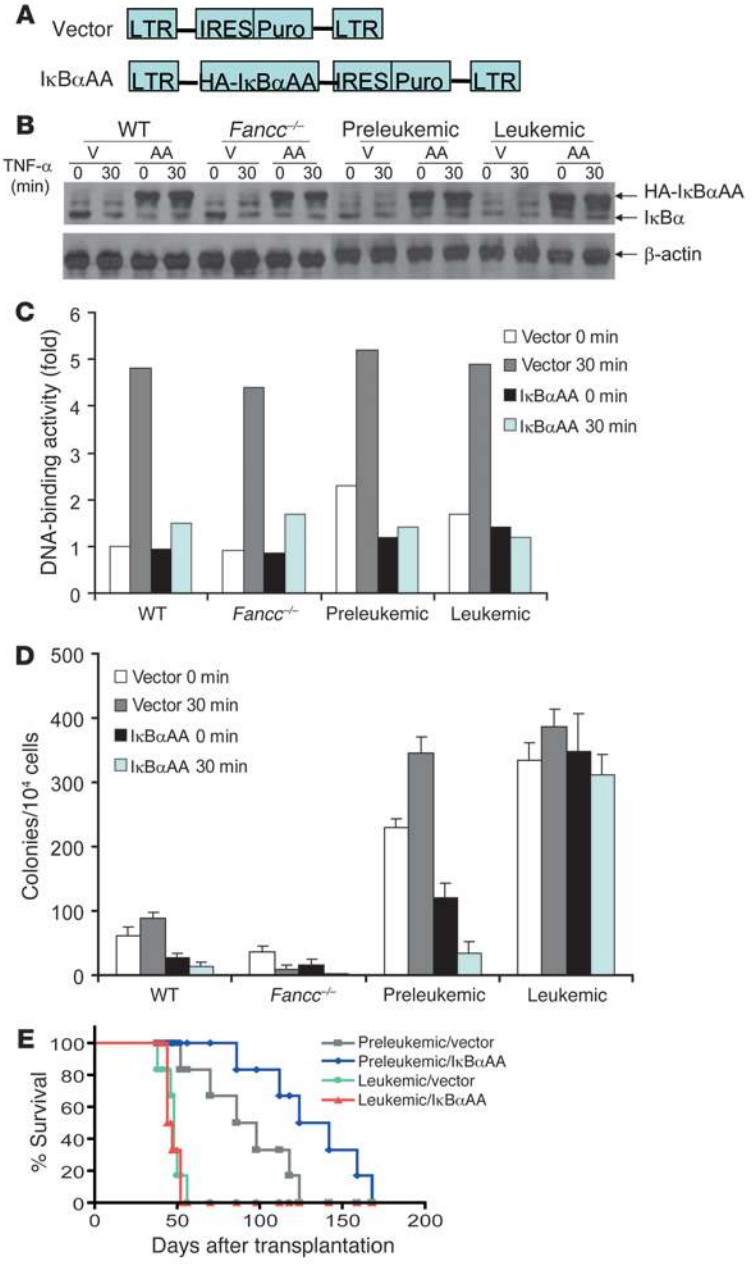

Figure 8
Inhibition of NF-κB fails to prevent leukemia development in *Fancc*$^{-/-}$ BM progenitors. (**A**) Retroviral constructs expressing an IκBα super-repressor mutant (IκBαAA). (**B** and **C**) IκBαAA effectively inhibited TNF-α–induced NF-κB activation as determined by assessing IκBα degradation (**B**) and NF-κB DNA-binding activity (**C**). (**D**) Inhibition of NF-κB activation suppresses clonal growth of preleukemic but not leukemic progenitor cells. Colony growth is shown for each group, including untreated (0 min) and TNF-α–treated (30 min) freshly isolated WT, *Fancc*$^{-/-}$, and *Fancc*$^{-/-}$ preleukemic and leukemic cells, each of which expressed the IκBα super-repressor IκBαAA or empty vector. Data represent the number (mean ± SD) of total colonies from 3 independent experiments. (**E**) Survival of recipient mice transplanted with preleukemic or leukemic cells transduced with the IκBα super-repressor IκBαAA or empty vector. Experiments were performed 2 times, each with 3 recipient mice (total 6 mice per group).

were incubated with the pan-caspase inhibitor Z-VAD-FMK (100 μM; Calbiochem) for 2 hours before H_2O_2 (10 μM; Sigma-Aldrich) was added to the culture in each medium change.

Retroviral vectors and infection. The full-length human *FANCC* cDNA (GenBank accession number NM000136) was amplified by PCR, using Pfu DNA polymerase (Stratagene) and subcloned into the *Not*I site of retroviral vector SFβ91 (a gift from Chris Baum, Cincinnati Children's Hospital Medical Center) to create SFβ91-FANCC. The cDNA of the IκBα super-repressor mutant IκBαAA (a gift from Michael Karin, UCSD, San Diego, California, USA) was subcloned into the MSCV-Puro retroviral vector (BD Biosciences — Clontech) to create MSCV-Puro-IκBαAA. The retroviral vector Flag-MKK7-KM has been reported elsewhere (36). Retroviruses were prepared by the Viral Vector Core of Cincinnati Children's Research Foundation. Retroviral supernatant was collected at 36 hours, 48 hours, and 60 hours after transfection. Cells were plated onto Retronectin-coated (Takara-Shuzo) non-tissue culture 24-well plates and prestimulated for 2 days in IMDM medium containing 20% FCS, 100 ng/ml SCF, 20 ng/mL IL-6, and 50 ng/mL Flt-3L (Peprotech). Cells were then exposed to the retroviral supernatant for 3 hours at 37°C in the presence of 4 μg/ml polybrene (Sigma-Aldrich). Cells were centrifuged at 600 *g* for 45 minutes. Infection was repeated 2 times, and infection efficiency was assessed by the detection of GFP$^+$ cells by flow cytometry.

Clonogenic progenitor assays. Unfractionated marrow cells were cultured in a 35-mm tissue culture dish in 4 ml of semisolid medium containing 3 ml of MethoCult M 3134 (Stem Cell Technologies) and the following growth factors: 100 ng/ml SCF, 10 ng/ml IL-3, 100 ng/ml G-CSF, and 4 U/ml erythropoietin (Peprotech). On day 10 after plating, erythroid and myeloid colonies were enumerated and photographed. Hematopoietic clonal growth results were expressed as means (of triplicate plates) ± SD.

BM transplantation. Age-matched congenic B6.SJL-PtrcaPep3b/BoyJ (B6.BoyJ; CD45.1$^+$) mice (The Jackson Laboratory) were used as transplant recipients. These mice were lethally irradiated (9.5 Gy, 110 cGy/min, ^{137}Cs) and injected i.v. with 1 × 10^6 test cells (CD45.2$^+$), mixed with 1 × 10^6 competitor cells (BoyJ; CD45.1$^+$). In some experiments, recipient mice were injected i.p. with 1 dose of TNF-α (100 μg/kg/) or both TNF-α and NAC (100 mg/kg/d) for 10 days starting at day 10 after transplantation. Donor-derived repopulation in recipients was assessed by the proportion of leukocytes in peripheral blood that expressed the CD45.2 (by staining the cells with CD45.1-PE and CD45.2-APC) marker by flow cytometry. Short- and long-term engraftment analysis of donor cells was performed at 1 month and 4 months after transplantation, respectively. For secondary BM transplantation, 2 × 10^6 BM cells from individual primary leukemic recipients were injected into cohorts of 6 lethally irradiated recipients.

Determination of ROS production. BM cells were incubated with CM-H_2DCFDA (Molecular Probes; Invitrogen) in the dark for 15 minutes at 37°C. After washing, the cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Data were analyzed using the CellQuest program (BD Biosciences).

Histology, cytology, and immunohistochemistry. At necropsy, organs were removed, preserved in formalin, and then embedded in paraffin blocks. Sections were stained with H&E. For cytological and morphological analysis, cytospin preparations were stained with Wright-Giemsa (Fisher Scientific). For immunohistochemistry, paraffin sections were deparaffinized,

rehydrated, incubated in 0.1 mM sodium citrate (pH 6.0), washed and incubated with peroxidase blocking reagent (VectaStain Elite ABC kit; Vector Laboratories). After washing in PBS, the slides were incubated with the primary antibodies 8-oxodG (Alpha Diagnostic). Following 3 PBS washes, slides were incubated with secondary antibody and then detected with the VectaStain Elite ABC reagents.

NF-κB activation. Nuclear protein extracts were prepared from BM cells using a Transfactor Extraction kit (BD Biosciences). Nuclear extracts were incubated with DNA specific for the NF-κB consensus sequence, and the DNA binding activity of NF-κB was measured using a Transfactor kit (BD Biosciences).

Immunoblotting. Cells were solubilized in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet NP-40) containing a cocktail of protease inhibitors (Calbiochem). Equal amounts of protein were separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and blotted with antibodies against IγBγ, p65, TRAIL, $p53^{Ser20}$ (all from Santa Cruz Biotechnology Inc.), γH2AX (Upstate), caspase-3 and caspase-8 (Cell Signaling Technology), and Flag (M2) and γ-actin (Sigma-Aldrich).

RNA isolation and RT-PCR. Total RNA was prepared with an RNeasy kit (QIAGEN) following the manufacturer's procedure. Reverse transcription was performed with random hexamers and Superscript II RT (Invitrogen) and was carried out at 42°C for 60 minutes and stopped at 95°C for 5 minutes. These reactions were followed by PCR using primers listed in Supplemental Table 3, with the following thermal cycling parameters: 95°C for 5 minutes; 15, 20, or 30 cycles at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes.

Serum levels of cytokines. The serum levels of inflammatory cytokines were measured using ELISA kits (R&D Systems) according to the manufacturer's instructions.

LM-PCR. LM-PCR was performed as described previously (58). Briefly, 200–400 ng of genomic DNA was digested with RNase (Gentra Systems) and *Tsp509* I (New England Biolabs) at 37°C for 30 minutes, followed by 65°C for 5 hours. For primer extension (PE 95°C for 5 minutes, 64°C for 30 minutes, 72°C for 15 minutes), restriction-digested DNA was added to the reaction mixture containing 2 U of Native Pfu polymerase (Stratagene), 1× final concentration of reaction buffer, 200 μM dNTPs (Invitrogen), and 0.25 pmol/μl biotinylated retroviral primer rvLTRI (5′-CTGGGGACCATCTGTTCTTGGCCTC-3′). The extension product was cleaned using Qia Quick PCR kit (QIAGEN) and eluted in 40 μl water. 200 μg of streptavidin-coated magnetic beads (Invitrogen) washed and diluted in 2× BW buffer (10 mM Tris pH 7.5, 1 mM EDTA, 2 M NaCl) was rotated with the eluted sample for 5 hours at room temperature. The captured DNA was exposed to a magnetic particle concentrator. The supernatant was discarded, and the captured DNA was washed twice with 100 μl of H_2O and then resuspended in 5 μl H_2O. In the presence of 80 U of T4 DNA Ligase (New England Biolabs), 40 pmol of annealed linker cassette was added to the captured DNA and incubated at 16°C overnight. Then the samples were washed 3 times with H_2O and finally resuspended in 10 μl H_20. For the first exponential PCR (initial denaturation: 94°C 2 minutes, then 94°C 15 seconds, 60°C 30 seconds, 68°C 1 minute for 30 cycles, final extension: 68°C for 10 minutes), 1 μl of the ligation product was added to the 2× Extensor High Fidelity PCR master mix (Abgene) with 25 pmol of retroviral primer rvLTRII (5′-GCCCTTGATCTGAACTTCTC-3′) and linker-specific primer OCI (5′-GACCCGGGAGATCTGAATTC-3′). Nested PCR was done with 25 pmol of retroviral primer rvLTRIII (5′-CCATGCCTTGCAAAATGGC-3′) and linker-specific primer OCII (5′-AGTGGCACAGCAGTTAGG-3′) under conditions identical to those used for the first PCR.

Comet assay. The generation of DNA strand breaks was assessed by the single-cell gel electrophoresis (comet) assay (38), using a Fpg-FLARE (fragment length analysis using repair enzymes) comet assay kit in accordance with the manufacturer's instructions (Trevigen). For each experimental point, at least 3 different cultures were analyzed, and 50 cells were evaluated from each sample. Comet tail length and tail moment were measured under a fluorescence microscope (Nikon model 027012) using an automated image analysis system based on a public domain NIH image program (38) (http://rsb.info.nih.gov/nih-image/).

Cytogenetic analysis. BM cells were incubated in 0.1 mg/ml Colcemid for 30–60 minutes and then incubated in 75 mM KCl. Chromosomes were subsequently fixed in 3:1 methanol/acetic acid and dropped onto glass slides. Metaphase chromosomes were stained with Giemsa and examined for abnormalities. For spectral karyotype (SKY) analysis, metaphase spreads were incubated with a mouse SkyPaint kit probe (Applied Spectral Imaging), followed by counterstain with DAPI. Raw spectral images were visualized by assigning red, green, and blue colors to specific spectral ranges. Chromosomes were identified using SKY View software (Applied Spectral Imaging). Chromosome aberrations were defined using the nomenclature rules from the International Committee on Standard Genetic Nomenclature for Mice. Approximately 30 metaphases were analyzed for each cell line.

Statistics. Data were analyzed statistically using a 2-tailed Student's *t* test or Kaplan-Meier survival analysis. Statistical significance was presumed when P was less than 0.05.

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