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Leukemia and chromosomal instability in aged *Fancc*$^{-/-}$ mice

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Fanconi anemia (FA) is an inherited disorder of genomic instability associated with high risk of myelodysplasia and acute myeloid leukemia (AML). Young mice deficient in FA core complex genes do not naturally develop cancer, hampering preclinical studies on malignant hematopoiesis in FA. Here we describe that aging *Fancc*$^{-/-}$ mice are prone to genomically unstable AML and other hematologic neoplasms. We report that aneuploidy precedes malignant transformation during *Fancc*$^{-/-}$ hematopoiesis. Our observations reveal that *Fancc*$^{-/-}$ mice develop hematopoietic chromosomal instability followed by leukemia in an age-dependent manner, recapitulating the clinical phenotype of human FA and providing a proof of concept for future development of preclinical models of FA-associated leukemogenesis.

The Fanconi anemia (FA) signaling network protects genomic integrity and prevents cancer by facilitating interphase DNA repair and orchestrating cell division [1–3]. Germline biallelic mutations of any FA genes cause Fanconi anemia, an inherited bone marrow failure syndrome associated with myelodysplasia (MDS) and acute myeloid leukemia (AML). The overall risk of leukemia in FA is increased six hundredfold [4].

Young mice deficient in core FA genes do not spontaneously recapitulate clinical hematopoietic manifestations of Fanconi anemia [5]. *Fancc*$^{-/-}$ mice demonstrate hypersensitivity to cross-linking agents [6], decreased hematopoietic stem cell repopulating ability [7,8], and hypersensitivity to interferon-γ [8], reflecting disruption of the FA signaling network during hematopoiesis. However, young *Fancc*$^{-/-}$ mice do not develop spontaneous leukemia or bone marrow failure [6,8]. One observation study of a small *Fancc*$^{-/-}$ mouse cohort ($n = 8$) did not detect decreased survival [9]. However, FA patients rarely develop AML in their first year of life [10], and two soft tissue tumors (adenocarcinoma and histiocytic sarcoma) have been reported in >13-month-old *Fancc*$^{-/-}$ mice [11]. Thus, we hypothesized that aging *Fancc*$^{-/-}$ mice may be predisposed to hematopoietic malignancies. If the absolute time to the onset of leukemia is similar in FA humans and mice, long-term observation of FA$^{-/-}$ mice may be crucial to detection of cancer predisposition. To address this translationally relevant question, we asked whether Fancc$^{-/-}$ mice develop malignancies as they age.

Methods

Mice

C57Bl/6J Fancc$^{-/-}$ mice were a gift of David W. Clapp (Indiana University). Mice were genotyped by polymerase chain reaction (PCR), as described [9]. B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ mice were purchased from the Indiana University In Vivo Therapeutics Core. All studies were approved by the Institutional Animal Care and Use Committee at Indiana University.

Marrow harvest and transplantation

Bone marrow cells were flushed from mouse femurs using a 23-gauge needle/syringe (Becton Dickinson). Light-density mononuclear cells (LDMNCs) were isolated by density gradient using Histopaque-1119 (Sigma) and centrifuging for 30 min at 1,800 rpm with no brake. After centrifugation, LDMNCs were removed from the interface and used for experiments. Cytospins were made by resuspending LDMNCs in phosphate-buffered saline (PBS) and centrifuging onto slides at 450 rpm for 5 min on a Shandon Cytospin 3 Cytocentrifuge (Thermo Scientific). Donor test LDMNCs (1.5×10^6, C57Bl/6J background) and donor

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competitor BoyJ LDMNCs (1.5×10^6) were transplanted into recipients via tail vein injection. Recipients were 8-week-old female B6.SJL-Ptprc^{a}Pepc^b/BoyJ mice that underwent whole-body split-dose 1,100-rad irradiation (700 rads/400 rads, 4 hours apart). For chimerism analysis, peripheral blood was collected from lateral tail veins into EDTA-coated tubes, incubated with red blood cell lysis solution (Qiagen) for 10 min at room temperature, washed, stained with anti-Cd45.2-flluorescein isothiocyanate (BD Biosciences) and anti-Cd45.1-phycoerythrin (BD Biosciences), as described [9], and analyzed on a FacsCalibur machine (Becton-Dickinson). At least 10,000 events/sample were acquired and analyzed using FlowJo Software.

Metaphase spreads

Bone marrow cells flushed from tibias were cultured in Iscove's modified Dulbecco's medium (IMDM) plus 20% fetal bovine serum, murine stem cell factor (100 ng/mL), and interleukin-6 (200 ng/mL) for 2 days. Cells were then exposed to 0.2 μg/mL colcemid (Life Tech) for 4 hours and pelleted at 800 rpm for 5 min. Cells were resuspended dropwise in prewarmed (37°C) 75 mM KCl and incubated at 37°C for 15 min. After pelleting, cells were resuspended in a 3:1 methanol:glacial acetic acid fixative. Cells were pelleted and resuspended in fixative two additional times before being dropped onto slides and dried overnight. Spreads were stained with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories).

Histology and flow cytometry

Murine tissues obtained postmortem were fixed in 10% formalin, paraffin-embedded, sectioned (5-μm sections), and stained with hematoxylin and eosin. Peripheral blood smears and bone marrow cytospins were stained with Giemsa, using the automated Siemens Hematek 3000 (Fisher) system. For flow cytometry, peripheral blood cells were incubated in RBC lysis solution, and bone marrow LDMNCS were isolated as described above. Cells were stained with either Gr-1-APC (Ly6G, clone: RB6-8C5) or B220-FITC (clone: RA3-6B2) and analyzed on a FacsCalibur machine, using live gating followed by data quantification with FlowJo software. Leukemia diagnoses were made using criteria established in the Bethesda proposal for classification of nonlymphoid neoplasms in mice [12], and were independently validated by a veterinary pathologist at the Indiana University School of Medicine.

Microscopy

Images of smears, cytospins, and histologic sections were obtained using a Zeiss Axiolab microscope with a color camera. Metaphase spreads were imaged on a Deltavision personalDx deconvolution microscope (Applied Precision). Image stacks (distance between *z*-sections: 0.2 μm) were deconvolved using Softworx and analyzed using Imaris software suite (Bitplane).

Statistics

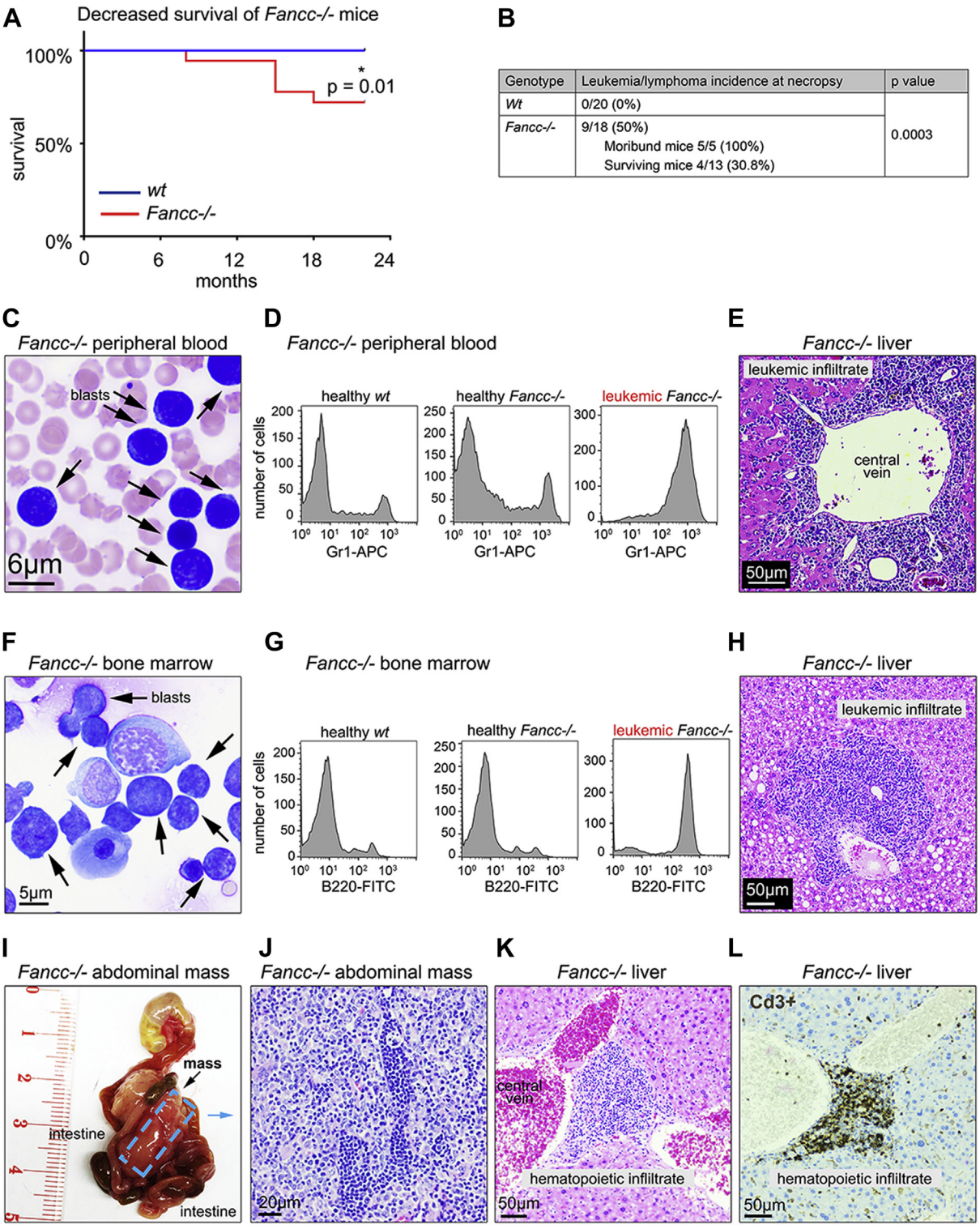
Statistical analysis was performed with GraphPad Prism 6 software (GraphPad Software, La Jolla, CA).

Results

We observed cohorts of wild-type (WT; $n = 20$) and *Fancc*$^{-/-}$ ($n = 18$) mice for 24 months and noticed decreased survival of *Fancc*$^{-/-}$ mice ($p = 0.01$) (Fig. 1A). Five *Fancc*$^{-/-}$ mice (27.8%) died between 8 and 24 months of age from leukemia or lymphoma (Fig. 1B–L). Specifically, we diagnosed AML in two moribund *Fancc*$^{-/-}$ mice with peripheral blasts, predominance of Gr-1$^+$ (Ly-6G) peripheral blood LDMNCs, and myeloid infiltrates around the liver vessels (Fig. 1C–E). One *Fancc*$^{-/-}$ mouse developed lethal B-cell acute lymphoblastic leukemia (ALL), as evidenced by expansion of B220$^+$ blasts that replaced >90% of bone marrow and infiltrated the liver (Fig. 1F–H). Additionally, two *Fancc*$^{-/-}$ mice died from metastatic abdominal T-cell lymphoma manifested by massive mesenteric lymph node conglomerates (Fig. 1I–J) and accompanied by Cd3$^+$ liver infiltrates (Fig. 1K–L) in the absence of bone marrow or peripheral blood abnormalities. After 24 months of observation, all surviving *Fancc*$^{-/-}$ and WT mice were sacrificed and examined by necropsy. Four of 13 (30.8%) 2-year-old *Fancc*$^{-/-}$ animals had hematopoietic solid tumors and/or peripheral blasts, consistent with leukemia/lymphoma. Serial blood counts did not reveal progressive pancytopenia in aging *Fancc*$^{-/-}$ mice, suggesting that the development of leukemia may not be preceded by bone marrow failure in this animal model of FA. Nine of 18 *Fancc*$^{-/-}$ mice developed hematopoietic malignancies by 2 years of age (including 5 animals that died prematurely from disease), compared with zero of 20 control WT mice ($p = 0.0003$) (Fig. 1B). Thus, aging Fancc$^{-/-}$ mice are prone to hematopoietic neoplasms, reflecting the age-dependent risk of leukemia in FA patients [4,10,13].

We next asked whether Fancc$^{-/-}$ AML can be propagated in WT mice via competitive stem cell transplantation. We mixed donor *Fancc*$^{-/-}$ Cd45.2$^+$ LDMNCs isolated from a moribund AML *Fancc*$^{-/-}$ mouse (Fig. 1C–E) with WT Cd45.1$^+$ competitor LDMNCs at a 1:1 ratio and transplanted the mixed cells into three lethally irradiated WT recipients. Three WT recipients of age-matched WT Cd45.2$^+$ LDMNCs mixed with WT Cd45.1$^+$ LDMNCs served as controls (Fig. 2A). By 50 days posttransplantation, all recipients of *Fancc*$^{-/-}$ LDMNCs had died of AML, whereas control recipients of WT LDMNCs remained healthy (Fig. 2B). The diagnosis of AML was confirmed in all recipients by flow cytometry, peripheral blood smears (Fig. 2C), and splenomegaly ($p = 0.0216$) (Fig. 2D). Peripheral blood flow cytometry revealed increased Cd45.2$^+$ chimerism ($p = 0.0436$) in recipients of leukemic *Fancc*$^{-/-}$ LDMNCs compared with controls at 1 month posttransplant (Fig. 2E, F), highlighting the malignant potential of leukemic *Fancc*$^{-/-}$ LDMNCs to outcompete WT hematopoietic cells in the host marrow.

The FA signaling network maintains genomic integrity during *Fancc*$^{-/-}$ hematopoiesis in vivo [14], and genomic instability promotes cancer [15]. Thus, we asked whether leukemic *Fancc*$^{-/-}$ mice exhibit increased chromosomal instability and whether chromosomal instability precedes



Genotype	Leukemia/lymphoma incidence at necropsy	p value
Wt	0/20 (0%)	0.0003
Fancc-/-	9/18 (50%) Moribund mice 5/5 (100%) Surviving mice 4/13 (30.8%)	

Figure 1. Aging *Fancc*$^{-/-}$ mice develop hematologic malignancies. (**A**) Kaplan–Meier survival curve of WT (n = 20) and *Fancc*$^{-/-}$ (n = 18) cohorts. (**B**) Table outlining incidence of leukemias and lymphomas in WT and *Fancc*$^{-/-}$ mice by 24 months of age. Statistical significance for (**A**) and (**B**) was determined using a log-rank (Mantel–Cox) test. (**C**) Peripheral blood smear of a moribund *Fancc*$^{-/-}$ mouse reveals leukemic blasts (*arrows*). (**D, E**) Diagnosis of acute myeloid leukemia was confirmed with flow cytometry, which revealed (**D**) increased expression of the Gr1 myeloid marker in the peripheral blood compared with WT and healthy *Fancc*$^{-/-}$ controls (**E**) and the presence of leukemic infiltrates in the liver. (**F**) Bone marrow cytospin of another moribund *Fancc*$^{-/-}$ mouse revealed multiple blasts (*arrows*). (**G, H**) Flow cytometry indicated increased expression of the B220 B-cell marker on bone marrow blasts, (**G**) and necropsy revealed leukemic infiltrates in the liver (**H**) consistent with B-cell ALL. (**I, J**) Necropsy of another *Fancc*$^{-/-}$ mouse revealed conglomerates of mesenteric lymph nodes. (**K, L**) Liver infiltrates in this mouse were Cd3^{+}, consistent with T-cell lymphoma.

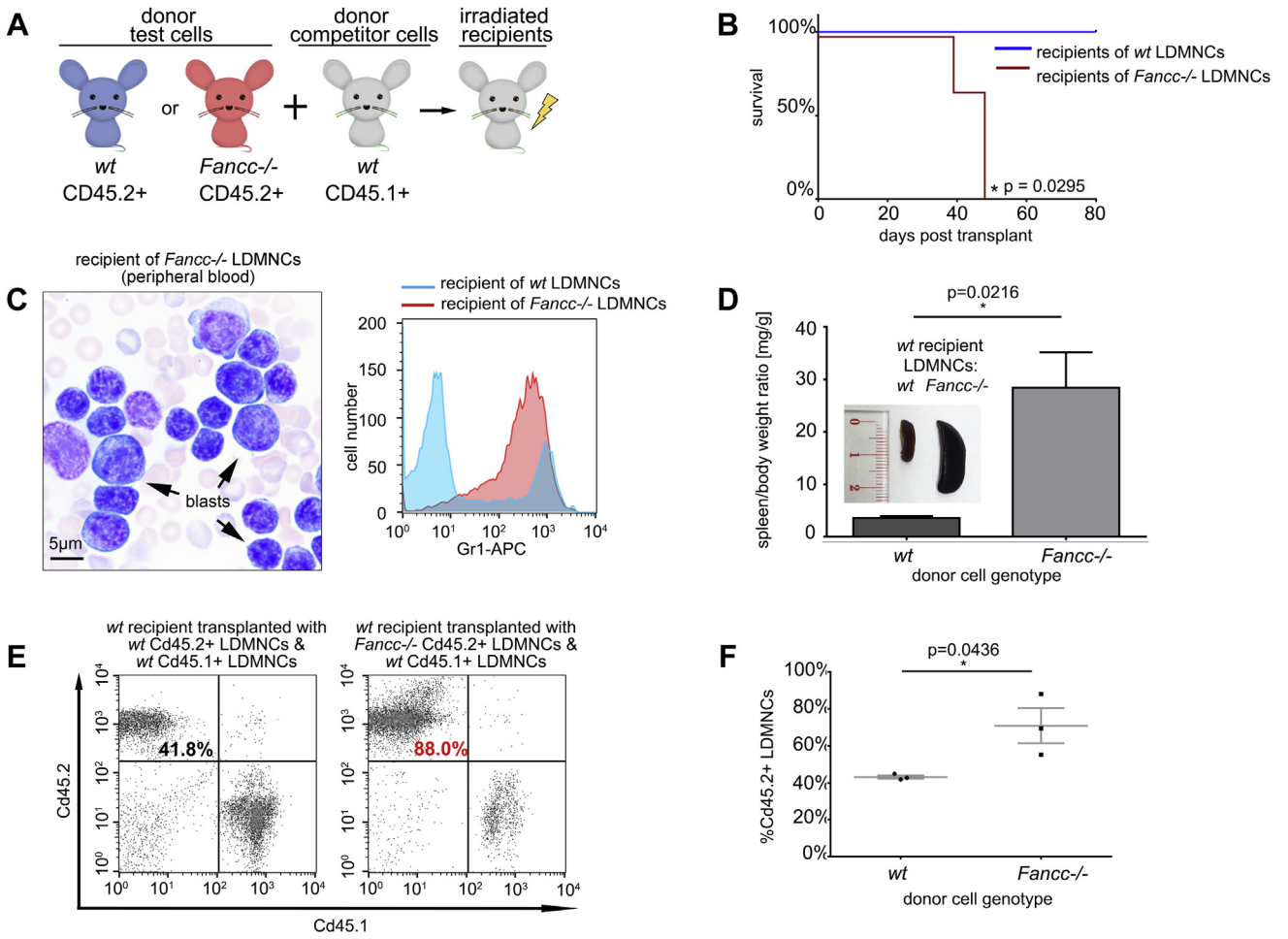


Figure 2. Transplanted *Fancc*$^{-/-}$ AML LDMNCs induce aggressive leukemia in WT recipients. (**A**) Schematic of competitive repopulation transplantation. Cd45.2^{+} LDMNCs obtained from a leukemic *Fancc*$^{-/-}$ mouse or WT control were mixed 1:1 with Cd45.1^{+} WT competitor LDMNCs and transplanted into lethally irradiated WT recipients (n = 3 recipients per genotype). (**B**) Recipients of *Fancc*$^{-/-}$ LDMNCs died within 2 months of transplantation. A log-rank (Mantel–Cox) test was used to assess significance. (**C**) AML in WT recipients of *Fancc*$^{-/-}$ LDMNCs. Gr1 was used as a myeloid marker for flow cytometry (right panel). (**D**) Splenomegaly in moribund WT mice transplanted with *Fancc*$^{-/-}$ LDMNCs. (**E, F**) Leukemic Cd45.2^{+} *Fancc*$^{-/-}$ LDMNCs overpopulate recipient bone marrow compared with WT Cd45.1+ competitor LDMNCs. Statistical significance was determined using an unpaired Student t test; error bars represent SEM.

the onset of leukemia during *Fancc*$^{-/-}$ hematopoiesis. We compared karyotypes of LDMNCs isolated from leukemic *Fancc*$^{-/-}$ with those of age-matched WT and healthy *Fancc*$^{-/-}$ marrows. Bone marrow cells isolated from healthy *Fancc*$^{-/-}$ mice had a higher incidence of aneuploidy and an increased frequency of abnormal mitotic figures compared with WT LDMNCs (Fig. 3A–D), indicating that *Fancc*$^{-/-}$ hematopoietic cells become chromosomally unstable before overt leukemogenesis occurs. Similarly, FA patients develop hematopoietic chromosomal and nuclear abnormalities prior to the onset of leukemia [14,16,17]. Leukemic *Fancc*$^{-/-}$ bone marrows were more aneuploid (Fig. 3B) with higher mitotic index compared with both age-matched WT ($p = 0.001$) and *Fancc*$^{-/-}$ nonleukemic ($p < 0.0001$) (Fig. 3D) marrows. This observation is consistent with further exacerbation of genomic instability and acquisition of bizarre karyotypic abnormalities reported in human FA-associated AML [18,19].

Discussion

Fancc$^{-/-}$ mice develop chromosomally unstable hematopoietic malignancies as they age, recapitulating clinical and genomic abnormalities seen in patients with Fanconi anemia (Figs. 1 and 3). Interestingly, a similar incidence of tumors has been reported in old mice deficient in another FA core gene, *Fanca*, although that observation has not reached statistical significance because of small sample sizes [20]. Thus, late-onset carcinogenesis may be a common phenotype of murine FA core gene knockouts. AML arising in *Fancc*$^{-/-}$ mice can be propagated via hematopoietic stem cell transplant and can produce rapid onset of lethal leukemia in WT transplant recipients

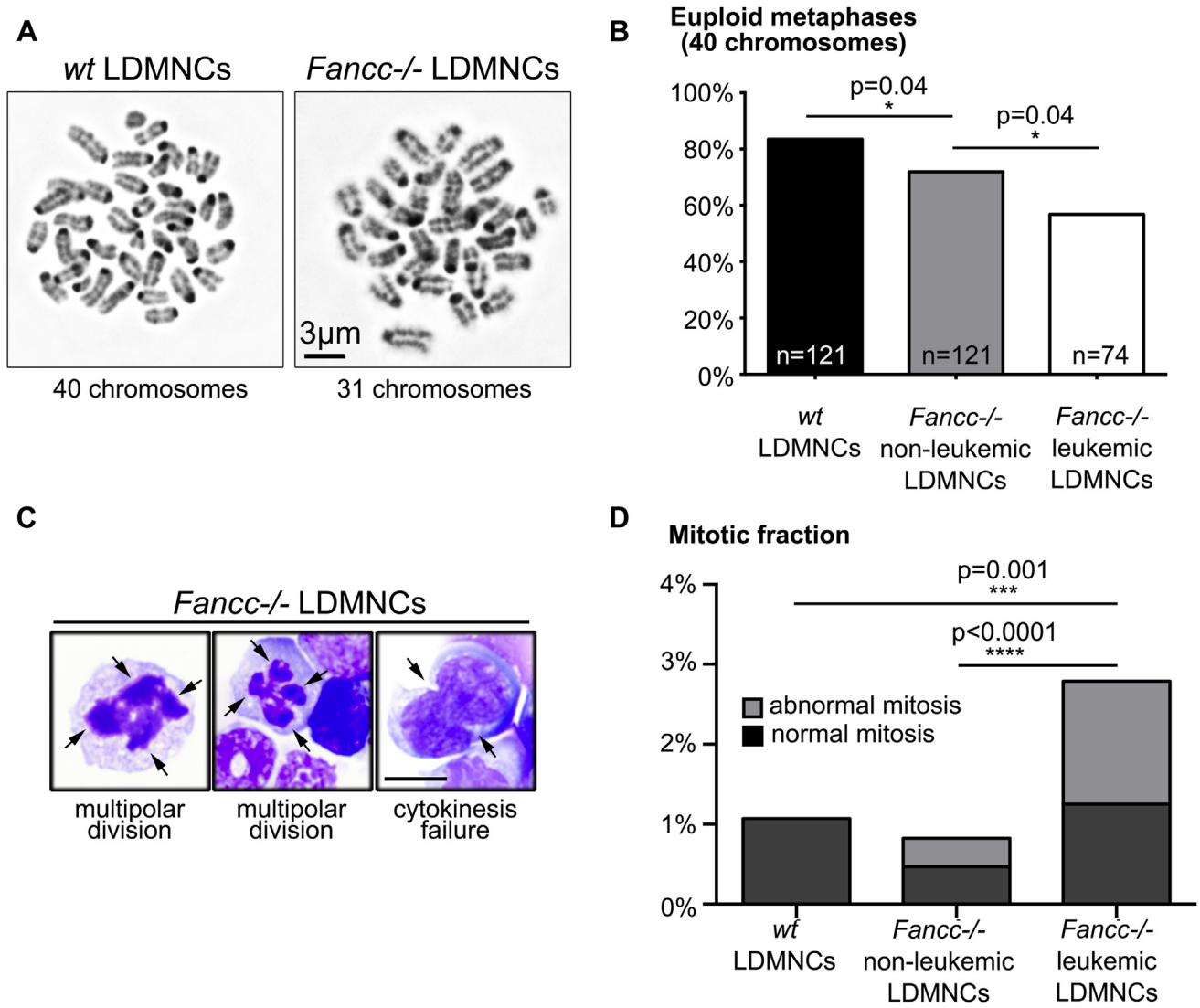


Figure 3. Genomic instability and abnormal mitosis in leukemic and preleukemic *Fancc*$^{-/-}$ mice. (**A**) Representative images of LDMNC metaphase spreads from WT and leukemic *Fancc*$^{-/-}$ mice. (**B**) Increased aneuploidy in leukemic *Fancc*$^{-/-}$ LDMNCs. At least 74 spreads were counted from WT, *Fancc*$^{-/-}$ nonleukemic, and *Fancc*$^{-/-}$ leukemic mice. Note increased chromosomal instability in nonleukemic *Fancc*$^{-/-}$ LDMNCs compared with age-matched WT controls. Fisher's exact test was used to determine statistical significance. (**C, D**) Leukemic *Fancc*$^{-/-}$ LDMNCs undergo abnormal mitosis (**C**) and have a higher mitotic index (**D**) compared with LDMNCs from WT and *Fancc*$^{-/-}$ nonleukemic mice (n = 3 mice/genotype; at least 500 cells were counted per genotype). Statistical analyses were performed using χ^2 tests with Yates correction.

(Fig. 2). As large-scale cohorts of leukemic mice are essential for preclinical drug testing, our observations may facilitate the development of future preclinical models of FA$^{-/-}$ AML.

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Conflict of interest disclosure

No financial interests/relationships with financial interest relating to the topic of this article have been declared.

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