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Fancd2 and p21 function independently in maintaining the size of hematopoietic stem and progenitor cell pool in mice

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Abstract

Fanconi anemia patients suffer from progressive bone marrow failure. An overactive p53 response to DNA damage contributes to the progressive elimination of Fanconi anemia hematopoietic stem and progenitor cells (HSPC), and hence presents a potential target for therapeutic intervention. To investigate whether the cell cycle regulatory protein p21 is the primary mediator of the p53-dependent stem cell loss, p21/*Fancd2* double-knockout mice were generated. Surprisingly double mutant mice displayed even more severe loss of HSPCs than *Fancd2*^{-/-} single mutants. p21 deletion did not rescue the abnormal cell cycle profile and had no impact on the long-term repopulating potential of *Fancd2*^{-/-} bone marrow cells. Collectively, our data indicate that p21 has an indispensable role in maintaining a normal HSPC pool and suggest that other p53-targeted factors, not p21, mediate the progressive elimination of HSPC in Fanconi anemia.

Keywords

Bone Marrow Failure; Fanconi Anemia; Hematopoietic Stem Cell; p21

Introduction

Fanconi anemia (FA) is a bone marrow failure disorder caused by the disruption of FA-BRCA network, which consists of at least 15 FA genes (including *FANCD2* and *FANCC*) and FA-associated genes (Bagby and Alter, 2006; Kim and D'Andrea, 2012).

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Authorship

Contributions: Q.S.Z. designed the study, performed research, analyzed and interpreted data, and wrote the manuscript; K.W.S., K.S., L.M.L., A.M., E.B., A.E.N., and E.J. performed research; A.M.S. and S.O. interpreted data and wrote the manuscript; and M.G. designed the study, interpreted data, and wrote the manuscript.

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Bone marrow failure is the primary cause of early mortality for FA patients (Kutler et al., 2003). It has been reported that a subset of FA patients with lower levels of CHK1 and p53 expression and abrogation of the G2 cell cycle checkpoint display much milder bone marrow deficiency (Ceccaldi et al., 2011). Further mechanistic studies established that an overactive p53 response to cellular stress and DNA damage drives the progressive elimination of hematopoietic stem and progenitor cells (HSPC) in FA patients, suggesting p53 down-regulation as a potential target for FA drug design (Ceccaldi et al., 2012).

It is also known that loss of p53 promotes carcinogenesis in both FA mouse models and human FA patients (Ceccaldi et al., 2011; Houghtaling et al., 2005; Rajagopalan et al., 2009). Given the detrimental tumorigenic effects of p53 loss, it is desirable to shut down only those p53-target genes specifically involved in HSPC elimination in FA. Further understanding the molecular targets of p53-mediated HSPC elimination could guide the future design of therapeutic regimens exploiting this approach. p21, encoded by the *Cdkn1a* gene, is a key p53-target gene and the main factor responsible for p53-mediated cell cycle arrest and apoptosis (Abbas and Dutta, 2009). p21 could even be the sole mediator of the overactive p53 response to DNA damage in FA HSPC, pointing to p21 inhibition as a way to prevent the progressive HSPC loss in FA (Murray et al., 2010; Sax et al., 2002). Interestingly, p21 deletion is already known to rescue stem cell self-renewal from mice suffering from DNA damage provoked by dysfunctional telomerase (Choudhury et al., 2007). On the other hand, it has also been reported that p21 has an important role in the regulation of FA-BRCA pathway activation (Rego et al., 2012). Therefore, the precise outcome of p21 deletion in FA patients warrants further investigation.

Fancd2^{-/-} mice on the 129S4 genetic background recapitulate major FA patient phenotypes, displaying tumor susceptibility and hematopoietic defects (Houghtaling et al., 2003; Zhang et al., 2010). Here we characterized p21 and *Fancd2* double-knockout mice to understand whether p21 is involved in the FA pathway and whether the rescue effects of p53 deletion on HSC maintenance are p21-related.

Methods

Mice

Fancd2 and *ROSA26* transgenic mice were maintained on the 129S4 background. *Cdkn1a* deficient mice were originally ordered from Jackson Laboratories (Bar Harbor, Maine) and backcrossed to the 129S4 background for more than 10 generations (Brugarolas et al., 1995). Heterozygotes were crossed inter se to generate mutant mice and littermate controls. All mice used were 4 months old. All animals were treated in accordance with the guidelines of the Institutional Animals Care and Use Committee.

Complete blood count (CBC)

Comprehensive CBC tests were measured by IDEXX Laboratories to monitor hematological parameters.

Histopathology

Testis sections were fixed in 10% phosphate-buffered formalin and stained with hematoxylin and eosin (H&E) as reported previously (Zhang et al., 2008).

Flow cytometry

Bone marrow cells were isolated from *Fancd2*^{-/-}/*Cdkn1a*^{-/-} mice and littermate controls, immunostained, and analyzed by flow cytometry, as previously described (Zhang et al., 2010).

In vivo competitive repopulation assay

Competitive repopulation assays were done as previously described (Zhang et al., 2010). Briefly, donor bone marrow cells were isolated from *Fancd2*^{-/-}/*Cdkn1a*^{-/-} mice (or littermate controls) and *ROSA26*^{Tg/O} mice. Test donor cells were mixed with *ROSA26*^{Tg/O} bone marrow cells at a 1:1 ratio and then transplanted into lethally irradiated *Fancc*^{-/-} recipient mice. Quantitative real-time PCR (qPCR) analysis of the peripheral blood was performed 6 months after transplantation.

Statistical analysis

Two-tailed, unpaired student's *t*-tests were performed to calculate *P* values using Prism 4.0 software (GraphPad Software Inc). A *P* value less than 0.05 was considered significant.

Results and Discussion

We reported previously that *Fancd2*^{-/-} mice have readily detectable hematopoietic defects, including fewer cKit⁺Sca1⁺Lin⁻ (KSL) hematopoietic stem and progenitor cells, compromised HSC repopulating capacity, and abnormal cell cycle status of KSL cells (loss of quiescence and increased cycling) (Zhang et al., 2010). To determine whether lack of p21 has an impact on any of these parameters in *Fancd2*^{-/-} mice, we characterized hematopoietic phenotypes of *Fancd2*^{-/-}/*Cdkn1a*^{-/-} mice first by measuring the size of their KSL pool. As compared to wild-type controls, both *Fancd2*^{-/-} mice and *Cdkn1a*^{-/-} mice suffered a significant reduction in the size of the KSL HSPC pool (Figure 1A). *Fancd2*^{-/-}/*Cdkn1a*^{-/-} mice had the smallest HSPC population among the different genotypes. This observation indicates that p21 and *Fancd2* deletion independently cause loss of hematopoietic stem and progenitor cells, suggesting that both *Fancd2* and p21 are important for maintaining the normal size of HSPC pool.

The ability to maintain quiescence is critical for HSC function (Orford and Scadden, 2008). Considering that p21 is an important cell cycle regulator, we next analyzed the cell cycle profiles of *Fancd2*^{-/-}/*Cdkn1a*^{-/-} KSL cells. As shown in Figure 1B, 1C & Figure 2, p21 deficiency did not significantly correct the abnormal cell cycle status of *Fancd2*^{-/-} KSL cells.

To further assess how *Fancd2*^{-/-}/*Cdkn1a*^{-/-} HSCs perform functionally in a stressful environment, we next performed *in vivo* competitive repopulation assays, in which test donor bone marrow cells competed with wild-type bone marrow bearing the *ROSA26*^{Tg/O} marker. We transplanted equal numbers of nucleated whole bone marrow cells into each lethally irradiated recipient and analyzed the recipient mice 6 months after transplantation. *Fancd2*^{-/-}/*Cdkn1a*^{-/-} bone marrow cells performed similarly to their single mutant *Fancd2*^{-/-} counterparts (Figure 1D). This is in stark contrast to the effect of p53 deletion, which significantly enhanced hematopoietic stem cell activity in *Fancd2*^{-/-} mice (Ceccaldi et al., 2012). Since *Fancd2*^{-/-} mice show germ cell loss (Houghtaling et al., 2003), we further examined whether p21 deletion would have any impact on this phenotype. As shown in Figure 3, testes from both *Fancd2*^{-/-} mice and *Fancd2*^{-/-}/*Cdkn1a*^{-/-} mice displayed tubules with vacuolated Sertoli cell cytoplasm and decreased numbers of spermatocytes and spermatids, once again demonstrating a lack of substantial rescue by p21 loss.

We also performed complete blood counts from 4-month old *Fancd2*^{-/-}/*Cdkn1a*^{-/-} mice and controls. Hematologic parameters of *Cdkn1a*^{-/-} mice were slightly higher than those of wild-type mice (Figure 4). However, the changes were insignificant. Overall *Fancd2*^{-/-}/*Cdkn1a*^{-/-} double mutant mice showed the worst hematopoietic parameters such as lowest platelet counts among all four genotype groups tested.

In summary, we observed that p21 deficiency did not rescue any of the major hematopoietic phenotypes of *Fancd2*^{-/-} mice, such as abnormal cell cycle status of HSPC and long-term repopulating potential. To the contrary, p21 deletion caused a further reduction in the HSPC pool. These results are in clear contrast to the obvious rescue effects from p53 deletion in mice on the same genetic background (Ceccaldi et al., 2012) and argue against the possibility that p21 functions as a primary mediator of p53-driven HSPC elimination in FA. Clearly other factors regulated by p53 play a more important role. It is noteworthy that p21 depletion in FA patient HSPCs is able to rescue their clonogenic growth defects (Ceccaldi et al., 2012). This discrepancy to our *in vivo* results may be partly due to the differences in the p53-mediated responses between human and mice. It is also possible that an acute knockdown of p21 may provide temporary benefits to hematopoiesis but that this is not sustained over time.

It has been under debate whether p21 is involved in normal HSC function (Tesio and Trumpp, 2011). In this study, we also uncovered an indispensable role of p21 in maintaining the normal size of the HSPC pool in 129S4 mice. This novel role was not seen in previous studies using p21 murine models on other genetic backgrounds (Cheng et al., 2000; van Os et al., 2007). Further investigation is needed to reconcile these discrepancies.

Acknowledgments

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Abbreviations

FA	Fanconi anemia
HSPC	hematopoietic stem and progenitor cells
H&E	hematoxylin and eosin
CBC	complete blood count
KSL	cKit ⁺ Sca1 ⁺ Lin ⁻
FITC	fluorescein isothiocyanate
qPCR	quantitative real-time PCR

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Highlights

1. Hyperactive p53 activity in Fanconi anemia stem cells is not mediated by p21.
2. p21 is important for maintaining the normal size of HSPC pool.
3. Fancd2 and p21 work independently in maintain normal hematopoiesis.

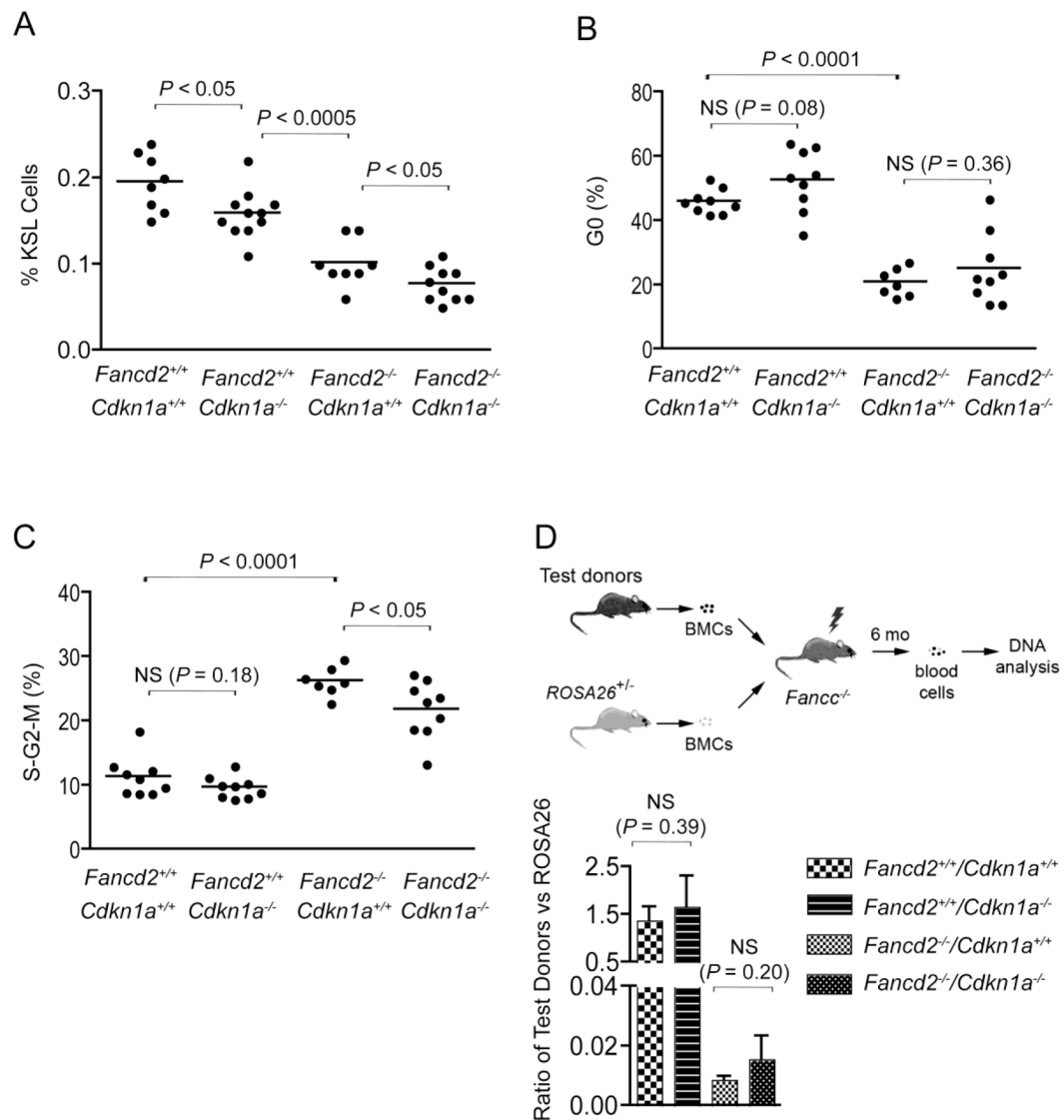


Figure 1. p21 deletion reduced KSL pool but did not change stem cell function

(A) Quantification of KSL hematopoietic stem and progenitor cell frequencies in the bone marrow of *Fancc2^{-/-}/Cdkn1a^{-/-}* mice and controls. The percentage of the KSL gate refers to the proportion of KSL cells in the whole nucleated bone marrow. n=8 for *Fancc2^{+/+}/Cdkn1a^{+/+}* mice, n=11 for *Fancc2^{+/+}/Cdkn1a^{-/-}* mice, n=8 for *Fancc2^{-/-}/Cdkn1a^{+/+}* mice, and n=10 for *Fancc2^{-/-}/Cdkn1a^{-/-}* mice. (B, C) Cell cycle profiles of bone marrow KSL cells. n=9 for *Fancc2^{+/+}/Cdkn1a^{+/+}* mice, n=9 for *Fancc2^{+/+}/Cdkn1a^{-/-}* mice, n=7 for *Fancc2^{-/-}/Cdkn1a^{+/+}* mice, and n=9 for *Fancc2^{-/-}/Cdkn1a^{-/-}* mice. NS denotes not significant. (D) *Upper panel*: Strategy used in the competitive repopulation experiment. BMCs denotes bone marrow cells. *Lower panel*: *In vivo* competitive repopulation of *Fancc2^{-/-}/Cdkn1a^{-/-}* (or control) donor and *ROSA26^{Tg/O}* bone marrow cells. qPCR analyses were performed to evaluate donor contribution to the peripheral blood cells from each donor. Three independent qPCR analyses were performed for each sample and results from 5 animals were pooled together for each experimental group. Data are presented as mean \pm SD. NS denotes not significant.

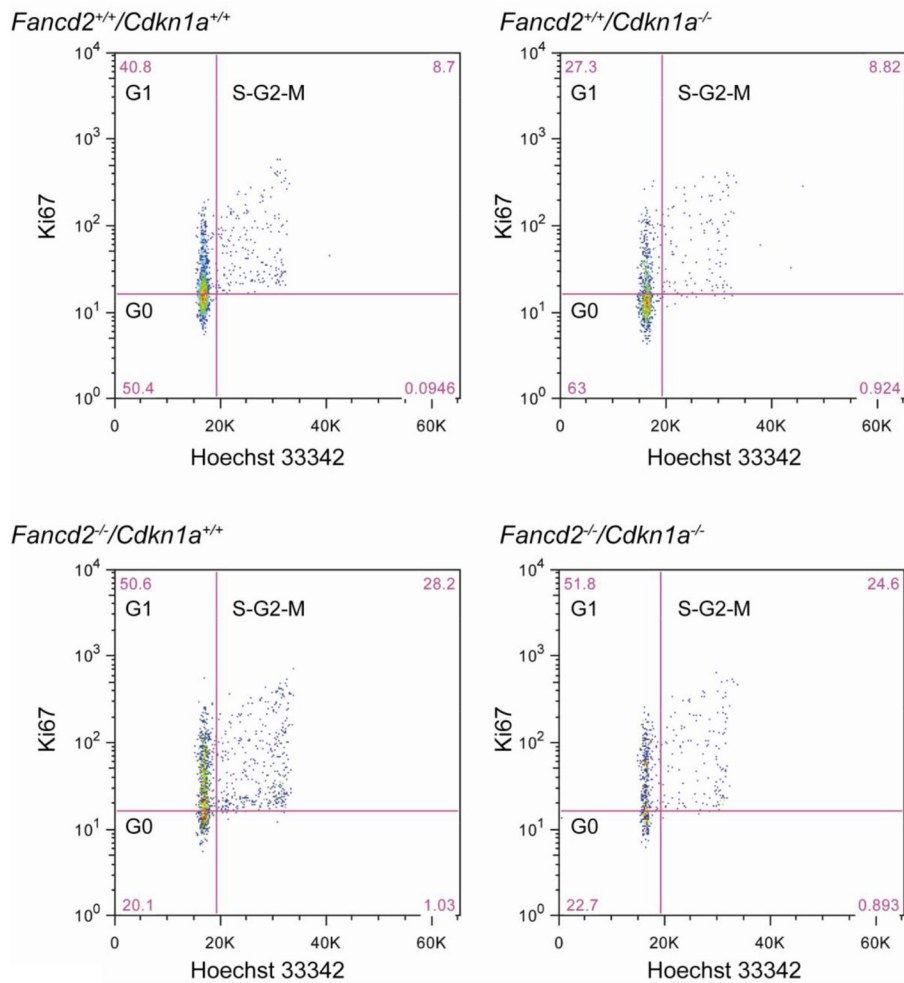


Figure 2. Representative flow cytometry profile of cell cycle analysis
 p21 deletion did not change the abnormal cell cycle status of *Fancd2*^{-/-} mice. Hoechst 33342 and FITC-conjugated anti-mouse Ki67 (BD Sciences) were used in combination to distinguish cells in G0, G1, and S-G2-M phases of the cell cycle.

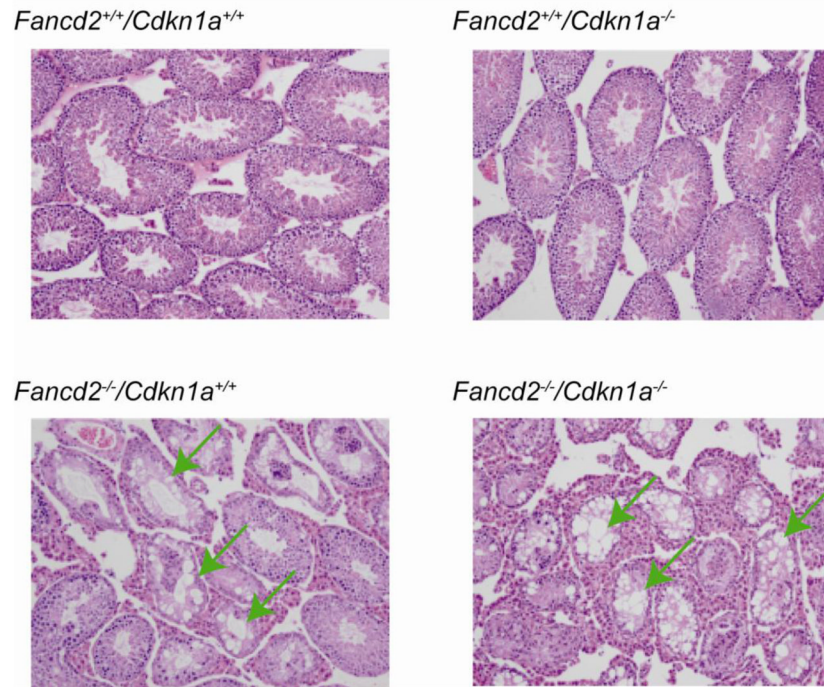


Figure 3. Representative pictures after H&E staining of testis sections
p21 deletion did not correct the germ-cell-loss phenotype of *Fancd2*^{-/-} mice. The mice used were 4 months old. The arrows indicate tubules with vacuolated Sertoli cell cytoplasm. Original magnification x100.

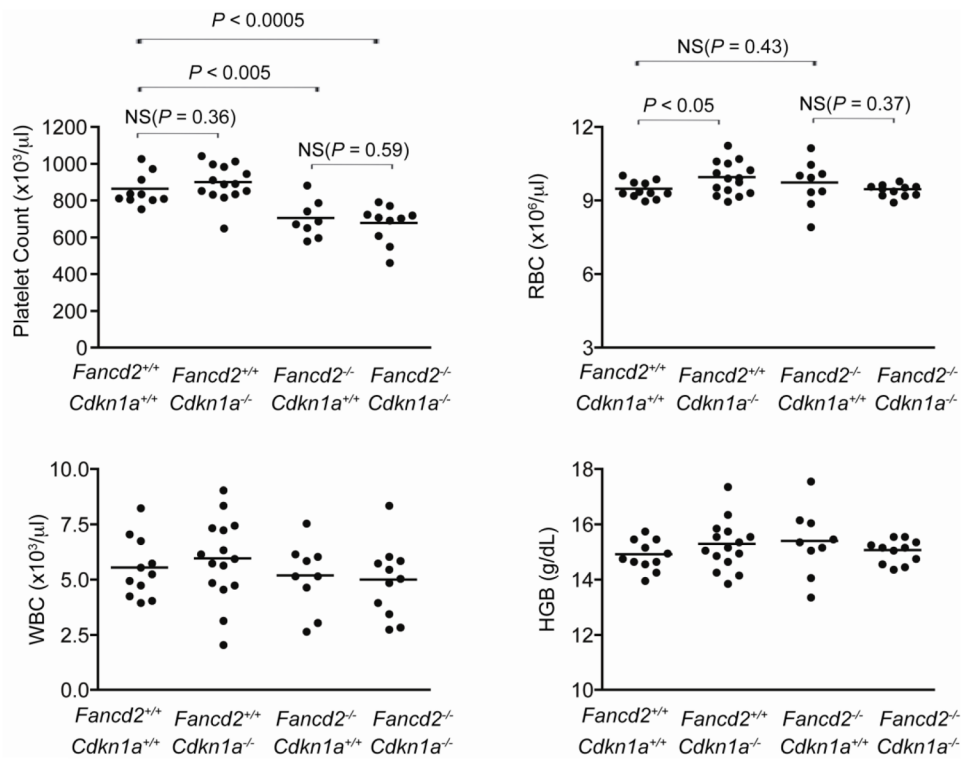


Figure 4. p21 deletion did not affect hematologic parameters of peripheral blood

Comprehensive CBC tests of peripheral blood from *Fancd2*^{-/-}/*Cdkn1a*^{-/-} mice and controls. n=11 for *Fancd2*^{+/+}/*Cdkn1a*^{+/+} mice, n=15 for *Fancd2*^{+/+}/*Cdkn1a*^{-/-} mice, n=9 for *Fancd2*^{-/-}/*Cdkn1a*^{+/+} mice, and n=11 for *Fancd2*^{-/-}/*Cdkn1a*^{-/-} mice. WBC denotes white blood cells; RBC, red blood cells; HGB, hemoglobin; and NS, not significant. Unless specified otherwise on the figure, *P* values were not significant.