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The immune receptor Trem1 cooperates with diminished DNA damage response to induce preleukemic stem cell expansion

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Abstract

Fanconi anemia (FA) is an inherited bone marrow failure syndrome with extremely high risk of leukemic transformation. Here we investigate the relationship between DNA damage response (DDR) and leukemogenesis using the *Fanca* knockout mouse model. We found that chronic exposure of the *Fanca*$^{-/-}$ hematopoietic stem cells to DNA crosslinking agent mitomycin C *in vivo* leads to diminished DDR, and the emergence/expansion of pre-leukemia stem cells (pre-LSCs). Surprisingly, although genetic correction of *Fanca* deficiency in the pre-LSCs restores DDR and reduces genomic instability, but fails to prevent pre-LSC expansion or delay leukemia development in irradiated recipients. Furthermore, we identified transcription program underlying dysregulated DDR and cell migration, myeloid proliferation, and immune response in the *Fanca*$^{-/-}$ pre-LSCs. Forced expression of the downregulated DNA repair genes, *Rad51c* or *Trp53i13*, in the *Fanca*$^{-/-}$ pre-LSCs partially rescues DDR but has no effect on leukemia, whereas shRNA knockdown of the upregulated immune receptor genes *Trem1* or *Pilrb* improves leukemia-related survival, but not DDR or genomic instability. Furthermore, Trem1 cooperates with diminished DDR *in vivo* to promote *Fanca*$^{-/-}$ pre-LSC expansion and leukemia development. Our study implicates diminishing DDR as a root cause of FA leukemogenesis, which subsequently collaborates with other signaling pathways for leukemogenic transformation.

INTRODUCTION

Fanconi anemia (FA) is an autosomal recessive disorder caused by the defects in at least 18 genes (*FANCA–T*).[1–6] Patients with mutations in any of these genes lead to an FA

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AUTHOR CONTRIBUTIONS

WD designed the research, performed the research, analyzed the data and wrote the paper; SA performed the research and analyzed the data; AFW performed the research; QP designed the research and wrote the paper.

CONFLICT OF INTEREST

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phenotype, manifested by the cellular and phenotypic consequences of genetic instability, growth retardation, congenital malformations, bone marrow (BM) failure, high risk of neoplasia and premature aging.[1–4] In response to DNA damage or replicative stress, eight FA proteins (FANCA, -B, -C, -E, -F, -G, -L and -M) form the FA core complex, which acts as an ubiquitin ligase. This FA core complex monoubiquitinates two downstream FA proteins, FANCD2 and FANCI, which then recruit other downstream FA proteins including FANCD1/BRCA2, FANCN/PALB2, FANCJ/BRIP1, FANCO/RAD51C, FANCP/SXL4 and FANCQ/XPF, and possibly other DNA repair factors, to nuclear loci containing damaged DNA and consequently influence important cellular processes such as DNA replication, cell cycle control, and DNA damage response (DDR) and repair.[7,8] Therefore, it is believed that the FA pathway is required for genome maintenance.

One of the common clinical features of FA is hematologic manifestations, possibly due to the defects in hematopoietic stem cells (HSCs). A majority of FA patients invariably experience progressive BM failure, and oftentimes progress to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).[1,2,9–11] Marrow dysfunction occurs at early stage, is associated with HSC loss and accounts for the majority of FA childhood mortality.[1,12–16] On the other hand, rapid hematopoietic cell loss then forces compensatory chronic proliferation. It is in this context that a selective pressure of DNA damage-induced apoptosis coupled with genomic instability during BM failure–myelodysplastic syndrome–acute myeloid leukemia progression may contribute to FA leukemia transformation.[11,12–14] The role of DDR in this process is not well understood.

Recent studies in both solid tumors and hematologic malignancies have demonstrated a DDR barrier as a critical mechanism in tumorigenesis.[17–23] It is believed that the most significant event in the multistep pathogenesis of leukemia features the emergence of a pool of leukemia stem cells (LSCs) with the limitless self-renewal and disease maintenance capacity.[24,25] In this study, we used the *Fanca* knockout mouse model ($Fanca^{-/-}$) to investigate the relationship between DDR and leukemia development. We demonstrate that diminished DDR is correlated with the emergence and expansion of pre-leukemia stem cells (pre-LSCs) in the $Fanca^{-/-}$ mouse model.

MATERIALS AND METHODS

Mice

$Fanca^{+/+}$, $Fanca^{-/-}$ and $Fancc^{+/+}$, and $Fancc^{-/-}$ mice were generated by interbreeding the heterozygous $Fanca^{+/-}$ or $Fancc^{+/-}$ mice,[26,27] respectively. Luc-$Fanca^{+/+}$, Luc-$Fanca^{-/-}$ and Luc-$Fancc^{+/+}$ mice were generated by interbreeding the heterozygous Luc-*Gadd45β* mice[28] with $Fanca^{+/-}$ mice. All the animals including BoyJ mice were maintained in the animal barrier facility at Cincinnati Children's Hospital Medical Center. For *in vivo* mitomycin C (MMC; Sigma-Aldrich, St Louis, MO, USA) treatment, 6–8-week-old mice or their male wild-type littermates were weekly intraperitoneal injected with 0.3 mg/kg of MMC.[29]

Flow cytometry analysis

The lineage marker (Lin) mixture (BD Biosciences, San Jose, CA, USA) for BM cells from treated or untreated mice included the following biotinylated antibodies: CD3ε (145–2C11), CD11b (M1/70), CD45R/B220 (RA3–6B2), mouse erythroid cells Ly-76 (Ter119), Ly6G and Ly-6C (RB6–8C5). Other conjugated antibodies (BD Biosciences) used for surface staining included: CD45.1 (A20), CD45.2 (A104), Sca1 (D7), c-kit (2B8), CD48 (HM48–1) and CD150 (9D1). Biotinylated primary antibodies were detected by incubation of antibody coated cells with streptavidin-PerCP or FITC (BD Biosciences) in a two-step staining procedure. For some of the experiments, pacific blue conjugated CD45.2 (A104, BioLegend, San Diego, CA, USA) was used to determine donor-derived cells.

Chromosomal breakage analysis

Chromosome breakage analysis was performed on LSK cells from MMC-treated *Fanca*$^{+/+}$ and *Fanca*$^{-/-}$ mice as previously described.[30] In brief, cells were treated with 0.05 mg/ml colcermid (Gibco, Grand Island, NY, USA) for 90 min, followed by 0.4% KCl hypotonic solution at 37 °C for 20 min, fixed with methanol and acetic acid at 4 °C for 15 min and dropped onto microscope slides. The cells were then rinsed with isoton, stained with Giemsa for 5 min and rinsed with Gurr Buffer (CTL Scientific, Deer Park, NY, USA) and Milli-Q-filtered deionized water. A total of 50 cells from each sample were scored for chromosome aberrations.

BM transplantation

To monitor DDR *in vivo*, 1000 LSK cells from Luc-WT or 2000 LSK cells from Luc-*Fanca*$^{-/-}$ mice (CD45.2^{+}) were transplanted into lethally irradiated CD45.1^{+} mice. Twenty weeks later, the recipient mice were subjected to weekly MMC injection for 6 weeks. The expression of *Gadd45β*-driven luciferase was analyzed by IVIS imaging. In other experiments, lentiviral vector transduced Luc-WT or Luc-*Fanca*$^{-/-}$ LSK cells from the recipient mice treated with MMC for 6 weeks, or sorted HSC (SLAM), multipotential progenitor (MPP) and HPC-1 fractions of leukemic mice were transplanted into lethally irradiated BoyJ mice. The recipients were subjected to MMC injection followed by IVIS imaging or analysis of leukemia development.

RESULTS

Diminished DDR is associated with the emerge and expansion of pre-LSCs

To link DDR to leukemogenesis, we first examined DDR *in vivo* by crossing the *Fanca*$^{+/-}$ mice to the Luc-*Gadd45β* mice, which express the luciferase transgene under the control of the promoter of the stress-responsive gene *Gadd45β* and allow for non-invasive *in vivo* imaging stress-induced expression of the luciferase marker.[28] *Gadd45β* is well established for its diverse roles in cell cycle control, cell survival, apoptosis, DNA damage repair and the maintenance of genomic stability.[31] *Gadd45β* can also act as a stress sensor in the development of hematopoietic malignancies such as leukemia.[32] To assess exclusively the response of HSCs without the effect of different (WT and *Fanca*$^{-/-}$) BM microenvironment, we first established WT and *Fanca*$^{-/-}$ BM chimeras in WT mice. By transplanting LSK (Lin

$^{-}$Sca1^{+}c-kit^{+}) cells isolated from Luc-*Fanca*$^{+/+}$ or Luc-*Fanca*$^{-/-}$ mice into lethally irradiated BoyJ mice, we were able to establish stable donor-derived chimera 20 weeks post BM transplantation (Supplementary Figure 1). The recipients were then exposed to DNA damage by intraperitoneally injection of low dose of the DNA crosslinker, MMC (0.3 mg/kg)[29] weekly for 6 weeks. We monitored *Gadd45β*-driven expression of the luciferase transgene after each injection by IVIS live imaging as a surrogate of DDR. MMC induced approximately same levels of luciferase signal after each injection during the 6 weeks of the experimental period in the recipients transplanted with Luc-WT LSK cells (Figure 1a). Interestingly, MMC induced high levels of *Gadd45β*-driven luciferase expression in the Luc-*Fanca*$^{-/-}$ LSK cell-transplanted recipients during the first 2 weeks of treatment; however, the luciferase signal progressively declined afterwards (Figure 1a). We also performed analysis of the donor LSK cells at 0, 3 and 6 weeks post MMC treatment for the well-established DNA damage marker γ-H2AX by flow cytometry and several well-known DDR genes by RT-PCR, including the p53 transcriptional target *p21*^{CIP1}, homologous recombination repair (*Rad51* and *Brca1*) and non-homologous end joining (*DNA-PKcs* and *Trp53bp1*). We found that MMC induced robust expression of γ-H2AX and the DDR genes *p21*^{CIP1}, *Rad51* and *Trp53bp1* at 3 weeks post treatment (Figures 1b and c). However, although DNA damage (γ-H2AX) exhibited persistent, the expression of the DDR genes was markedly diminished after 6 weeks of treatment. These results indicate a diminished DDR in the progeny of the transplanted *Fanca*$^{-/-}$ LSK cells during the chronic MMC treatment.

To determine the effect of the altered DDR on HSCs and progenitor cell, we next analyzed the frequency of each of the four phenotypic fractions of CD45.2^{+}LSK cells in the course of 6-week period of MMC treatment: HSC (LSKCD150^{+}CD48^{-}; SLAM), MPP (LSKCD150 $^{-}$ CD48^{-}), HPC-1 (LSKCD150 $^{-}$ CD48^{+}) and HPC-2 (LSKCD150^{+}CD48^{+}). As shown in Figure 1d, a significant expansion of HSCs and MPPs was evidenced starting at week 4 of MMC treatment in in the recipients transplanted with the Luc-*Fanca* $^{-/-}$ LSK cells. On the other hand, chronic MMC treatment did not cause expansion in the HPC compartments (Figure 1d). Consistently, a significant increase in the frequencies of HSCs and MPPs in total donor-engrafted (CD45.2^{+}) cells was detected in mice transplanted with *Fanca*$^{-/-}$ LSK cells starting at week 4 post MMC treatment (Supplementary Figure 1B). Interestingly, the expansion of *Fanca*$^{-/-}$ HSCs and MPPs was seen from MMC treatment at week 4 and did not progress further. To address this, we analyzed apoptosis and cell cycle in the four cell populations and found that there was an increase in both AnnexinV^{+}7-AAD^{-} apoptotic cells and S/G2/M phases in the *Fanca* $^{-/-}$ HSC and MPP populations at week five and week six post-MMC treatment (Supplementary Figures 1C and D). Thus, it is possible that increased apoptosis due to oncogenic stress could potentially offset the increased proliferation, thus limiting the further expansion of these pre-LSCs. Taken together, the observed diminished DDR in *Fanca*$^{-/-}$ LSK cells is accompanied by the expansion of phenotypic HSCs and MPPs.

To assess the consequence of diminished DDR and HSC/MPP expansion, we monitored the transplanted mice for leukemia development. Although all primary recipients of both WT and *Fanca*$^{-/-}$ cohorts treated with MMC for 6 weeks survived for >12 months without signs of leukemia, the secondary recipients of *Fanca*$^{-/-}$ cohorts gave rise to lethal leukemias

within 1 year (Figure 1e). This result indicates that the expanded *Fanca*$^{-/-}$ HSC/ MPP compartment contains pre-LSCs that induced leukemia in the secondary recipients.

To determine whether the development of *Fanca*$^{-/-}$ leukemia was due to the transformation of a specific target cell, we performed fractionation of the donor leukemic cells into three fractions: HSC, MPP and HPC-1 (Figure 1f). We transplanted 50 HSCs, 500 MPPs or 2000 HPC-1 cells, along with 200 000 protector (CD45.1^{+}) cells, into lethally irradiated recipients (CD45.1^{+}). Both HSC and MPP fractions produced leukemia (Figure 1g). However, recipients transplanted with MPP cells died significantly more quickly than the HSC cohort (Figure 1g). Further characterization of the leukemic mice showed that the secondary recipients transplanted with the *Fanca*$^{-/-}$ HSC and MPP cells developed myeloid leukemia, as characterized by increased white blood cells in the peripheral blood, anemia, splenomegaly and infiltration of myeloid blasts in the spleen and BM (Supplementary Figures 2A–D). Furthermore, the leukemia induced by the MPP fraction was associated with unilineage myeloid engraftment, whereas transplantation of the HPC-1 fraction did not result in any engraftment (Supplementary Figure 2E). Notably, in two of six recipients transplanted with the HSC fraction yielded long-term multilineage engraftment with no leukemia observed within 4 months (Supplementary Figure 2E). These results suggest that preleukemic expansion of the *Fanca*$^{-/-}$ HSCs may have initiated leukemogenesis by generating a large number of MPPs for transformation.

Genetic correction of FA deficiency restores DDR and reduces genomic instability, but fails to prevent pre-LSC expansion or delay leukemia

As the FA pathway is known to have a crucial role in DNA damage repair and genomic maintenance,[4,33] we asked whether the correction of FA genetic deficiency could prevent pre-LSC expansion through restoring DDR. To this end, we transduced Luc-WT and Luc-*Fanca*$^{-/-}$ LSK cells isolated from the 6-week MMC-treated recipient mice, which expanded the pre-LSCs (Figures 1d and e), with lentiviral vector expressing Vector or *FANCA* (Figures 2a and b). The sorted Venus^{+}LSK cells were transplanted along with 200 000 congenic BM cells (CD45.1) into lethally irradiated BoyJ mice. At 16 weeks post transplantation, MMC was administrated to the recipients to induce DNA damage, and DDR was assessed by *in vivo* live imaging for *Gadd45β*-driven luciferase expression. As shown in Figure 2c, re-expression of the *FANCA* gene in Luc-*Fanca*$^{-/-}$ LSK cells restored MMC-induced DDR to nearly the levels observed in Luc-WT cells. Consistently, *FANCA* complementation also significantly reduced genomic instability in Luc-*Fanca*$^{-/-}$ LSK cells, as analyzed by chromosomal breakage assay (Figure 2d). Surprisingly, ectopically expression of the functional *FANCA* gene failed to prevent pre-leukemic HSC expansion in the recipients transplanted with *FANCA*-transduced *Fanca*$^{-/-}$ LSK cells (Figure 2e). Furthermore, genetic complementation of these *Fanca*$^{-/-}$ preleukemic LSK cells with *FANCA* fails to delay leukemia development in the secondary recipients (Figure 2f). These results suggest that, although the root cause of the *Fanca*$^{-/-}$ preleukemic HSC expansion lies in genomic instability, additional alterations in other signaling events, likely unrelated to DDR or genomic maintenance, are required for full leukemia transformation.

RNA-seq analysis identifies transcription program underlying dysregulated DDR and immune response pathways

To identify the molecular mechanisms responsible for the observed preleukemic HSC expansion, we performed RNA-sequencing (RNA-seq) analysis using LSK cells isolated from WT, *Fanca*$^{-/-}$ and *Fancc*$^{-/-}$ mice treated with or without MMC for 6 weeks (Figure 3a). We reasoned that employing two FA models for gene profiling would reduce the probability that the identified alterations in gene expression might be due to a specific effect of a particular FA complementation group. Sequencing data were aligned using Tophat and the mm9 version of the mouse genome.[34] Using GeneSpring GX analysis, we found 363 unique differentially expressed genes in both *Fanca*$^{-/-}$ and *Fancc*$^{-/-}$ LSK cells, of which 161 were upregulated and 202 were downregulated, as compared with WT cells (Moderate *T*-test, FC ⩾ 2.0; Figure 3b).

Further pathway analysis revealed top 10 affected pathways shared in *Fanca*$^{-/-}$ and *Fancc*$^{-/-}$ LSK cells after DNA damage, including cell migration, immune response, DNA repair, cell activation, myeloid differentiation, inflammatory response, cell cycle, cellular response to DNA damage stimulus, cell motility and cell proliferation (Figure 3c). Not surprisingly, we observed that the expression of some DDR and repair-associated genes, particularly *Rad51c*[35] and *Trp53i13*,[36] was significantly decreased in expanding (*Fanca*$^{-/-}$ and *Fancc*$^{-/-}$) relative to homeostatic (WT) LSK cells (Figure 3d, left). Interestingly, many of genes in the cell migration, myeloid proliferation and immune response pathways were markedly upregulated in the expanding *Fanca*$^{-/-}$ and *Fancc*$^{-/-}$ LSK populations compared with WT LSK cells (Figure 3d, right). This gene profiling indicates that pre-leukemic HSC expansion and subsequent leukemogenic initiation in the FA LSK compartment involves transcriptional alterations not only of genes in DDR/repair but also of those involved in DDR/repair-unrelated cell signaling.

Overexpression of DDR genes fails to prevent pre-leukemic expansion of *Fanca*$^{-/-}$ HSCs

To address whether the observed transcriptional alterations in *Fanca*$^{-/-}$ LSK cells were associated with pre-leukemic HSC expansion and leukemogenesis, we chose two downregulated DDR genes (Figure 3d, left), *Rad51c*[35] and *Trp53i13*,[36] and three upregulated genes functioning in the cell migration, myeloid proliferation and immune response pathways (Figure 3d, right), *Trem1*,[37] *Nlrp12*(ref.38) and *Pilrb*,[33] for further study. We first performed quantitative RT-PCR to validate the altered expression of these genes identified in the RNA-seq analysis (Figure 3e).

We then asked whether manipulation of the expression of these genes could restore DDR or prevent leukemogenesis. We first examined the effect of overexpression of the DDR genes in preleukemic *Fanca*$^{-/-}$ HSCs. We transduced WT and *Fanca*$^{-/-}$ LSK cells from the 6-week MMC-treated recipient mice (Figure 1), with a lentiviral vector expressing the WT *Rad51c* or *Trp53i13* and transplanted the transduced cells into lethally irradiated WT recipients (Figure 4a). MMC-induced DDR was monitored by IVIS imaging. As shown in Figure 4b, overexpression of *Rad51c* or *Trp53i13* in *Fanca*$^{-/-}$ pre-LSCs partially rescued DDR in response to MMC treatment. This was associated with reduced chromosomal aberrations in *Rad51c*- or *Trp53i13*-expressing cells compared with vector-transduced cells (Figure 4c).

However, ectopic expression of *Rad51c* or *Trp53i13* was unable to prevent pre-leukemic HSC (LSKCD150^{+}CD48^{-} cell) expansion (Figure 4d) or leukemia development (Figure 4e) in secondary recipients. Thus, partial restoration of DDR in pre-leukemic *Fanca* $^{-/-}$ HSCs is not sufficient to prevent leukemogenesis.

Knockdown of immune response genes delays leukemia

The observation that diminished DDR and pre-leukemic HSC expansion in the *Fanca*$^{-/-}$ LSK compartment was associated with the upregulation of genes involved in the immune response/ migration/myeloid differentiation pathways prompted us to determine whether the repression of these genes in *Fanca*$^{-/-}$ pre-leukemic HSCs could rescue DDR or prevent leukemic transformation. Among the top upregulated genes, Trem1 belongs to the TREMs (triggering receptor expressed on myeloid cells) family of recently discovered receptors of the immunoglobulin superfamily, which have important roles in innate immune responses.[37,39] Nlrp12α, which is a member of the Nod-like receptor family of proteins, has a pivotal role in regulating inflammation and tumorigenesis.[38,40] Pilrb (paired immunoglobulin-like type 2 receptor beta) is a member of the paired immunoglobin-like type 2 receptors, which consist of highly related activating and inhibitory receptors that are involved in the regulation of many aspects of the immune system.[33,41] The reason for choosing these three top upregulated genes for further analysis is that they are involved in cell migration, myeloid proliferation and immune response outside of the DDR pathways, and that they are not known to function in hematological malignancy. We used lentiviral shRNA to knockdown *Trem1, Nlrp12* or *Pilrb* in LSK cells isolated from the 6-week MMC-treated recipient mice (Figure 5a), and transplanted the transduced LSK cells into lethally irradiated recipients to monitor DDR and leukemia development. We found that knockdown of *Trem1*, *Nlrp12* or *Pilrb* did not improve MMC-induced DDR (Figure 5b) or genomic instability (Figure 5c) in pre-leukemic *Fanca*$^{-/-}$ LSK cells, confirming that these genes are not involved in DDR. Interestingly, knockdown of *Trem1* or *Pilrb*, but not *Nlrp12*, significantly decreased pre-leukemic *Fanca*$^{-/-}$ HSC expansion (% SLAM cells in LSK cells: 7.15 ± 1.2% for sh*Trem1* vs 12.28± 2.02% for sh*Scramble*, $P=0.0011$; 8.33 ± 1.64% for sh*Pilrb* vs 12.28 ± 2.02% for sh*Scramble*, $P=0.0132$; 11.05 ± 1.21% for sh*Nlrp12* vs 12.28 ± 2.02% for sh*Scramble*, $P=0.2034$), whereas this inhibitory effect mediated by the knockdown was not observed in WT cells (Figure 5d). Furthermore, the recipients transplanted with pre-leukemic *Fanca*$^{-/-}$ LSK cells expressing the *Trem1* or *Pilrb* shRNA showed delayed leukemia development (Figure 5e). Interestingly, knockdown of *Trem1* or *Pirlb* caused a significant increase in apoptosis (Supplementary Figure 3A) without effect on cell cycle status of the donor-derived LSK cells (Supplementary Figure 3B). These data suggest that de-regulated migration/myeloid/ immune response pathways may collaborate with diminished DDR in mediating pre-leukemic HSC expansion and leukemogenesis in the *Fanca*$^{-/-}$ LSK cells.

Trem1 functionally cooperates with diminished DDR *in vivo*

Because we observed that knockdown of *Trem1* exhibited the most significant effect among the three de-regulated migration/myeloid/immune response genes, we investigated whether Trem1 functionally cooperated with diminished DDR. We first determined the kinetics of *Trem1* upregulation in pre-leukemic *Fanca*$^{-/-}$ HSCs. Interestingly, *Trem1* transcript was progressively increased from week 3 during MMC treatment (Figure 6a), which was

inversely correlated with declined DDR in pre-leukemic *Fanca*$^{-/-}$ HSCs (Figure 1). To determine the effect of Trem1 on the proliferation of pre-leukemic *Fanca*$^{-/-}$ HSCs, WT and *Fanca*$^{-/-}$ LSK cells were isolated from MMC-treated recipient mice at week 3, at which time both DDR and *Trem1* expression started showing the progressive changes (Figure 1a; Figure 6a), and transduced the cells with lentiviruses expressing the *Trem1* and *eGFP* transgenes. Two days post transduction, 100 GFP^{+} LSKCD150^{+} cells were sorted and plated per well for colony forming unit assay. Expression of Trem1 significantly enhanced colony generation of the pre-leukemic *Fanca*$^{-/-}$ HSCs in first plating and, to a greater extent, in the second plating compared with the empty vector control (Figure 6b).

To determine whether Trem1 functionally cooperates with diminished DDR *in vivo*, we isolated LSK cells from the MMC-treated recipient mice at week 1, 3 and 6 (designated as week-1, week-3 and week-6 LSK cells, respectively), which represents different stages of MMC-induced DDR (Figure 1). We transduced the cells with *Trem1* lentiviruses and transplanted the transduced LSK cells into lethally irradiated mice for assessment on preleukemic HSC expansion and leukemia development. Expression of Trem1 in week-1 *Fanca*$^{-/-}$ LSK cells did not lead to expansion of SLAM cells (Figure 6c). However, Trem1-transduced week-3 LSK cells in the *Fanca*$^{-/-}$ background significantly increased the frequency of SLAM cells in the LSK compartment compared with the corresponding vector control (Figure 6c). Trem1 also enhanced expansion of the week-6 *Fanca*$^{-/-}$ SLAM cells (Figure 6c), albeit not statistically significant compared with the Vector control, probably because its expression at the week-6 stage was already high enough (Figure 6a) for this specific effect. Of note, expression of Trem1 in WT LSK cells at all three stages did not result in expansion of SLAM cells (Figure 6c). Moreover, expression of Trem1 in week-3 and week-6 *Fanca*$^{-/-}$ LSK cells led to leukemia with a shorter latency compared with the Vector controls (Figure 6d). In particular, four out of nine (44%) the mice transplanted with *Trem1*-transduced week-3 *Fanca*$^{-/-}$ LSK cells succumbed to lethal leukemia within 60 weeks post transplant, whereas all mice transplanted with vector-control cells (9/9) were alive and healthy at this time point (Figure 6d). These data suggest that Trem1 may collaborate with diminished DDR in promoting pre-leukemic HSC expansion and leukemogenic transformation in the *Fanca*$^{-/-}$ LSK compartment.

DISCUSSION

In this study, we investigated the relationship between DDR and leukemogenesis using a mouse model deficient for the core complex of the FA DNA repair pathway. We demonstrated a temporal correlation of diminished DDR with the emergence and expansion of preleukemic *Fanca*$^{-/-}$ HSCs and MPPs. Expansion of preleukemic HSCs/MPPs may be an essential initiating event in leukemogenesis, as it can enlarge the pool of transformable stem cells and progenitors. There are several findings that highlight the significance of impaired DDR, as a function of FA deficiency, in leukemogenesis: (1) DDR undergoes progressive decline in *Fanca*$^{-/-}$ HSCs after 3 weeks of MMC treatment; (2) this diminished DDR is correlated with expansion of premalignant *Fanca*$^{-/-}$ HSCs and MPPs and leukemia development in secondary recipients; (3) global gene profiling identifies downregulated DDR/repair pathways and upregulated migration/myeloid differentiation/immune response signaling, as potential underlying mechanisms for FA leukemic transformation; (4) re-

expression of the downregulated *Rad51c* and *Trp53i13* rescues DDR and genomic instability but fails to prevent pre-LSC expansion or leukemia; (5) knockdown of *Trem1* or *Pilrb* delays leukemia, but is unable to rescue DDR and genomic instability; and (6) Trem1 functionally cooperates with diminished DDR *in vivo* to promote pre-leukemic *Fanca*$^{-/-}$ HSC expansion and leukemogenic transformation. Our study provides novel insights into the mechanism of leukemic transformation with emphasis on corroborative pathways as barriers to the emergence of pre-leukemic HSCs in a mouse model of genomic instability *in vivo*.

Among cellular components, the genome is particularly susceptible to damage, which can be resulted from spontaneous reactions in the nucleus, oxidative damage due to metabolic byproducts or from extrinsic agents, or replication associated defects.[42] It is recently shown that maintenance of genomic integrity is a limiting factor in maintaining HSC function.[43] On the other hand, accumulated DNA damage is essential in the development of malignancies, and DNA damage accumulated in HSCs and progenitors is responsible, at least in part, for hematological malignancies.[42,44] In addition, stem cells, including HSCs, may be particularly susceptible to DNA damage due to their longevity.[45] The FA pathway has important role in DDR/repair and genome maintenance.[46,47] We show that loss of DDR, due to FA deficiency, is correlated with the emergence and expansion of pre-leukemic *Fanca*$^{-/-}$ HSCs that give rise to leukemia in secondary transplanted recipients. These results implicate the DDR checkpoint machinery as a crucial mechanism that guards against the initiation and progression of FA HSCs into leukemic transformation. Indeed, the critical importance of the DDR in hematopoiesis is well demonstrated by the severe clinical consequences, including BM failure, immunodeficiency and high incidence of leukemia, observed in patients with inherited mutations in DNA damage signaling and repair components.[48] Our finding is in line with the proposal that impaired DDR and subsequently improperly repaired DNA damage, resulting in genetic instability, may ultimately lead to deregulated self-renewal, heralding the emergence of LSCs and leukemic transformation.[49,50]

In solid cancers, DDR activation has been proposed as an inducible barrier against tumorigenesis at early pre-invasive stages.[17–20] DNA damage accumulation in HSCs is associated with broad attenuation of DNA repair and response pathways that are dependent upon HSC quiescence.[51] Studies conducted using mutant mice have underscored the importance of DNA repair pathways in maintaining the functionality of HSCs.[43,52,53] One novel finding of the study is that genetic complementation of pre-leukemic *Fanca*$^{-/-}$ HSCs can rescue DDR and genomic instability, but is unable to prevent clonal expansion or leukemic transformation. Consistently, ectopic expression of *Rad51c* or *Trp53i13*, the two DDR/repair genes identified in our RNA-seq analysis, partially restores DDR and reduces genomic instability of pre-leukemic *Fanca*$^{-/-}$ HSCs. However, overexpression of *Rad51c* or *Trp53i13* is unable to prevent the expansion of these FA pre-leukemic HSCs and leukemia development in secondary recipients. These results appear to be in conflict with the conventional model that HSCs must possess effective DDR to balance long-term regeneration with protection from malignant transformation.[54] It is in this context that our study argues the effectiveness of the cellular DDR machinery in preventing the transition of the initiating pre-leukemic HSC population into a LSC population with transformed properties.

It is intriguing that the loss of DDR is accompanied by upregulation of a large number of genes involved in cell migration, myeloid differentiation and immune response pathways in pre-leukemic *Fanca*$^{-/-}$ HSCs. More interestingly, although knockdown of the top three genes functioning in these signaling pathways, *Trem1*, *Nlrp12* and *Pilrb*, fails to rescue the compromised DDR or reduce chromosomal instability in the pre-leukemic *Fanca*$^{-/-}$ HSCs, knockdown of *Trem1*, and to a lesser degree, *Pilrb* inhibits expansion of the FA pre-leukemic HSCs and delays the development of lethal leukemia in secondary recipients. Moreover, our *in vitro* and *in vivo* studies indicate that Trem1 collaborates with diminished DDR in promoting pre-leukemic HSC expansion and leukemogenic transformation in the *Fanca*$^{-/-}$ LSK compartment. These results suggest a model for FA leukemogenesis in which diminished DDR as a root cause of FA leukemogenesis, which subsequently collaborates with other signaling pathways for leukemia transformation. This FA model of leukemic transformation resembles the classical 'two-hit' model of tumorigenesis,[55] in which FA genetic deficiency leads to DDR loss followed by differentiation blockage and upregulation of proliferative signaling. Therefore, our study contributes to better understanding of the anti-leukemic role of DDR and collaborating signaling events that regulate the fate of pre-leukemic HSCs and multistep development of FA leukemia under *in vivo* conditions.

Although further studies are required to define the underlying mechanism, our results suggest a potential linkage between Trem1 overexpression and diminished DDR resulted from FA deficiency. We speculate that the FA pathway may have a role in cellular immune response checkpoint in restricting the expansion of HSCs and progenitor cells. The crosstalk between DDR and immune response has been reported.[56,57] On one hand, stimulation of DDR by exogenous or endogenous insults triggers innate and adaptive immune response to favor the immunogenicity of the incipient cells.[56] On the other hand, microbial infection is known to be sufficient to damage the DNA in the nucleus and activate some or all of the DNA repair pathways.[57] In addition, disruption of DDR immune response crosstalk compromises multicellular integrity and has been linked to cell cycle-related and immune defects.[56,58,59] Therefore, it has been proposed that targeting these DDR immune response collaborating signaling events opens up a new avenue of therapeutic options.[56,60–62] In this content, the FA pathway may have an important role in DDR maintenance, thereby preventing the disruption of the cellular DDR immune response crosstalk.

Supplementary Material

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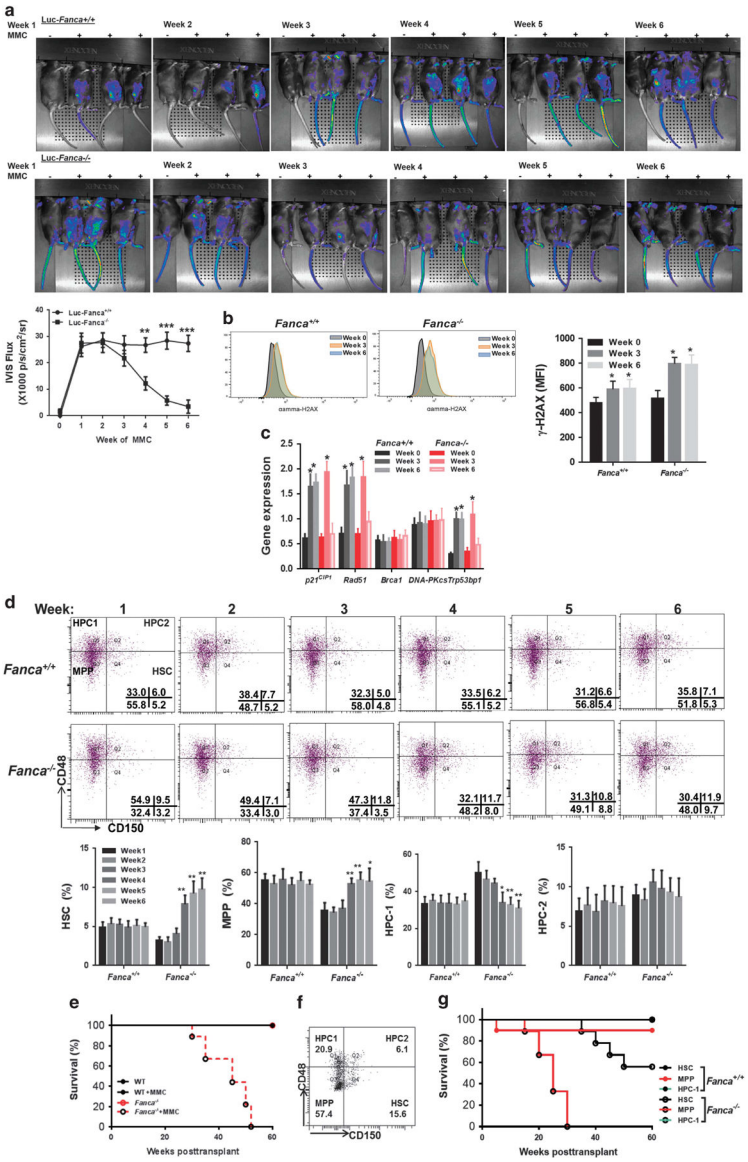

Figure 1.

Diminished DDR is associated with the expansion of *Fanca*$^{-/-}$ pre-LSCs. (**a**) MMC treatment leads to diminished DNA damage response (DDR) in *Fanca*$^{-/-}$ hematopoietic stem and progenitor cells (HSPCs). 1,000 Lin^{-} Sca1^{+}c-kit^{+} (LSK) cells from Luc-WT mice or 2000 LSK cells from Luc-*Fanca*$^{-/-}$ mice were transplanted into lethally irradiated BoyJ (CD45.1^{+}) recipients. Twenty weeks after bone marrow transplantation, the recipients were intraperitoneal injected with low dose of MMC (0.3 mg/kg) weekly for 6 weeks. Luc images were taken 1 h after each MMC injection. The bioluminescent image signals were quantified using LiveImage Pro. 2.0 software (right, Caliper Life Science, Hopkinton, MA, USA). Results are means±s.d. of three independent experiments. (**b**) Persistent DNA damage in MMC-treated *Fanca*$^{-/-}$ HSPCs. Donor-derived LSK cells from the recipients described in **a** at the indicated time points post MMC treatment were subjected to flow cytometric analysis for γ-H2AX. Representative flow plots (left) and quantification of MFI (right) are shown.

(**c**) Altered expression of DDR genes in *Fanca*$^{-/-}$ HSPCs. RNA from donor-derived LSK cells isolated from recipient mice described in a at the indicated time points post MMC treatment were subjected to RT-PCR analysis for the expression of the indicated DDR genes. Samples were normalized to the level of *GAPDH* mRNA. (**d**) MMC treatment leads to *Fanca*$^{-/-}$ HSC expansion. The recipient mice described in **a** were injected with low dose of MMC weekly for 6 weeks. Two days after each injection, donor-derived (CD45.2^{+}) WT or *Fanca*$^{-/-}$ BM LSK cells were gated for analysis of HSC (LSKCD150^{+}CD48^{-}), MPP (LSKCD150^{-}CD48^{-}), HPC-1 (LSKCD150^{-a}CD48^{+}) and HPC-2 (LSKCD150^{+}CD48^{+}) cell fractions. Representative flow plots and quantification are shown. (**e**) *Fanca*$^{-/-}$ secondary recipients develop lethal leukemia. Two million BM cells from the 6-week MMC-treated mice described in **a** were transplanted into lethally irradiated secondary CD45.1^{+} recipient mice. Survival of the recipients was monitored and plotted by the Kaplan–Meier curve method. $n = 8$–10 mice for each group. *$P<0.05$, *Fanca*$^{-/-}$ vs WT control. (**f**) Gating strategy for sorting HSC (LSKCD150^{+}CD48^{-}), MPP (LSKCD150^{-}CD48^{-}) and HPC-1 (LSKCD150^{-}CD48^{+}) cell fractions isolated from leukemic mice. (**g**) *Fanca*$^{-/-}$ HSCs and MPPs but not HPC-1 cells give rise to lethal leukemia. Fifty HSC, 500 MPP or 2000 HPC-1 cells from the secondary recipient mice were transplanted, along with 200 000 c-Kit-depleted protector cells, into lethally irradiated BoyJ recipient. Survival of the recipients was monitored and plotted by the Kaplan–Meier curve method. $n = 9$ mice for each group. *$P<0.05$, HSC vs HPC-1; **$P<0.01$, MPP vs HPC-1; *$P<0.05$, HSC vs MPP.

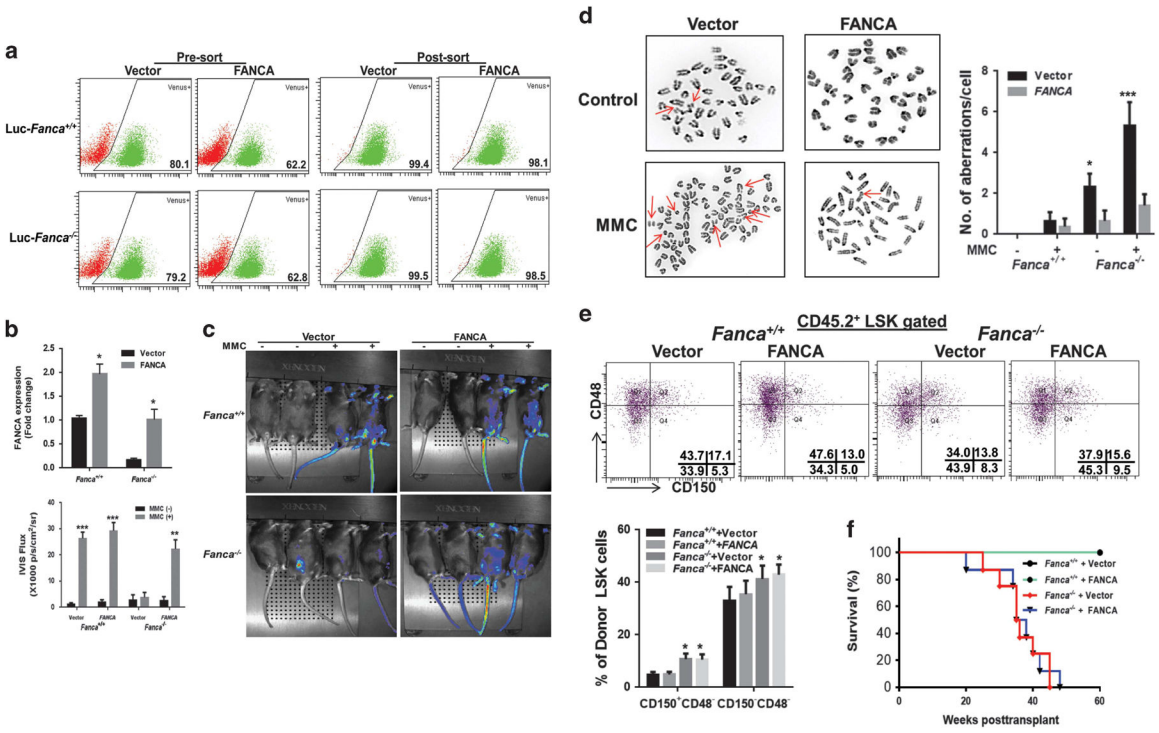

Figure 2.

Genetic correction of FA deficiency restores DDR but fails to prevent pre-LSC expansion and leukemia. (**a**) Luc-WT or Luc-*Fanca*$^{-/-}$ LSK cells isolated from the recipient mice treated with MMC for 6 weeks were transduced with lentivirus expressing Venus only (Vector) or Venus plus *FANCA* (*FANCA*), and then subjected to fluorescence-activated cell sorting for Venus^{+} cells. The percentages of pre-sorted and post-sorted Venus^{+} cells are shown. (**b**) The level of *FANCA* mRNA in genetic corrected LSK cells. RNA from cells described in **a** were subjected to RT-PCR analysis for *FANCA* expression. Samples were normalized to the level of *GAPDH* mRNA. (**c**) Re-expression of *FANCA* in pre-leukemic *Fanca*$^{-/-}$ HSCs restores DDR. A total of 2000 sorted transduced LSK cells described in **a**, along with 200 000 c-Kit-depleted protector cells, were transplanted to lethally irradiated BoyJ mice. Sixteen weeks later, recipients were treated with MMC followed by IVIS live image. Representative Luc images (right) and quantification (left) are shown. Results are means ±s.d. of three independent experiments. (**d**) Re-expression of *FANCA* in pre-leukemic *Fanca*$^{-/-}$ HSCs reduces genomic instability. Transduced LSK cells described in **a** were subjected to chromosomal breakage analysis. Representative images and quantification of 50 cells in random fields are shown. Red arrows denote chromosomal aberrations. (**e**) Re-expression of *FANCA* in pre-leukemic *Fanca*$^{-/-}$ HSCs fails to prevent pre-LSC expansion. BM cells from the recipients were subjected to flow cytometric analysis for donor-derived HSC (LSKCD150^{+}CD48^{-}) and MPP (LSKCD150^{-}CD48^{-}) 16 weeks post bone marrow transplantation. Representative flow plots (upper) and quantification (lower) are shown. (**f**) Re-expression of *FANCA* in pre-leukemic *Fanca*$^{-/-}$ HSCs fails to delay leukemia development. Survival of the secondary recipients was monitored and plotted by the Kaplan–Meier curve method. n= 6–8 mice for each group. P>0.05, *Fanca*$^{-/-}$/Vector vs *Fanca*$^{-/-}$/*FANCA*. *P<0.05; **P<0.01; ***P<0.001.

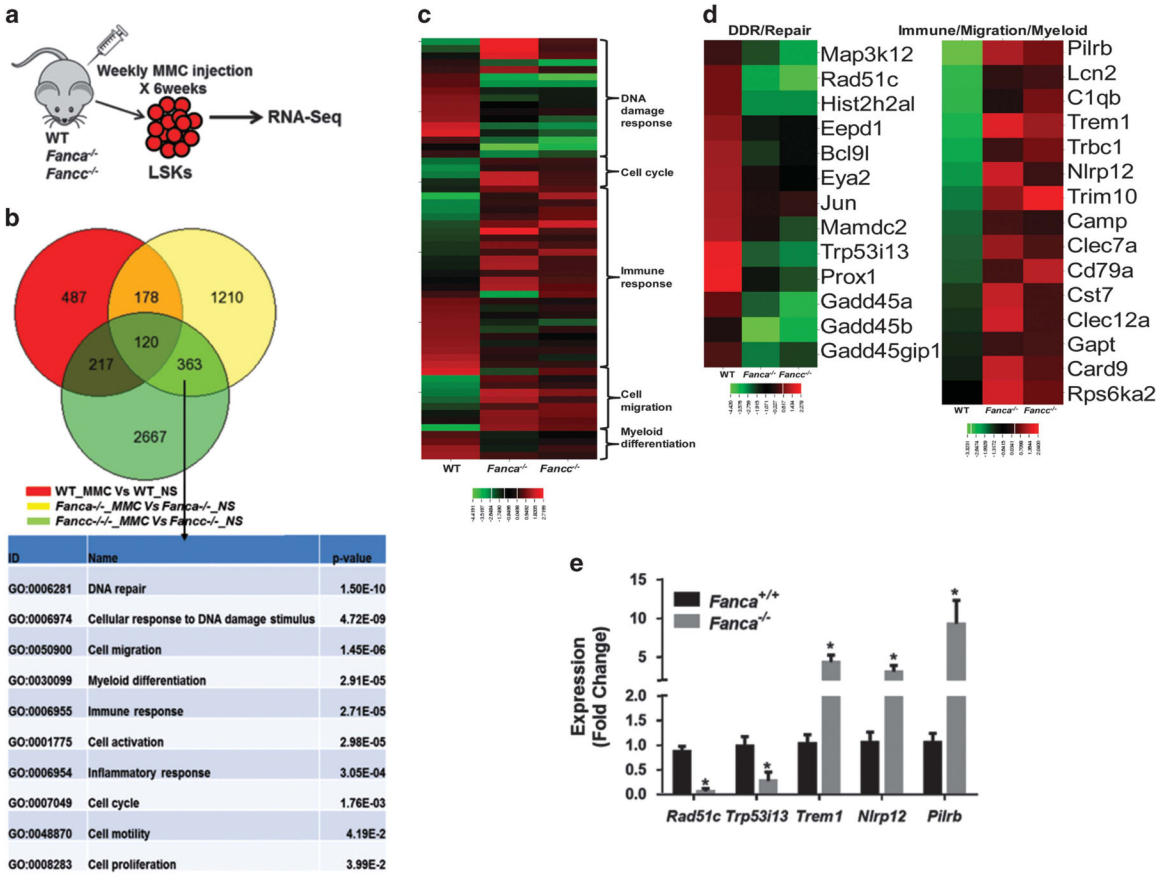

Figure 3.

Gene expression profiling identified genes altered in expression. (**a**) Schematic representation of experimental design. LSK cells isolated from WT, *Fanca*$^{-/-}$ and *Fancc*$^{-/-}$ mice treated with or without MMC for 6 weeks were subjected to RNA-seq analysis. (**b**) Analysis of differential gene expression in LSK cells from the indicated groups. Differentially expressed genes (0.05 *P*-value cutoff, 2.0-fold change cutoff) in each group were used for pairwise comparison with identify unique and shared genes. Numbers depicted in the Venn diagram (left) represent the up- or downregulated genes in unique sections or shared genes in each intersection. Shared genes in *Fanca*$^{-/-}$ and *Fancc*$^{-/-}$ group were further selected to run the pathway analysis module of GeneSpring GX v12 using the curated WikiPathway database. Top 10 pathways are shown (right). (**c, d**) Heatmap presentation of differential expression of selected genes across the indicated groups. Row represents individual genes and columns represent indicated groups. (**e**) Quantitative RT-PCR validation of selected genes identified in RNA-seq analysis. RNA was extracted from LSK cells isolated from 6 weeks MMC injected WT, *Fanca*$^{-/-}$ and *Fancc*$^{-/-}$ mice followed by RT-PCR using primers listed in Supplementary Table S1. **P*<0.05; ***P*<0.01; ****P*<0.001.

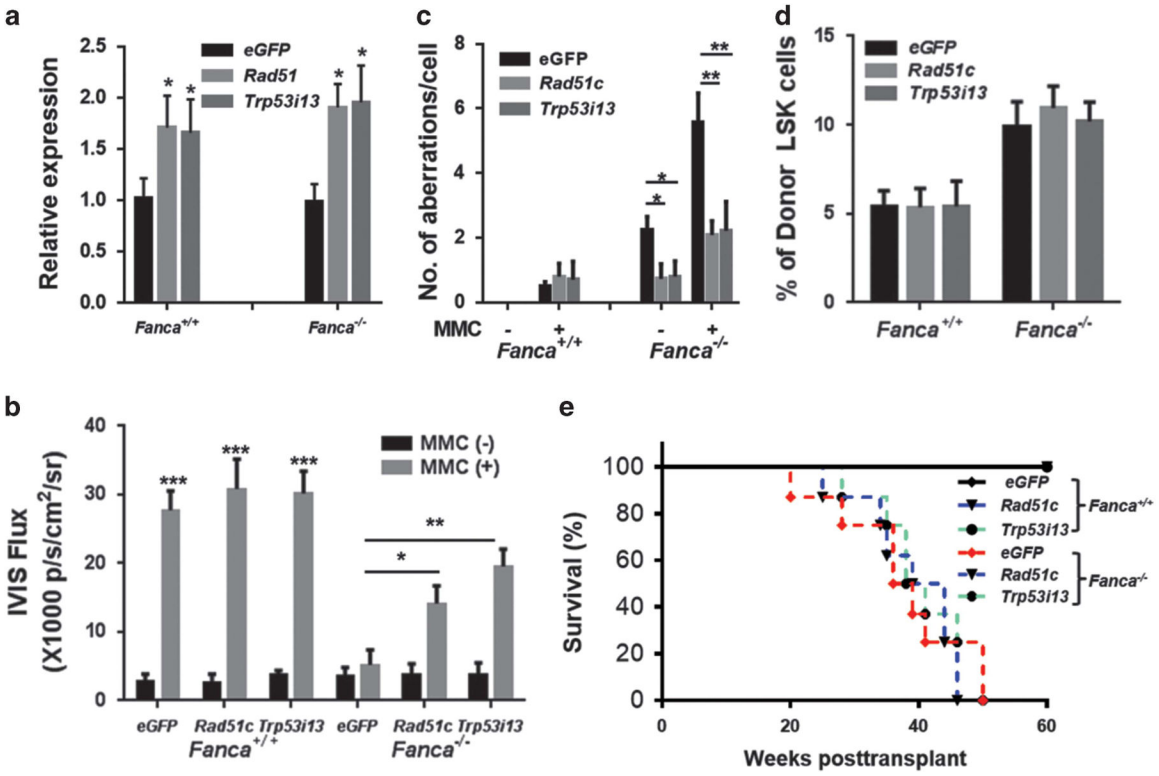

Figure 4.

Forced expression of the downregulated DDR/repair genes in pre-leukemic *Fanca*$^{-/-}$ HSCs restores DDR but fails to prevent leukemia. (**a**) The level of *Rad51c* and *Trp53i13* mRNA in lentiviral vector-transduced LSK cells. Luc-WT or Luc-*Fanca*$^{-/-}$ LSK cells isolated from the recipient mice treated with MMC for 6 weeks were transduced with lentivirus expressing eGFP only (Vector), eGFP plus *Rad51c* (*Rad51c*) or *Trp53i13* (*Trp53i13*) and then subjected to fluorescence-activated cell sorting sorting for GFP^{+} cells. RNA was then extracted from the sorted GFP^{+} cells for RT-PCR analysis for *Rad51c* and *Trp53i13*. Samples were normalized to the level of *GAPDH* mRNA. (**b**) Ectopic expression of *Rad51c* or *Trp53i13* in pre-leukemic *Fanca*$^{-/-}$ HSCs partially restores DDR. A total of 2000 sorted eGFP^{+} cells described in **a**, along with 200 000 c-Kit-depleted protector cells, were transplanted to lethally irradiated BoyJ mice. Sixteen weeks later, recipients were treated with MMC followed by IVIS live image. (**c**) Ectopic expression of *Rad51c* or *Trp53i13* in pre-leukemic *Fanca*$^{-/-}$ HSCs reduces genomic instability. Cells described in **a** were treated with MMC and subjected to chromosome breakage analysis. Quantification of 50 cells in random fields is shown. (**d**) Forced expression of *Rad51c* or *Trp53i13* fails to prevent expansion of the pre-leukemic *Fanca*$^{-/-}$ HSCs. Low density bone marrow cells from the recipients described in **a** were subjected to flow cytometric analysis for donor-derived HSCs (LSKCD150^{+}CD48^{-}) 16 weeks after bone marrow transplantation. (**e**) Forced expression of *Rad51c* or *Trp53i13* fails to delay leukemia development. The recipients described in **a** were analyzed for survival and plotted by the Kaplan–Meier curve method. $P>0.05$, *eGFP* vs *Trp53i13* or *eGFP* vs *Rad51c*. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

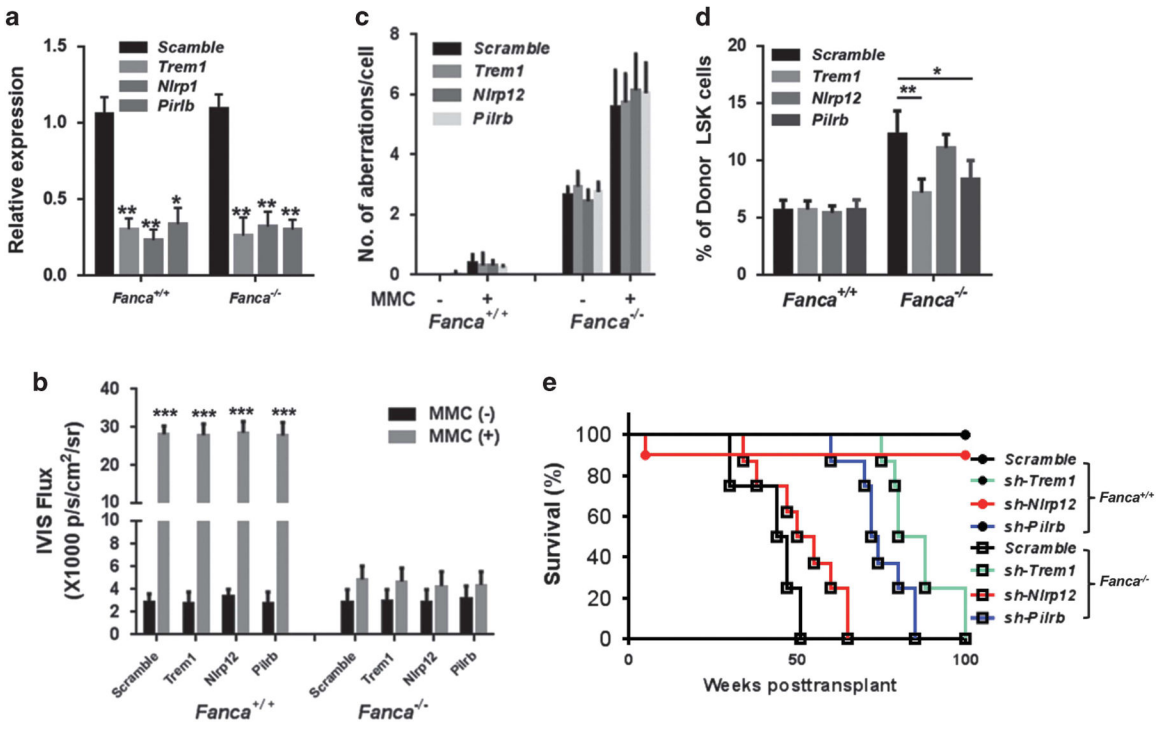

Figure 5.

Knockdown of migration/myeloid differentiation/immune response genes inhibits pre-leukemic *Fanca*$^{-/-}$ HSC expansion and delays leukemia. (**a**) The level of *Trem1, Nlrp12* and *Pirlb* mRNA in shRNA-transduced LSK. Luc-WT or Luc-*Fanca*$^{-/-}$ LSK cells isolated from the recipient mice treated with MMC for 6 weeks were transduced with lentiviruses expressing scramble shRNA or shRNA targeting *Trem1*, *Nlrp12* or *Pilrb*. RNA was extracted from sorted GFP^{+} cells for RT-PCR analysis for *Trem1*, *Nlrp12* and *Pilrb*. Samples were normalized to the level of *GAPDH* mRNA. (**b**) Knockdown of *Trem1*, *Nlrp12* or *Pilrb* fails to restore DDR. A total of 2000 sorted eGFP^{+} cells described in **a**, along with 200 000 c-Kit-depleted protector cells, were then transplanted into lethally irradiated BoyJ mice. Sixteen weeks later, recipients were treated with MMC followed by IVIS live image. (c) Knockdown of *Trem1*, *Nlrp12* or *Pilrb* fails to reduce genomic instability. Cells described in a were treated with MMC and subjected to chromosome breakage analysis. Quantification of 50 cells in random fields is shown. (**d**) Knockdown of *Trem1* or *Pilrb* but not *Nlrp12* inhibits pre-leukemic HSC expansion. Low density bone marrow cells from recipients described in **a** were subjected to flow cytometric analysis for donor-derived HSCs (LSKCD150^{+}CD48^{-}) 16 weeks after bone marrow transplantation. (**e**) Knockdown of *Trem1* or *Pilrb* delays leukemia development. Survival of the recipients transplanted with *Fanca*$^{-/-}$ LSK expressing scramble shRNA or shRNA targeting *Trem1*, *Nlrp12* or *Pilrb* (n= 8–10) was monitored and plotted by Kaplan–Meier curve method. P<0.01, *Scramble* vs *Trem1*; P o0.05, *Scramble* vs *Pilrb*; P>0.05, *Scramble* vs *Nlrp12p*; P<0.01, *sh-Trem1* vs *sh-Nlrp12*; P<0.05, *sh-Trem1* vs *sh-Pirlb*. *P<0.05; **P<0.01; ***P<0.001.

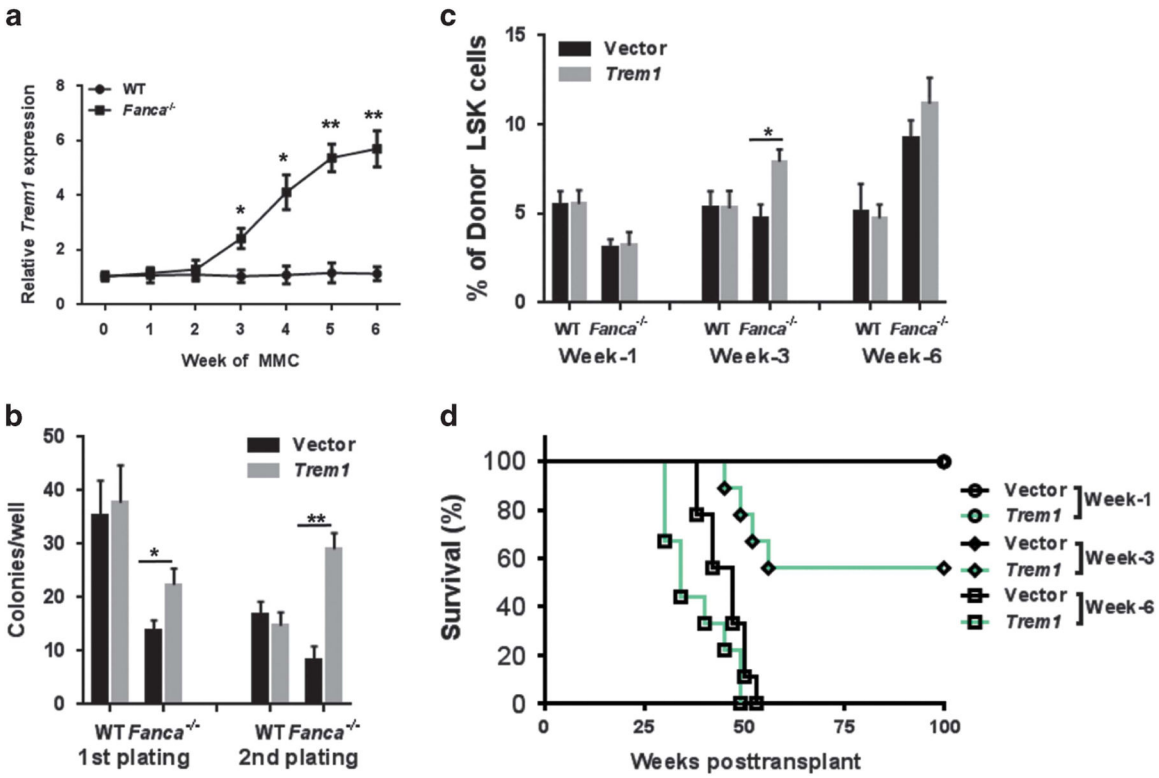

Figure 6.

Trem1 functionally cooperates with diminished DDR *in vivo* to promote *Fanca*$^{-/-}$ pre-LSC expansion and leukemia development. (a) Progressive increase of *Trem1* expression in pre-leukemic *Fanca*$^{-/-}$ HSCs. RNA was extracted from WT and *Fanca*$^{-/-}$ LSKCD150^{+} cells isolated from recipient mice treated with MMC for up to 6 weeks, and subjected to RT-PCR using the primers listed in Supplementary Table S1. (b) Trem1 enhances colony generation of the pre-leukemic *Fanca*$^{-/-}$ HSCs. WT and *Fanca*$^{-/-}$ LSK cells were isolated from MMC-treated recipient mice at week 3 and transduced with vector or *Trem1.* A total of 100 GFP^{+} LSKCD150^{+} cells were sorted and subjected to serial plating. Data represent mean±s.d. of three independent experiments. (c) Expression of *Trem1* promotes pre-leukemic HSC expansion in the week-3 and week-6 recipients. Low density bone marrow cells from week-3 and week-6 recipients were subjected to flow cytometric analysis for donor-derived HSCs (LSKCD150^{+}CD48^{-}) 4 months post bone marrow transplantation. (d) Expression of *Trem1* accelerates leukemia development in the week-3 and week-6 recipients. Survival of the recipients was monitored and plotted by Kaplan–Meier curve method. All transplant data are compiled from three independent cohorts. *P<0.05, Vector vs *Trem1* at week 3; P>0.05, Vector vs *Trem1* at week 1 or 6. *P<0.05; **P<0.01; ***P<0.001.