Fancd2 is required for nuclear retention of FOXO3a in hematopoietic stem cell maintenance

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Running Title: Fancd2 is required for FOXO3a nuclear retention

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Abstract

Functional maintenance of hematopoietic stem cells (HSCs) is constantly challenged by stresses like DNA damage and oxidative stress. Here we show that the Fanconi anemia (FA) protein Fancd2 and stress transcriptional factor Foxo3a cooperate to prevent HSC exhaustion in mice. Deletion of both Fancd2 and Foxo3a led to an initial expansion followed by a progressive decline of bone marrow (BM) stem and progenitor cells. Limiting dilution transplantation and competitive repopulating experiments demonstrated a dramatic reduction of competitive repopulating units and progressive decline in hematopoietic repopulating ability of double-knockout (dKO) HSCs. Analysis of the transcriptome of dKO HSCs revealed perturbation of multiple pathways implicated in HSC exhaustion. Fancd2 deficiency strongly promoted cytoplasmic localization of Foxo3a in HSCs and re-expression of Fancd2 completely restored nuclear Foxo3a localization. By co-expressing a constitutively active CA-FOXO3a and WT or a non-ubiquitinated Fancd2 in dKO BM stem/progenitor cells, we demonstrated that Fancd2 was required for nuclear retention of CA-FOXO3a and for maintaining hematopoietic repopulation of the HSCs. Collectively, these results implicate a functional interaction between the FA DNA repair and FOXO3a pathways in HSC maintenance.

Introduction

Hematopoietic stem cells (HSCs) are maintained in an undifferentiated quiescent state in a bone marrow (BM) niche (1). It appears that the HSC pool is maintained throughout the lifetime and this lifelong persistence of HSCs is likely due to their balance between quiescence and cycling, as well as to their localization in specialized niches in BM (2, 3). Increased proliferation or differentiation ultimately may lead to premature exhaustion of the stem cell pool, which is defined by a decrease in the number of HSCs caused by their enhanced cell cycling (4). Whereas numerous molecular factors that contribute to quiescence exist in the HSCs and the BM niche, HSCs are inevitably exposed to stress such as accumulation of reactive oxygen species (ROS) and DNA damage, which can drive HSCs into uncontrolled cell-cycle entry and excessive proliferation. DNA damage or oxidative stress can actually result in HSC exhaustion (5, 6). Studies in ataxia telangiectasia mutated (ATM) deficient mice showed progressive BM failure resulting from a defect in HSC function that was associated with elevated ROS that induce oxidative stress (7). Prolonged treatment of HSCs with an antioxidant can extend the life span of HSCs and protect HSCs against loss of self-renewal capacity (7).

Fanconi anemia (FA) is an inherited disorder characterized by progressive bone marrow failure, developmental defects, and predisposition to cancer (8, 9). Most FA patients demonstrate BM failure during childhood and are at risk of developing acute myelogenous leukemia (10, 11). FA is caused by a deficiency in any of the sixteen FA genes (FANCA-Q) (12, 13), which cooperate in a DNA repair pathway for resolving DNA interstrand cross-link (ICL) encountered during replication or generated by DNA-damaging agents (14, 15). Several FA proteins form a large nuclear complex that modifies the key downstream FA gene product FANCD2, by conjugation of a single ubiquitin molecule (monoubiquitination). This critical modification could be stimulated by DNA damage or genotoxic stress, resulting in the localization of the FANCD2 protein to sites of nuclear DNA lesions (16). Patients with FANCD2 mutations have earlier onset and more rapid progression of hematologic manifestations (17). It has been reported that Fancd2-deficient mice had

Capsule:

Background: Maintenance of HSCs is challenged by DNA damage and oxidative stress.

Results: Fancd2 deficiency promoted cytoplasmic localization of Foxo3a in HSCs, re-expression of Fancd2 restored nuclear Foxo3a localization and prevented HSC exhaustion.

Conclusion: Fancd2 is required for nuclear retention of FOXO3a and maintaining hematopoietic repopulation of HSCs.

Significance: Our results implicate an interaction between FA DNA repair and FOXO3a pathways in HSC maintenance.
multiple hematopoietic defects, including HSC and progenitor loss in early development, abnormal cell-cycle status and loss of quiescence in hematopoietic stem and progenitor cells, and compromised functional capacity of HSCs (5). Mammalian forkhead members of the class O (FOXO) transcription factors, including FOXO1, FOXO3a, FOXO4 and FOXO6, are implicated in the regulation of diverse physiologic processes, including cell cycle arrest, apoptosis, DNA repair, stress resistance, and metabolism (18). Loss of FOXOs results in elevated ROS, which in turn negatively regulates cellular responses (19). FOXO proteins are normally present in an active state in a cell's nucleus. Activated AKT phosphorylates FOXO3a proteins at three consensus Akt phosphorylation sites (Thr-32, Ser-253, and Ser-315), triggering the inactivation of FOXO3a and its export from the nucleus into the cytoplasm (20). Mouse knockout studies have shown that Foxo factors, particularly Foxo3a, function to regulate the self-renewal of HSCs and contribute to the maintenance of the HSC pool during aging by providing resistance to oxidative stress (21).

We recently reported a functional interaction between FOXO3a and the FA protein FANCD2 in response to oxidative stress (22). However, the functional consequence of this interaction is not known. In this study, we have exploited the genetic relationship between the two proteins by generating Fancd2−/−Foxo3a−− double knockout (dKO) mice and demonstrating that Fancd2 cooperates with nuclear Foxo3a to prevent HSC exhaustion.

Experimental procedures

Animals

All protocols of animal experiments described in this study were approved by the Institutional Animal Care and Use committee at Cincinnati Children’s Hospital Medical Center. Fancd2−/− mice were provided by Dr. Markus Grompe (Oregon Health & Sciences University) (23). Double heterozygotes Fancd2+/-Foxo3a+/- mice were first generated from interbreeding Fancd2+/- heterozygotes with the Foxo3a+/- mice (18), and WT and double-knockout (dKO) mice were generated from interbreeding of the double heterozygotes. All the animals including BoyJ (C57BL/6: B6, CD45.1+) mice were maintained in the animal barrier facility at Cincinnati Children’s Hospital Medical Center.

Flow analysis and cell sorting

Femurs and tibias were flushed to dissociate the BM fraction. Cells were resuspended in 5mL PBS/0.5% BSA and filtered through a 70-µm filter (BD Biosciences). The mononuclear cells were isolated by Ficoll (GE Healthcare) gradient centrifugation. The following antibodies were used for flow cytometry analyses: APC-cy7-anti-cKit, PE-cy7-anti-Sca-1, Pacific blue-anti-CD150, FITC-anti-CD48, FITC-anti-CD34, PE-anti-CD45.1, APC-anti-CD45.2, APC-anti-Ki67, and APC-anti-BrdU antibodies. The lineage antibody cocktail included the following biotin-conjugated anti-mouse antibodies: Mac1, Gr-1, Ter119, CD3e, and B220 (BD Biosciences). Secondary reagent used included streptavidin-PerCP-Cy5.5 (BD Biosciences). Initially, for LSK (Lineage−Sca-1−c-Kit+) staining, cells were stained by using biotin-conjugated anti-lineage antibody cocktail followed by staining with a secondary Perp-cy5.5-anti-Streptavidin antibody (BD Biosciences). For HSC (SLAM; Lin−ckit+Sca-1+CD150+CD48−) subpopulation, cells were stained with antibodies for LSK cells in addition to pacific blue-CD150 (Biolegend) and FITC-CD48 (Biolegend). Flow cytometry was performed on a FACS-LSR II (BD Biosciences) and analysis was done with FACSDiva Version 6.1.2 software (BD Biosciences). For cell sorting, lineage negative cells were enriched using lineage depletion columns (StemCell Technologies) according to the manufacturer’s instruction. The LSK population or HSCs (Lin−ckit−Sca-1−CD34−) were acquired by using the FACSArria II sorter (BD Biosciences).

Limiting-dilution cobblestone area-forming cell (CAFC) assay

Limiting dilution CAFC assay using five dilutions (0, 10, 30, 90, 270, and 810) of LSK cells was performed as described previously (24). Briefly, cells were plated on confluent OP9 stromal cells in
96 well plates with 10 wells per cell concentration, and numbers of CAFC were counted after 4 weeks.

BM transplantation
For the limiting dilution assay, graded numbers of donor BM cells (CD45.2+) harvested from WT, *Fancd2*−/−, *Foxo3a*−/− and dKO mice were mixed with 2× 10^5 protector BM cells (CD45.1+) and transplanted into lethally irradiated (split dose of 700 Rad + 475 Rad with 3 hrs apart) BoyJ mice (CD45.1+). Plotted are the percentages of recipient mice containing less than 1% CD45.2+ blood nucleated cells at 16 weeks after transplantation. The frequency of functional HSCs was calculated according to Poisson statistics.

For competitive repopulation assay, 50 LSK CD150+CD48+ (SLAM) cells or 1000 LSK cells (CD45.2+) plus 4×10^5 recipient BM cells (CD45.1+) were transplanted into lethally irradiated BoyJ mice (CD45.1+). Five mice were transplanted for each genotyping group. Blood samples were collected from the recipients every 4 weeks after BM transplantation. The donor blood chimerism was determined by staining peripheral blood samples with APC-anti-CD45.2 (BD Biosciences) and analyzed on a FACS Canto instrument (BD Biosciences).

For serial BM transplantation, 2000 sorted LSKs were transplanted into sublethally irradiated (6 Gy) primary recipients. At 8 weeks, a portion of the primary recipient mice was sacrificed, and CD45.2+ LSKs were sorted again and transplanted into lethally irradiated secondary recipients at 1,500 cells/mouse.

Cell-cycle and apoptosis Analysis
To analyze the cell cycle status of HSCs, BM cells were stained with antibodies against Lin cells, c-kit, Sca-1, CD150 and CD48, followed by fixation and permeabilization with transcription factor buffer set (BD Biosciences) according to the manufacturer’s instruction. After fixation, cells were incubated with APC-anti-Ki67 (Biolegend), washed and stained with PI (BD Bioscience). Cells were analyzed by flow cytometry. For apoptosis detection, BM cells were stained with the SLAM antibodies described above, and then stained with APC-Annexin V (BD Biosciences) and PI. Annexin V-positive cells were determined as apoptotic cells using the FACS LSR II (BD Biosciences).

In vivo Bromodeoxyuridine (BrdU) incorporation assay
Mice were injected intraperitoneally with a single dose of BrdU (Sigma-Aldrich; 1mg/6g mouse weight) 48 hours prior to sacrifice. BM cells were harvested and stained with biotin-conjugated antilineage antibody cocktail followed by staining with a secondary Percp-cy5.5-anti-Streptavidin, PE-cy7-anti-Sca-1, APC-cy7-anti-cKit and FITC-anti-CD34 (eBiosciences) antibodies, and then fixed and stained with APC-anti-BrdU antibody (BD Biosciences) using the Cytofix/Cytoperm Kit (BD Biosciences), according to the manufacturer’s instructions. Analysis was performed on a FACS LSR II (BD Biosciences).

RNA Isolation, Quantitative PCR and Microarray analysis
Total RNA from SLAM cells isolated from mice with the indicated genotypes was prepared with 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 min and stopped at 95 °C for 5 min. First-strand cDNA was used for real-time polymerase chain reaction (PCR) using primers listed in Table 1. Samples were normalized to the level of β-actin mRNA, and the relative expression levels were determined by the standard curve method.

For microarray analysis, cDNA was synthesized from total RNA and hybridized to Affymetrix Mouse gene 2.0 ST Array (Affymetrix Inc.). The RNA quality and quantity assessment, probe preparation, labeling, and hybridization were carried out in the Cincinnati Children’s Hospital Medical Center Affymetrix Core using standard procedures. Hybridization data were sequentially subjected to normalization, transformation, filtration and functional classification. Data analysis was performed with Genespring GX11 (Agilent Technologies). Gene set enrichment analysis was performed using GSEA v2.0 software (http://www.broadinstitute.org/gsea/index.jsp) as described (25).
Lentiviral vector construction, virus production and transduction

The SF-LV-cDNA-eGFP lentiviral vector (26) was generously provided by Dr. Lenhand Rudolph (Institute of Molecular Medicine and Max-Planck-Research Department of Stem cell Aging, Germany). The CA-FOXO3a cDNA carrying alanine substitutions at T32A, S253A and S315A (20) was amplified (forward primer: 5’-ATTACC CGTGATGGCAGAGGCACCGGCTTC-3’ and reverse primer: 5’-AAAGT TAACCTAGCTGGCCACCAGC-3’) from the Addgene plasmid 8361 (Addgene) and inserted into SF-LV-cDNA-eGFP. The SF-LV-cDNA-mCherry lentiviral vector was created by replacing the IRES-eGFP cassette with an IRES-mCherry cassette, which was amplified from the Addgene plasmid 45766 (Addgene) using the following primer sets: forward primer, 5’-ATAAGAATGCGGCCGCCCCCTCTCCCTCCC CCCCCCTAAC-3’ and reverse primer, 5’- GCGACGCGTGTTCTGACATTACTTGTAGC CTC GTCCATG-3’. The Flag-tagged mouse Fancd2 cDNA was amplified by two step PCR using primers (forward primer: 5’- TTGCAACC CGGTATGATTCCAAAGAGACTCG GCTAGATTCC-3’ and reverse primer 1: 5’- AAATATGCGGCCGCTCAAGCGTAGTGCCGG CACGTGTAAGGGTAGCTGGTGCCCTGAGCCTT GTATCCTATCAGCTCATCCTGTAATCGA-3’; reverse primer 2: 5’- TGTCACTCGTATCCTGTAGATCATGATCAT GATCTTCTAATACCGCTATGCTTTATGT AGTCGGAATCGCTGCTGCTGCTGCTTGT CATGACTGCC-3’) from a mouse Fancd2 cDNA clone (provided by Dr. Markus Grompe from Oregon Health & Sciences University) and inserted into SF-LV-cDNA-mCherry empty vector. The K559R mutant form of mouse Fancd2 plasmid was created with the QuickChange site-directed mutagenesis kit (Agilent Technologies). Lentivirus was produced in 293 T cells after transfection of 20 µg cDNA plasmid, 15 µg pCMVΔR8.91 helper plasmid and 6 µg pMD.G, using standard calcium phosphate transfection procedures (27). Medium was replaced with fresh medium 12 hours after transfection. To harvest viral particles, supernatants were collected 48 hours after transfection, filtered through 0.45 µm-pore-size filters, and concentrated by the PEG-it™ Virus precipitation solution (System Biosciences) according to the manufacturer’s protocol. Virus pellet was resuspended in sterile PBS and stored at -80°C.

For lentivirus transduction, the sorted CD34-LSK cells or LSK cells were maintained in StemSpan™ Serum-Free Expansion Media (Stemcell Technologies) with 50 ng/ml SCF (Peprotech) and 50 ng/ml TPO (Peprotech) for 24 hours before transduction. Transduction medium consisted of concentrated lentivirus suspension diluted 1:30 and 6 µg/ml polybrene (Millipore). Lentivirus transduction was performed in round bottom 96-well plates, using 50 µl reaction volumes, for 24 h at 37 °C. Cells were then resuspended in 100 µl and incubated for 3 days.

Immunofluorescent analyses

CD34-LSK cells were sorted from mice with the indicated genotype, and then cytospun on slides, fixed by 4% paraformaldehyde, permeabilized with blocking solution (1XPBS / 0.25% Triton X-100 / 5% BSA), and subsequently processed for anti-FOXO3a monoclonal antibody (2497; Cell signaling), or anti-pS473AKT antibody (05-1003; Millipore). Nuclear DNA was stained by using Nuclei were visualized using 4’, 6-diamidino-2-phenylindole (DAPI, Life Technologies). Images were collected on a Nikon C2 confocal microscope.

Fluorescence intensity in the nuclear and cytoplasmic regions was quantified using Image J (http://rsbweb.nih.gov/ij/). Background-corrected cytoplasmic to nuclear (C/N) ratios were calculated from mean fluorescence intensity measured within a small square or circular region of interest placed within the nucleus, cytoplasm, and outside of each cell. 20 cells per sample and 3 to 4 samples per group were analyzed. Quantitative fluorescence data were exported from ImageJ generated histograms into Graphpad software for further analysis and presentation.

Statistics

Statistical significance was assayed by Student’s t test and one-way Anova. Values are presented as Mean ± SD. A P value of < 0.05 was considered significant. Limiting dilution assay used a
Poison-based probability statistic to calculate frequencies through the use of serial dilutions.

**Results**

**Deletion of Fancd2 and Foxo3a causes HSC exhaustion**

We previously reported an oxidative damage-specific interaction between FANCD2 and FOXO3a in human cells (22). To further investigate the genetic relationship between the two proteins, we generated *Fancd2-/-Foxo3a-/-* double knockout (dKO) mice. Because mice deficient for *Fancd2* or *Foxo3a* show defect in HSC function (5, 21), we focused the effect of simultaneous loss of Foxo3a and Fancd2 on HSC maintenance. Surprisingly, deletion of both *Fancd2* and *Foxo3a* in mice led to an initial expansion followed by a progressive decline of BM stem and progenitor cells. Specifically at one month of age, dKO mice showed a significant increase in both progenitor (Lin-ckit+Sca-1+; LSK; Figure 1A and B) and HSCs (LSK CD150+CD48-; SLAM; (28); Figure 1C and D). However, at 5 months of age, dKO BM stem and progenitor cells declined significantly (11.8±1.6 at 1 month vs 4.53±1.1 at 5 months for LSK; 6.9±0.8 at 1 month vs 1.16±0.60 at 5 months for SLAM; Figure 1). The results described above suggest that the dKO HSCs might have undergone replicative exhaustion. To test this notion, we determined the number of functional competitive repopulating units (CRUs) in aged (5 months old) dKO mice by performing limiting dilution BM transplantation assay. Graded numbers of test BM cells (CD45.2+) from WT, *Fancd2-/-*, *Foxo3a-/-* or dKO mice were mixed with 2×10^5 protector BM cells (CD45.1+) and transplanted into lethally irradiated congenic recipients (CD45.1+). At 4 months after transplant, the frequency of functional CRUs dKO mice was 1 in 107,151 (p<0.0001 vs WT), significantly lower than the frequencies in those of WT (1 in 23,114), *Fancd2-/-* (1 in 44,458; p=0.0005 vs dKO), or *Foxo3a-/-* (1 in 37,428; p=0.005 vs dKO) mice (Figure 1E).

We next performed competitive repopulating experiments by transplanting 50 SLAM cells (CD45.2+) from WT, *Fancd2-/-*, *Foxo3a-/-* or dKO mice, along with 4×10^5 recipient BM cells (CD45.1+) into lethally irradiated recipient mice. We determined the repopulating ability of donor HSCs by periodically measuring the percentage of donor-derived cells in the peripheral blood of the transplant recipients. The percentage of donor-derived cells in the recipients of WT SLAM cells was steadily increased during the period of 4-40 weeks after transplantation (Figure 1F). The donor chimerism in the peripheral blood of the recipients of *Fancd2-/-* or *Foxo3a-/-* cells was decreased 4 weeks after transplantation compared to that of recipients transplanted with the WT cells, but remained relatively steady afterwards. Significantly, donor chimerism in the recipients of dKO SLAM cells underwent progressive decline after 4 weeks post-transplantation (23.9±2.58% at 4 weeks vs 14.5±0.44% at 16 weeks and 4.07±1.15% at 40 weeks; Fig. 1F). Furthermore, we performed serial transplantation to ascertain the phenomenon of dKO HSC exhaustion. As shown in Figure 1G, seven of 10 secondary recipients of dKO cells died within 4 months post-transplantation, while majority of the recipients of WT, *Fancd2-/-* or *Foxo3a-/-* cells survived beyond 160 days (Figure 1G). Taken together, these results indicate that simultaneous loss of Foxo3a and Fancd2 results in HSC exhaustion.

**Loss of Fancd2 and Foxo3a increases proliferation of HSCs**

To determine the mechanism underlying dKO HSC exhaustion, we analyzed apoptosis and cell cycle of the phenotypic HSC (SLAM) cells from WT, *Fancd2-/-*, *Foxo3a-/-* and dKO mice. We first examined whether increased apoptosis could be responsible for the observed HSC exhaustion in dKO mice. No increase in the frequency of Annexin V-positive (Apoptotic) cells was found in WT or *Foxo3a-/-* SLAM cells; although a moderate but statistically not significant increase in the apoptotic HSCs was detected in *Fancd2-/-* and dKO mice (Figure 2A and B). We next examined the cell cycle status of WT, *Fancd2-/-*, *Foxo3a-/-* and dKO SLAM cells using Ki67 and PI staining. Remarkably, we observed a significant decrease in the frequency of quiescent (G0) cells in dKO SLAM cells compared to that in WT, *Fancd2-/-* or *Foxo3a-/-* SLAM cells (Figure 2C and D).
Moreover, we observed a significant increase in the frequency of BrdU-positive cells in the CD34+ LSK cells from dKO mice compared to those from WT, Fancd2−/− or Foxo3a−/− mice, as detected by in vivo BrdU incorporation (Figure 2E and F). Together, these results indicate that Fancd2-Foxo3a deficiency leads to increased cycling in HSCs.

The transcriptome of dKO HSCs implicates perturbation of multiple pathways in HSC function

To identify the molecular mechanism underlying dKO HSC exhaustion, we performed global gene expression profiling analyses on phenotypic HSC (SLAM) cells freshly isolated from WT, Foxo3a−/−, Fancd2−/− and dKO mice. Analysis of gene expression data revealed 788 unique differentially expressed genes, of which 461 were up-regulated and 327 down-regulated in dKO HSCs when compared to WT, Foxo3a−/− and Fancd2−/− HSCs with fold changes larger than 1.3 (Figure 3A). Microarray results were confirmed by qRT-PCR for a subset of the genes (Table 2). Classification based on functional annotation revealed that a large proportion of differentially expressed genes in dKO HSCs belonged to cell cycle control (Ccnb1, Ccnd1, Aurkb, Atr, Chek2) (Figure 3B and C), consistent with the observed increase in cycling HSCs. Another group of up-regulated genes were those involved in the differentiation of HSCs (Mafb, Msc, Apoe, Hoxa9) (Figure 3B and C). Other differentially regulated transcripts included genes known to be involved in oxidative stress response (Cat, Txnrd2, Prdx3) or to have roles in DNA repair (Ercc5, Rpa3, Rfc1) (Figure 3B and C). In addition, Foxo3a target genes Cdkn1b, which is a cell cycle inhibitor protein, and Pdk1, which encodes the Pten-induced kinase 1, were downregulated in the Fancd2 KO HSCs. In contrast, other Foxo3a target genes, such as Id1, that encodes the DNA-binding protein inhibitor ID-1, were upregulated in Fancd2 KO HSCs. (Table 2). These transcriptional changes suggest that multiple pathways might have been perturbed in HSC exhaustion caused by simultaneous loss of Foxo3a and Fancd2. To test this notion, we performed gene set enrichment analysis (GESA), which revealed a strong correlation of genes downregulated in dKO SLAM cells with gene sets that identify cell cycle checkpoints, DNA repair, and DNA binding (Figure 3D; Table 3). Conversely, the genes upregulated in dKO HSCs strongly correlated with gene sets typical of hematopoietic lineage differentiation (Figure 3D; Table 3).

Fancd2 is required for nuclear localization of Foxo3a in HSCs

To understand how simultaneous loss of Fancd2 and Foxo3a affects transcriptional program in HSCs, we asked whether Fancd2 deficiency affected nuclear localization of Foxo3a in HSCs. We isolated BM CD34+LSK cells from the WT and Fancd2−/− mice and examined the distribution of Foxo3a by immunofluorescence. The majority of Foxo3a was present in the nucleus of WT CD34+LSK cells (Figure 4A), consistent with previously report (21). However, we observed increased cytoplasmic Foxo3a staining in Fancd2−/− CD34+LSK cells (Figure 4A). To address whether this abnormal cytoplasmic localization of Foxo3a was due to the loss of Fancd2, we performed a rescue experiment with genetically corrected Fancd2−/− CD34+LSK cells by lentivirus expressing the WT mouse Fancd2 gene (Figure 4B). It appeared that re-expression of the mouse Fancd2 could completely restore nuclear Foxo3a localization (Figure 4C). These data suggest that a functional Fancd2 may be required for nuclear localization of Foxo3a in HSCs.

It has been shown that FOXO3a nuclear localization and thus transcriptional activity is regulated by phosphatidylinositol-3 kinase (PI3K)-mediated activation of AKT, which directly phosphorylates FOXO3a, leading to its nuclear export and inactivation (20). To exclude the possibility that increased cytoplasmic localization of Foxo3a in Fancd2−/− HSCs was a phenomenon secondary to AKT activation, we treated the WT and Fancd2−/− CD34+LSK cells with AKT inhibitor (124005; Calbiochem; 5 µM) for 2 hours and examined the phosphorylated AKT (pS473AKT) and Foxo3a subcellular localization. Treatment of WT CD34+LSK cells with the Akt inhibitor showed increased nuclear Foxo3a compared to vehicle controls (Figure 4D). However, we did not...
observe a significant increase in nuclear localization of Foxo3a in Fancd2-/- CD34-LSK cells treated with the Akt inhibitor (Figure 4D). Thus, increased cytoplasmic Foxo3a in Fancd2-/- HSCs is not driven by Akt-mediated phosphorylation.

A role of Fancd2 in the retention of nuclear Foxo3a in HSCs

The observation that Fancd2 deficiency reduced Foxo3a in the nucleus of HSCs prompted us to investigate the status of an active form of FOXO3a (CA-FOXO3a), which is constitutively active because it cannot be inactivated by phosphorylation and thus is resistant to nuclear export (20), in Fancd2-/- HSCs. We transduced Foxo3a-/- and Fancd2-/-Foxo3a-/- dKO CD34-LSK cells with lentivirus expressing the active CA-FOXO3a and eGFP (Figure 4E), and examined the subcellular localization of CA-FOXO3a in Foxo3a null cells with (Foxo3a+) or without (Fancd2-/- Foxo3a+) a Fancd2 gene. The transgene-encoded CA-FOXO3a was almost exclusively localized in the nucleus of the Fancd2-sufficient Foxo3a-/- CD34-LSK cells (Figure 4F). However, Fancd2-deficiency strongly promoted cytoplasmic localization of CA-FOXO3a in the Foxo3a-/- Fancd2-/- CD34-LSK cells (Figure 4F). Thus, it appears that Fancd2 plays a role in nuclear retention of the constitutively active CA-FOXO3a in HSCs.

Monoubiquitination is required for the role of Fancd2 in FOXO3a nuclear retention

Monoubiquitination of human FANCD2 at K561 is a critical step in the FA DNA repair pathway (29, 30). To determine whether FANCD2 monoubiquitination was required for FOXO3a nuclear retention, we identified the monoubiquitination site (K559) in mouse Fancd2, and generated a mutant that had arginine residue substituted for the lysine residue at position 559 (K559R; Figure 5A). We confirmed the loss of monoubiquitination of the mutant Fancd2 in response to replicative stress induced by hydrourea (HU; Figure 5B). We co-transduced the dKO LSK cells with lentivirus expressing the constitutively active CA-FOXO3a with an eGFP marker (CA-FOXO3a-eGFP) and the WT mouse Fancd2 with a mCherry marker (mFancd2 WT-mCherry) or its non-monoubiquitated mutant (mFancd2 K559R-mCherry), and sorted for double-positive (eGFP+ mCherry+) CD34-LSK cells (Figure 5C). Immunofluorescence analysis showed that expression of WT mFancd2 strongly promoted CA-FOXO3a localization in the nucleus (Figure 5D). In contrast, co-transduction of CA-FOXO3a and the non-monoubiquitated mutant mFancd2 K559R or vector alone led to increased cytoplasmic localization of CA-FOXO3a in dKO CD34-LSK cells (Figure 5D). Thus, monoubiquitination is essential for the effect of Fancd2 on FOXO3a nuclear retention.

Fancd2 and Foxo3a cooperate to maintain HSC function

To evaluate the functional consequence of FANCD2 in FOXO3a nuclear retention, we carried out two sets of experiments to examine whether Fancd2 and Foxo3a cooperate to maintain HSC function. For in vitro experiments, we used limiting-dilution cobblestone area-forming cell (CAFC) assay (24) to evaluate the self-renewal capacity of the dKO LSK cells co-expressing the WT FOXO3a and the WT or mutant Fancd2. Graded numbers of double-positive (eGFP+ mcherry+) LSK cells were plated on confluent OP9 stromal cells in 96 well plates, and numbers of CAFC were counted after 4 weeks. There was a significant decrease in CAFC colonies formed by dKO LSK cells co-expressing the mutant Fancd2-K559R or vector alone and FOXO3a in wells plated with 90, 270, and 810 LSK cells compared to those co-expressing the WT Fancd2 and FOXO3a (Figure 5E). For in vivo experiments, we performed competitive BM transplantation assay to determine the long-term hematopoietic repopulating ability of the test dKO LSK cells in lethally irradiated recipients. 1000 double-positive (eGFP+ mcherry+) LSK cells (CD45.2+) were co-transduced with FOXO3a-eGFP and mFancd2 WT-mCherry or mFancd2 K559R-mCherry lentivirus, along with 4×10^5 recipient BM cells (CD45.1+) were transplanted into lethally irradiated recipient mice. The repopulating capacity of donor HSCs was monitored by measuring donor-derived cells in the peripheral blood of the transplant recipients at 4
months post-transplantation. We observed significantly higher donor chimerism in the recipients of dKO LSK cells co-expressing Fancd2-WT and FOXO3a than those with cells co-expressing the mutant Fancd2-K559R or vector alone and FOXO3a (Figure 5F). Furthermore, there was a concomitant decrease in donor stem and progenitor (LSK) cells in the BM of the recipients transplanted with cells co-expressing FOXO3a and the mutant Fancd2-K559R or empty vector at 4 months after transplantation (Figure 5G). These results strongly argue a functional interaction between Fand2 and nuclear FOXO3a in HSC maintenance.

Discussion
In this study, we demonstrated that deletion of both Fancd2 and Foxo3a in mice induced HSC exhaustion. Increased HSC cycling may be one of the underlying mechanisms. In supporting this notion, we provided several pieces of evidence: (i) cell cycle analysis showed a significant decrease in quiescent HSCs in dKO mice compared to not only WT but also Fancd2-/- or Foxo3a-/- mice (Figure 2C and D); (ii) In vivo BrdU incorporation assay revealed a marked increase in proliferation in dKO CD34+LSK cells compared to those from mice with other three genotypes (Figure 2E and F); (iii) Global gene expression profiling on phenotypic HSC (SLAM) cells exhibited a deregulated cell cycle-associated transcriptional program that was unique to dKO HSCs (Figure 3B and C). (iv) Functionally, we demonstrated that a functional Fancd2 was required for nuclear retention of FOXO3a and for HSC maintenance (Figures 4 and 5). These data suggest that a functional interaction between the FANC2D and FOXO3a may be a novel mechanism by which HSCs maintain quiescence and avoid replicative exhaustion.

It has been reported that Foxo3a is not crucial for HSC differentiation but plays a role in HSC self-renewal (21). Deletion of Foxo3a impairs hematopoietic repopulating capacity of the HSCs, which is not caused by replicative exhaustion but at least in part by increased cellular accumulation of ROS (21). FANC2D plays critical roles in DNA damage repair of double-strand breaks (DSBs) (31, 32). Long-lived quiescent HSCs may be particularly susceptible to the accumulation of DSBs over time (33). Mice deficient for key DNA repair pathway components exhibit diminished HSC self-renewal capacity with age and early functional exhaustion (34, 35). In this context, we showed that in Fancd2-deficient BM, the content of phenotypic HSCs was reduced and their long-term repopulating activity was impaired compared to WT HSCs (Figure 1C, D and F), which is consistent with previously study (5). However, deletion of Fancd2 alone did not induce the phenotype typical of HSC exhaustion, as observed in dKO mice that showed an initial expansion followed by a progressive decline of both phenotypic HSCs and their long-term repopulating activity (Figure 1C, D and F). These results support the notion that Fancd2 and Foxo3a cooperate to prevent HSC exhaustion.

In quiescent WT murine HSCs, the majority of the Foxo3a protein is localized in the nucleus (36). It is also known that this nuclear localization is regulated by Akt, which phosphorylates Foxo3a and induces the export of Foxo3a from the nucleus to the cytoplasm (20). We showed that there was a marked increase in cytoplasmic Foxo3a accompanied by reduced nuclear Foxo3a in phenotypic Fancd2--/- HSCs (Figure 4A) and that this phenomenon was Akt-independent (Figure 4D), suggesting a role for Fancd2 in nuclear Foxo3a retention. Indeed, genetic correction of the Fancd2--/- HSCs with a WT mouse Fancd2 completely rescued Foxo3a nuclear retention (Figure 4C). Moreover, by co-expressing of the constitutively active CA-FOXO3a and the non-monoubiquitinated mutant mFancd2 K559R in dKO CD34+LSK cells, we showed that monoubiquitination is essential for the effect of Fancd2 on FOXO3a nuclear retention (Figure 5D). This is consistent with previous observation that the non-monoubiquitinated FANCD2 mutant failed to colocalized with FOXO3a on chromatin in response to oxidative DNA damage (22).

The mechanism responsible for FOXO3a sequestration in cytoplasm of the Fancd2 KO cells is not clear at this time. We speculate two possibilities. First, we previously showed that FOXO3a and FANC2D associated as a nuclear complex and co-localized on chromatin DNA (22). In this context, FANC2D might play a role...
in stabilizing FOXO3a in the nucleus. Consequently in Fancd2 KO HSCs, loss of Fancd2 might lead to increased cytoplasmic CA-FOXO3a. Second, sequestration of CA-FOXO3a in cytoplasm might be caused by abnormal activation of extracellular signal-regulated kinase (ERK) in Fancd2 KO HSCs. It is known that ERK regulates FOXO3a nuclear-cytoplasmic localization (37). ERK interacts with and phosphorylates FOXO3a predominantly at residues Ser294, Ser344 and Ser425, which are different from the Akt phosphorylation sites at T32, S253, and S315, leading to FOXO3a nuclear exportation. Several groups have reported constitutive activation of ERK in FA-deficient cells (38, 39). Thus, the constitutively activated ERK could act on the non-Akt phosphorylation sites leading to increased cytoplasmic localization of CA-FOXO3a in the Fancd2 KO HSCs.

In two well-established assays designed to evaluate self-renewal (limiting-dilution CAFC assay) and hematopoietic repopulating (competitive BM transplantation assay) ability of a HSC, we observed a significant decrease in both CAFC formation and long-term repopulation by dKO LSK cells co-expressing the non-monoubiquitinated Fancd2-K559R mutant and the WT FOXO3a compared to dKO LSK cells co-expressing the WT Fancd2 and FOXO3a (Figure 5E and F). Thus, it is tempting to speculate that a functional interaction between Fancd2 and FOXO3a is required for HSC maintenance. Moreover, that the observed an initial expansion followed by a progressive decline of both phenotypic HSCs and their long-term repopulating activity requires simultaneous inactivation of Foxo3a and Fancd2 indicates that the HSC exhaustion phenotype is resulted from a synergistic effect. In this context, we speculate a coordinate regulation of HSC functions by the FOXO3 and FA pathways. Indeed, Foxo3a or Fancd2 single-knockout mice did not experience HSC exhaustion, as judged by progressive decline in HSC pool and HSC self-renewal capacity, suggesting that the defect in dKO HSCs was not simply additive but synergistic.

In summary, the present studies demonstrate that simultaneous inactivation of Fancd2 and Foxo3a in mice induces HSC exhaustion and identify increased HSC cycling as one of the underlying mechanisms for the defect. These findings reveal functional interaction between the FA DNA repair pathway and the FOXO3a stress response pathway in HSC maintenance and may suggest new targets for therapeutically exploring the pathogenic role of stress-induced HSC exhaustion in blood diseases.
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Abbreviations: BM, Bone marrow; CAFC, Cobblestone area-forming cell; CRU, Competitive repopulating units; dKO, double-knockout; GESA, Gene set enrichment analysis; HSCs, Hematopoietic stem cells; ROS, Reactive oxygen species; FA, Fanconi anemia; ICL, Interstrand cross-link.
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Figure Legends

Figure 1. Deletion of Fancd2 and Foxo3a causes HSC exhaustion. A. Frequency of LSK (Lin−ckit+Sca-1−) cells in the BM of WT, Foxo3a−/−, Fancd2−/− and dKO mice at 1 and 5 months of age. Shown are representative flow plots. B. Quantitation of BM LSK cells in WT, Foxo3a−/−, Fancd2−/− and dKO mice at 1 and 5 months old. Each group comprises 4 to 6 mice. C. Frequency of SLAM (LSK CD150+CD48−) cells in BM LSK cells from WT, Foxo3a−/−, Fancd2−/− and dKO mice at 1 and 5 months of age. Shown are representative flow plots. D. Quantitation of BM SLAM cells in WT, Foxo3a−/−, Fancd2−/− and dKO mice at 1 and 5 months old. Each group comprises 4 to 6 mice. E. Competitive repopulating units (CRUs) determined by limiting dilution BM transplantation assay. Graded numbers of test BM cells (CD45.2+) were mixed with 2 × 10^5 protector BM cells (CD45.1+) and transplanted into irradiated congenic recipients (CD45.1+). Plotted are the percentages of recipient mice containing less than 1% CD45.2+ blood nucleated cells at 16 weeks after transplantation. Frequency of functional HSCs was calculated according to Poisson statistics. F. Competitive repopulation assay. 50 SLAM test (CD45.2+) and 4 x10^5 competitor (CD45.1+) whole BM cells were mixed and transplanted into irradiated CD45.1+ recipients. Donor-derived cells (CD45.2+) in the peripheral blood were determined at 4-40 weeks post-transplantation. Data are means ± s.e.m. (n=8-10 from two independent experiments). G. Kaplan-Meier survival curves of secondary recipients (n=8-10). 2,000 sorted LSKs from the indicated mice were transplanted into sublethally irradiated CD45.1+ mice, and 8 weeks later, the primary recipient mice was sacrificed, and CD45.2+ LSKs were sorted again and transplanted into lethally irradiated secondary recipients at 1,500 cells/mouse. Data shown are the survival rates expressed as a percentage. **p < 0.01; ***p < 0.001.

Figure 2. Loss of Fancd2 and Foxo3a increases proliferation of HSCs. A. Flow cytometric analysis of apoptotic cells within the phenotypic HSC (SLAM) population. BM cells of WT, Foxo3a−/−, Fancd2−/−, and Foxo3a−/− Fancd2−/− dKO mice were gated for the SLAM population, and analyzed for Annexin V-positive cells. B. Quantification of Annexin V-positive cells within the SLAM population. Each group comprises 6 mice. C. Cell cycle analysis of SLAM cells. BM cells of WT, Foxo3a−/−, Fancd2−/−, and Foxo3a−/− Fancd2−/− dKO mice were gated for the SLAM population, and analyzed for cell-cycle phases by flow cytometry. Representative dot plots of DNA content (PI) were plotted versus Ki-67 staining. G0: Ki-67− and 2n DNA; G1: Ki-67+ and 2n DNA; and G2/S: Ki67+ and DNA>2n. D. Quantification of quiescent (G0) cells within the SLAM population. Each group comprises 6 mice. (E). BrdU incorporation analysis of CD34− LSK cells. WT, Foxo3a−/−, Fancd2−/−, and Foxo3a−/− Fancd2−/− dKO mice at the age of 4-6 weeks were injected with a single dose of BrdU. 48 hours later, the mice were sacrificed, and BM cells were analyzed for BrdU positive cells by flow cytometry. (F). Quantification of BrdU positive cells in the CD34− LSK cells. Each group comprises 4 to 6 mice. **p < 0.01; ***p < 0.001; ****p <0.0001.

Figure 3. Global gene expression analysis of phenotypic HSCs. Whole-genome microarray data were obtained from freshly isolated SLAM (LSK CD150+CD48−) cells from WT, Foxo3a−/−, Fancd2−/− and dKO mice. A. Venn diagrams illustrating the overlap between genes upregulated and downregulated in WT, Foxo3a−/−, Fancd2−/− and dKO SLAM cells. B. Pie charts show the distribution of the 461 upregulated and the 327 downregulated genes in dKO SLAM cells into functional groups. C. Heat map displays the expression of genes with cell-cycle checkpoint, DNA repair, oxidative stress, and HSC differentiation-related functional annotations that are significantly downregulated and upregulated in dKO SLAM cells. The rows correspond to genes and the columns to samples. Gene expression values are indicated on a log2 scale according to the color scheme shown. Unregulated and downregulated genes are presented in green and red, respectively. D. GSEA analyses are shown for gene sets identified for cell-cycle checkpoints, DNA repair, DNA binding and HSC differentiation pathways. For each GSEA, the p-value and enrichment score (ES) are shown above each pathway graph.
Figure 4. Fancd2 is required for nuclear localization of Foxo3a in HSCs. A. Increased cytoplasmic Foxo3a staining in Fancd2<sup>−/−</sup> HSCs. Left panel: Freshly isolated CD34<sup>−</sup> LSK cells from WT and Fancd2<sup>−/−</sup> BM were immunostained to detect Foxo3a (red). Nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAPI; blue). Scale bar: 10 μm. Right panel: Ratio of fluorescence intensity of anti-Foxo3a staining in cytoplasm (C) and the nucleus (N). B. Flow cytometry of mCherry-positive cells before and after sorting. Mock: samples without virus. C. Re-expression of Fancd2 restores Foxo3a nuclear localization. Left panel: mFancd2-mCherry or empty-mCherry lentivirus transduced Fancd2<sup>−/−</sup> CD34<sup>−</sup> LSK cells were stained with anti-Foxo3a antibody (green) and DAPI (blue). Scale bar: 10 μm. Right panel: Ratio of fluorescence intensity of anti-Foxo3a staining in cytoplasm (C) and the nucleus (N). D. Enhance cytoplasmic localization of Foxo3a in Fancd2<sup>−/−</sup> HSCs is independent of Akt activation. Left panel: CD34<sup>−</sup> LSK cells from the WT and Fancd2<sup>−/−</sup> BM were treated with AKT inhibitor (5 µM) for 2h. Foxo3a, pS473AKT and nuclear DNA were visualized by red, green and blue, respectively. Scale bar: 10 μm. Right panel: Quantification of the fluorescence intensity of anti-Foxo3a staining in cytoplasm (C) and the nucleus (N). E. Flow cytometry of GFP-positive cells before and after sorting. Mock: samples without virus. F. Fancd2 is required for nuclear retention of the constitutively active CA-FOXO3a in HSCs. Left panel: Foxo3a<sup>−/−</sup> and Fancd2<sup>−/−</sup> Foxo3a<sup>−/−</sup> dKO CD34<sup>−</sup>LSK cells were transduced with lentivirus expressing the active CA-Foxo3a and eGFP (green). Transduced CD34<sup>−</sup> LSK cells were staining by anti-FOXO3a antibody (Red). Nuclei were visualized by DAPI (blue). Scale bar: 10 μm. Right panel: Quantification of the fluorescence intensity of anti-FOXO3a staining in the nucleus (N) and cytoplasm (C). Each group comprises 3 to 4 mice, and 20 cells per sample. Akt-i: Akt-inhibitor; ***p < 0.001.

Figure 5. Fancd2 and Foxo3a cooperate to maintain HSC function. A. Protein sequence alignment of human and mouse Fancd2, and generation of a mutant mFancd2 that had arginine residue substituted for the lysine residue at position 559. B. WT murine embryonic fibroblast cells were transduced with lentivirus carrying empty vector, mFancd2-WT or mFancd2-K559R, and then treated with or without hydrourea (HU) for 24 hours. Cell lysis were separated by SDS-PAGE gel, and immunoblotted with antibodies against Flag (mFancd2) and Topo1 (loading control). C. The dKO LSK cells were co-transduced with lentivirus expressing the CA-Foxo3a-eGFP and mFancd2 WT-mCherry or mFancd2 K559R-mCherry, and then sorted for double-positive (eGFP<sup>+</sup> mcherry<sup>+</sup>) CD34<sup>−</sup> LSK cells. Shown is flow cytometry analysis of the double-positive cells before and after sorting. Mock: samples without virus. D. Monoubiquitination is essential for the effect of Fancd2 on FOXP3a nuclear retention. dKO LSK cells were co-transduced with lentivirus expressing the constitutively active CA-FOXP3a with an eGFP marker (CA-FOXP3a-eGFP) (green) and the WT mouse Fancd2 with a mCherry marker (mFancd2 WT-mCherry) or its non-monoubiquitinated mutant (mFancd2 K559R-mCherry) (yellow). Transduced CD34<sup>−</sup> LSK cells were staining by anti-FOXP3a antibody (Red). Nuclei were visualized by DAPI (blue). Scale bar: 10 μm. Right panel: Quantification of the fluorescence intensity of anti-Foxo3a staining in the nucleus (N) and cytoplasm (C). Each group comprises 3 to 4 mice, and 20 cells per sample. E. Limiting-dilution cobblestone area-forming cell (CAFC) assay. dKO LSK cells were co-transduced with lentivirus expressing the WT FOXP3a with an eGFP marker and the WT mouse Fancd2 with a mCherry marker (mFancd2-WT) or its non-monoubiquitinated mutant (mFancd2-K559R) or an empty vector. Graded numbers of double-positive (eGFP<sup>+</sup> mcherry<sup>+</sup>) LSK cells were plated on confluent OP9 stromal cells in 96 well plates, and numbers of CAFC were counted after 4 weeks. (F-G). BM transplantation assay to determine the long-term hematopoietic repopulating ability. 1000 double-positive (eGFP<sup>+</sup> mCherry<sup>+</sup>) LSK cells (CD45.2), co-transduced with FOXP3a-eGFP and mFancd2 WT-mCherry or mFancd2 K559R-mCherry or empty-mCherry lentivirus, along with 4×10<sup>5</sup> recipient BM cells (CD45.1) were transplanted into lethally irradiated recipient mice. The repopulating capacity of donor HSCs was monitored by measuring percentage of GFP positive cells in the peripheral blood of the transplant recipients at 4 months post-transplantation (F). The percentage of GFP-positive LSK cells in the BM of
the transplant recipients was determined at 4 months post-transplantation (G). ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. 
| Table 1. Primer sequence                          |
|---------------------------------------------|
| **Ccnd1** Forward: 5'-TTTCTTTGAGCGGCCTGTGTGT-3'  |
| Reverse: 5'-CGGAACACTAGAAACCTAAGAG-3'          |
| **Atr** Forward: 5'-TGCGGTCTGTGCTAGACGGCCTGTGT-3' |
| Reverse: 5'-AGTGCTGGTGCCTGTGCTGTGCTGT-3'       |
| **Mafb** Forward: 5'-CAACAGCTACCCACTAGCA-3'    |
| Reverse: 5'-GCCCAGTTTCTGACACTTGAAGGTA-3'       |
| **Msc** Forward: 5'-GGAGGACCCGCTACAGGGACA-3'   |
| Reverse: 5'-ACCCACAGAAAGGCTATGCT-3'            |
| **Apoe** Forward: 5'-GCGGACATGGAGGATCTACTG-3'  |
| Reverse: 5'-CTTGTAACAGCTAGGCTAGGCTG-3'         |
| **Cat** Forward: 5'-CAGTACAGGAGATGCGACACCT-3'  |
| Reverse: 5'-TGTGGAGAACATCGAGCAGAAGGCA-3'       |
| **Rpa3** Forward: 5'-CACAGTATATCGACCGGCCC-3'   |
| Reverse: 5'-TTCTCCTCGTAAATCGGGCTTCC-3'         |
| **Prdx3** Forward: 5'-TGTCGTACAGCAAGCTAGTG-3'  |
| Reverse: 5'-TTGGGCTTGATATCGGAGGAC-3'           |
| **Ercc5** Forward: 5'-TGTTGCGTCGTAGTGCGACAGG-3' |
| Reverse: 5'-TGGAGCAGACCTGGCAGGATA-3'           |
| **Pink1** Forward: 5'-CTGACGGGCCTAGGCTGTT-3'   |
| Reverse: 5'-TTTGCTCCGCAAGCACAAG-3'             |
| **Id1** Forward: 5'-AGAAGCAGCTGCAGGGATG-3'     |
| Reverse: 5'-ACCTCCTCGCCACTCTGATGT-3'           |
| **Cdkn1b** Forward: 5'-CGGTTCCGAGGCTGCTTTTTC-3'|
| Reverse: 5'-CGTCACACTTTCATGGAATTGGA-3'         |
Table 2. qRT-PCR validation

| Gene name | Foxo3a vs WT (FC) | Fancd2 vs WT (FC) | dKO vs WT (FC) |
|-----------|-------------------|------------------|----------------|
| Ccnd1     | 0.89±0.25         | 1.33±0.1         | 1.84±0.2       |
| Atr       | 0.83±0.08         | 0.97±0.13        | 0.37±0.14      |
| Mafb      | 1.26±0.11         | 1.25±0.12        | 2.34±0.58      |
| Msc       | 1.29±0.08         | 1.06±0.08        | 1.82±0.33      |
| Apoe      | 1.07±0.16         | 1.12±0.16        | 2.58±0.2       |
| Cat       | 0.74±0.08         | 0.95±0.07        | 0.52±0.13      |
| Rpa3      | 0.86±0.04         | 1.08±0.07        | 0.52±0.13      |
| Prdx3     | 0.72±0.07         | 0.92±0.03        | 0.63±0.14      |
| Ercc5     | 0.99±0.09         | 1.01±0.12        | 0.66±0.1       |
| Pink1     | 0.64±0.14         | 0.66±0.13        | 0.58±0.21      |
| Cdkn1b    | 0.6±0.07          | 0.62±0.11        | 0.46±0.01      |
| Id1       | 1.47±0.04         | 1.40±0.11        | 1.52±0.19      |

FC: Fold Change
Table 3. Gene Sets with Strong Correlation to Genes Downregulated and Upregulated in dKO HSCs

| Gene Set Name                                      | Gene Set Description                                                                 | P-Value     |
|----------------------------------------------------|--------------------------------------------------------------------------------------|-------------|
| **Upregulated Genes**                              |                                                                                      |             |
| **Cell Differentiation**                           |                                                                                      |             |
| KEGG_HEMATOPOIETIC_CELL_LINEAGE                    | Hematopoietic cell lineage                                                           | 2.28 e^-4   |
| MATURE_B_LYMPHOCYTE_UP                             | Up-regulated genes in the B lymphocyte developmental signature                      | 5.44 e^-9   |
| HEMATOPOIESIS_MATURE_CELL                          | Up-regulated in mature blood cell populations from adult bone marrow and fetal liver.| 4.69 e^-8   |
| DENDRITIC_CELL_MATURATION_UP                       | Genes up-regulated during in vitro maturation of CD14+ monocytes (day 0) into immature and mature dendritic cells | 6.18 e^-7   |
| HEMATOPOIESIS_LATE_PROGENITOR                      | Up-regulated in hematopoietic late progenitor cells from adult bone marrow and fetal liver. | 3.8 e^-7    |
| REGULATION_OF_CELL_DIFFERENTIATION                 | Any process that modulates the frequency, rate or extent of cell differentiation     | 5.37 e^-4   |
| **Cell Cycle**                                     |                                                                                      |             |
| CELL_CYCLE                                         | Occur in a cell during successive cell replication or nuclear replication events.    | 3.72 e^-3   |
| POSITIVE_REGULATION_OF_CELL_CYCLE                  | Any process that activates or increases the rate or extent of progression through the cell cycle. | 4.34 e^-3   |
| **Downregulated Genes**                            |                                                                                      |             |
| **Stem Cell Sets**                                 |                                                                                      |             |
| LIM_MAMMARY_STEM_CELL_UP                           | Genes consistently up-regulated in mammary stem cells both in mouse and human species. | 6.98 e^-6   |
| JAATINEN_HEMATOPOIETIC_STEM_CELL_DN                | Genes down-regulated in CD133+ cells (HSC) compared to the CD133- cells.           | 4.87 e^-7   |
| IVANOVA_HEMATOPOIESIS_LATE_PROGENITOR              | Genes in the expression cluster up-regulated in hematopoietic late progenitor cells from adult bone marrow and fetal liver. | 3.24 e^-8   |
| GAL_LEUKEMIC_STEM_CELL                             | Genes down-regulated in leukemic stem cells (LSC) cells from AML                     | 8.53 e^-8   |
| WONG_ADULT_TISSUE_STEM_MODULE                      | Genes coordinately up-regulated in a compendium of adult tissue stem cells.          | 1.55 e^-9   |
| Term                                      | Description                                                                                           | Score  |
|-------------------------------------------|-------------------------------------------------------------------------------------------------------|--------|
| BYSTRYKH_HEMATOPOIESIS_STEM_CELL_QTL     | Transcripts in hematopoietic stem cells (HSC) which are trans-regulated                               | 3.41 e-8 |
| DNA Damage Control                        |                                                                                                       |        |
| REACTOME_DNA_REPAIR                       | Genes involved in DNA Repair                                                                         | 1.07 e-4 |
| REACTOME_REPAIR_SYNTHESIS_FOR_GAP_FILL    | Genes involved in Repair synthesis for gap-filling by DNA polymerase                                  | 2.45 e-5 |
| REACTOME_NUCLEOTIDE_EXCISION_REPAIR      | Genes involved in Nucleotide Excision Repair                                                         | 6.08 e-5 |
| DNA Binding                               |                                                                                                       |        |
| DNA_BINDING                               | Interacting selectively with DNA                                                                     | 1.58 e-6 |
| TRANSCRIPTION_FACTOR_ACTIVITY             | The function of binding to a specific DNA sequence in order to modulate transcription.                | 1.23 e-4 |
| TRANSCRIPTION_COFACTOR_ACTIVITY           | The function that links a sequence-specific transcription factor                                      | 3.95 e-4 |
| TRANSCRIPTION_FACTOR_BINDING              | Interacting selectively with a transcription factor                                                   | 1.83 e-3 |
| Protein Complex                           |                                                                                                       |        |
| REGULATION_OF_GENE_EXPRESSION            | Any process that modulates the frequency, rate or extent of gene expression.                          | 2.07 e-3 |
| PROTEIN_COMPLEX                           | Any protein group composed of two or more subunits, which may or may not be identical.               | 1.66 e-4 |
| HISTONE_DEACETYLASE_COMPLEX               | Complex that possesses histone deacetylase activity                                                  | 3.08 e-3 |
| TRANSCRIPTION_FACTOR_COMPLEX              | Any complex, distinct from RNA polymerase, including one or more polypeptides capable of binding DNA at promoters regulatory sequences, and regulating transcription. | 6.09 e-3 |
| PROTEIN_DNA_COMPLEX_ASSEMBLY             | The aggregation, arrangement and bonding together of proteins and DNA molecules to form a protein-DNA complex. | 1.11 e-1 |
Figure 1

A

1 month

WT

5.6% LSK

Foxo3a-/-

5.5% LSK

Fancd2-/-

4.5% LSK

dKO

10.2% LSK

5 months

WT

6.7% LSK

Foxo3a-/-

5.0% LSK

Fancd2-/-

3.4% LSK

dKO

5.7% LSK

B

% LSK in BMLDCs

WT

Foxo3a-/-

Fancd2-/-

dKO

1 month

5 month

p=0.003**

C

1 month

WT

3.3% CD150

Foxo3a-/-

3.2% CD150

Fancd2-/-

2.0% CD150

dKO

6.1% CD150

5 months

WT

6.4% CD150

Foxo3a-/-

3.6% CD150

Fancd2-/-

3.4% CD150

dKO

1.8% CD150

D

% SLAM in LSK cells

WT

Foxo3a-/-

Fancd2-/-

dKO

1 month

5 month

p=0.0006***

E

% negative recipients

Number of cells transplanted

WT: 1,23,114

Foxo3a-/-

Fancd2-/-

dKO: 1/107,151

F

Donor-derived cells

Weeks post-transplant

WT

Foxo3a-/-

Fancd2-/-

dKO

G

% Survival

Days post-treatment

WT

Foxo3a-/-

Fancd2-/-

dKO
Figure 3

A  

Up  

Down  

- 1228  665  1167  

- 461  464  1094  

- 1004  343  1138  

- 382  327  417  

- 847  

- dKO VS WT  

- dKO VS Foxo3a-/-  

- dKO vs Fancd2-/-  

B  

Up-regulated in dKO  

Down-regulated in dKO  

Functional Groups  

- Differentiation  

- Cell cycle  

- Stem cells  

- Oxidative stress  

- DNA binding  

- DNA repair  

- Development  

- Other  

C  

WT  Foxo3a-/-  Fancd2-/-  dKO  

D  

ES = -0.58; P<0.01  

ES = -0.43; P<0.01  

ES = -0.27; P<0.01  

ES = 0.54; P<0.01
Figure 5

A  Human 541  FSKQNEASSHIQDDMLVIRKQLSSTVF  
Mouse 539  FSQQPGTSNHIQDDMLVIRKQLSSTVF.  
**  *  **  *  **  **  *  **  **  **

Mutant 539  FSQQPGTSNHIQDDMLVIRKQLSSTVF.

K559R

B  

|      | Mock | WT | WT | Mutant | Mutant |
|------|------|----|----|--------|--------|
| HU   | -    | -  | +  | -      | +      |
| Flag |      |    |    |        |        |
| Topo1|      |    |    |        |        |


C  

Pre-sort

Post-sort

D  

E  

F  

G  

$p=0.0007^{***}$  

$p=0.003^{***}$  

$p=0.0001^{****}$
Fancd2 is required for nuclear retention of FOXO3a in hematopoietic stem cell maintenance
Xiaoli Li, Jie Li, Andrew Wilson, Jared Sipple, Jonathan Schick and Qishen Pang

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