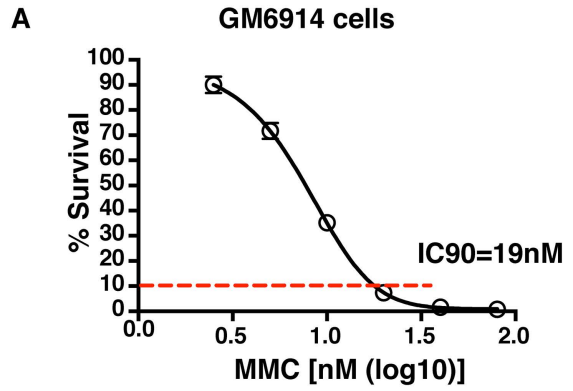
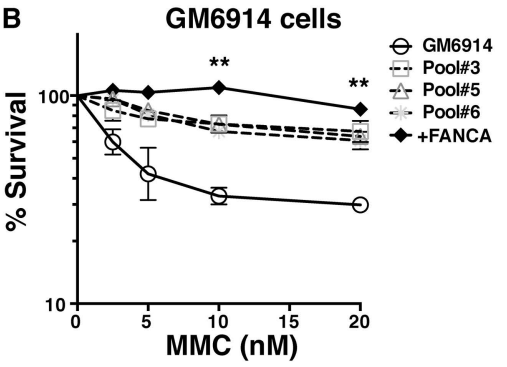
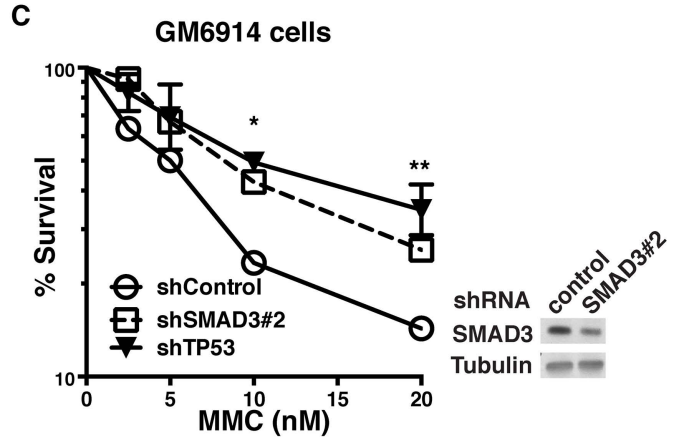
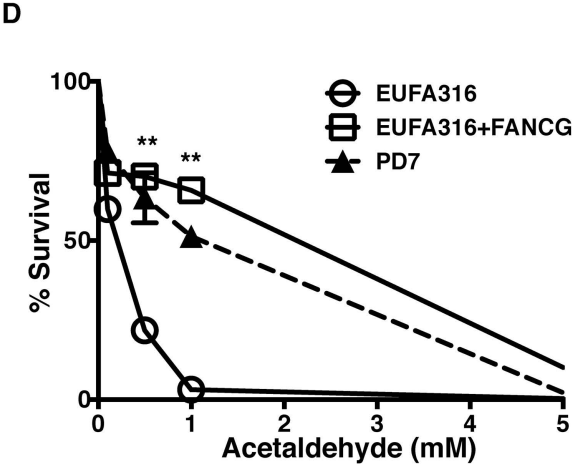
Figure S1


A
GM6914 cells
% Survival
100
90
80
70
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
IC90=19nM
MMC [nM (log10)]


B
GM6914 cells
% Survival
100
10
0
5
10
15
20
**
**
MMC (nM)
GM6914
Pool#3
Pool#5
Pool#6
+FANCA


C
GM6914 cells
% Survival
100
10
0
5
10
15
20
*
**
MMC (nM)
shControl
shSMAD3#2
shTP53
shRNA
control
SMAD3#2
SMAD3
Tubulin


D
% Survival
100
50
0
0
1
2
3
4
5
**
**
Acetaldehyde (mM)
EUFA316
EUFA316+FANCG
PD7

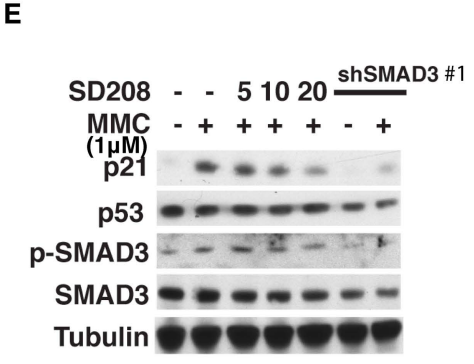

E
SD208
MMC
(1μM)
shSMAD3 #1
p21
p53
p-SMAD3
SMAD3
Tubulin

Figure S1. Genome-wide shRNA screening for MMC resistance in FA cells. Related to Figure 1.
(**A**) Identification of IC90 concentration of MMC in GM6914 (FA-A) fibroblast cells. Cells were treated with different doses of MMC for 7 days, and cell viability was measured using CellTiterGlo reagent. The IC90 of MMC was 19nM for GM6914 cells. (**B**) Reassessment of MMC sensitivity of cells after virus infection and MMC treatment. GM6914 (FA-A) cells were transduced with 6 pools of shRNA retrovirus libraries, and selected with puromycin for 48h. Cells were then treated with MMC for 7 days, and resistant cells were harvested to re-assess their MMC sensitivity by treating with MMC for 3 days. Data shown represent cells transduced by pool#3, 5 and 6. MMC sensitivity of FANCA-corrected cells is also shown. (**C**) MMC sensitivity of GM6914 (FA-A) cells was evaluated after SMAD3 and TP53 knockdown with shSMAD3#2 and shTP53. Survival was measured after 3 days exposure to indicated concentrations of MMC. Data shown are representative of two independent experiments. Immunoblot in the right panel shows the knockdown efficiency of shRNA#2 targeting SMAD3 in GM6914 cells. (**D**) Acetaldehyde sensitivity of EUFA316 (FA-G) cells. Survival was measured after 3 days exposure to indicated concentrations of acetaldehyde. FANCG-corrected EUFA316 and PD7 wild type cells were positive controls. Data shown are representative of two independent experiments. (**E**) Expression of p21 and p53 proteins in GM6914 (FA-A) cells with disruption of the TGF-β pathway by shSMAD3#1 or TGF-β inhibitor SD208.
Error bars represent mean ± s.e.m. (*, $p<0.05$; **, $p<0.01$).

Figure S2

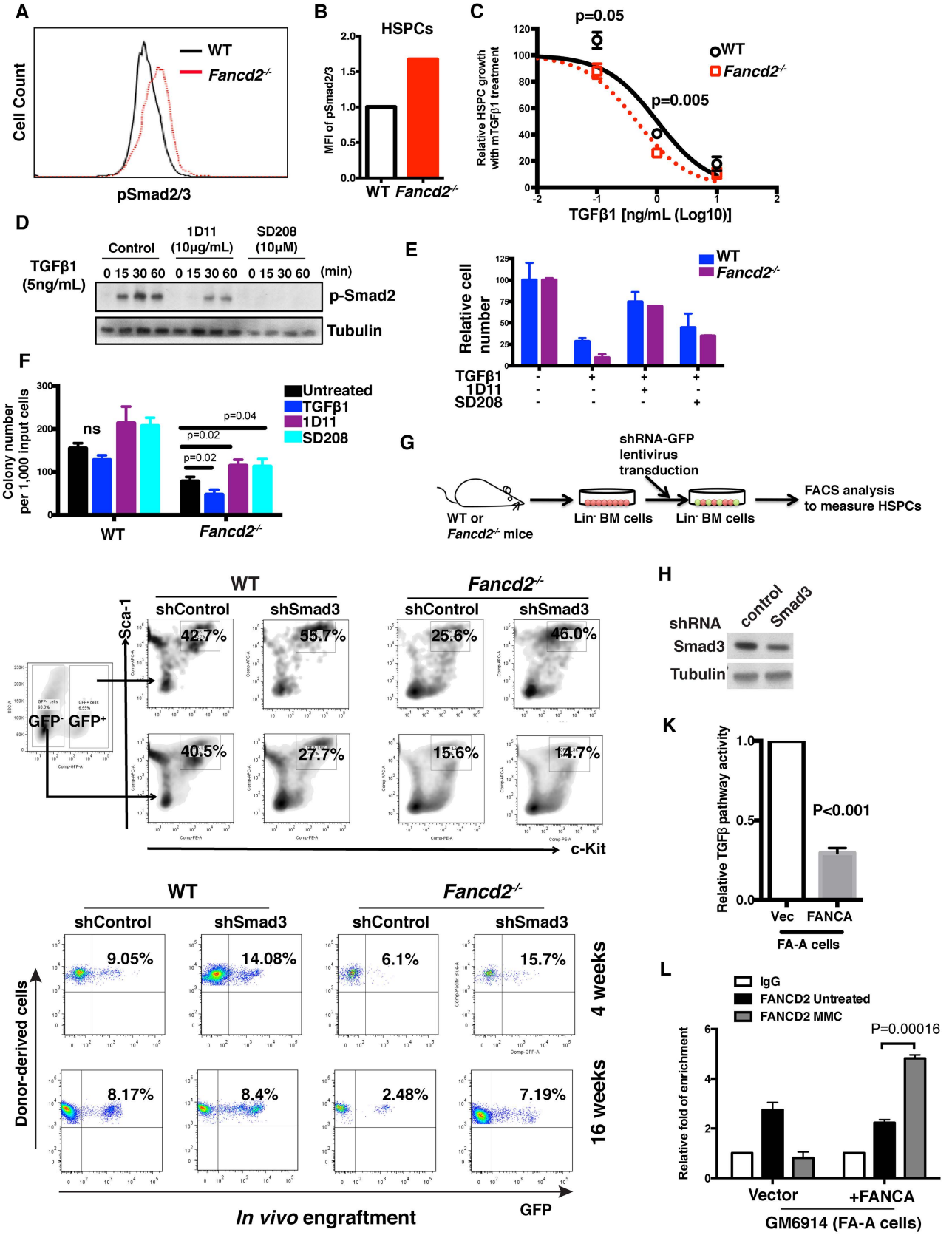

A
Cell Count
WT
Fancd2-/-
pSmad2/3
B
HSPCs
MFI of pSmad2/3
WT
Fancd2-/-
C
p=0.05
p=0.005
WT
Fancd2-/-
Relative HSPC growth with mTGFβ1 treatment
TGFβ1 [ng/mL (Log10)]
D
Control
1D11 (10μg/mL)
SD208 (10μM)
TGFβ1 (5ng/mL)
0 15 30 60 0 15 30 60 0 15 30 60 (min)
p-Smad2
Tubulin
E
WT
Fancd2-/-
Relative cell number
TGFβ1
1D11
SD208
F
Untreated
TGFβ1
1D11
SD208
Colony number per 1,000 input cells
ns
p=0.04
p=0.02
p=0.02
WT
Fancd2-/-
G
shRNA-GFP lentivirus transduction
WT or Fancd2-/- mice
Lin- BM cells
Lin- BM cells
FACS analysis to measure HSPCs
I
WT
Fancd2-/-
shControl
shSmad3
shControl
shSmad3
Sca-1
GFP-
GFP+
42.7%
55.7%
25.6%
46.0%
40.5%
27.7%
15.6%
14.7%
c-Kit
H
shRNA
control
Smad3
Smad3
Tubulin
K
Relative TGFβ pathway activity
P<0.001
Vec
FANCA
FA-A cells
J
WT
Fancd2-/-
shControl
shSmad3
shControl
shSmad3
9.05%
14.08%
6.1%
15.7%
8.17%
8.4%
2.48%
7.19%
4 weeks
16 weeks
Donor-derived cells
In vivo engraftment
GFP
L
IgG
FANCD2 Untreated
FANCD2 MMC
P=0.00016
Relative fold of enrichment
Vector
+FANCA
GM6914 (FA-A cells)

Figure S2. HSPCs from *Fancd2*$^{-/-}$ mice display hyperactive TGFβ activity and inhibition of TGFβ signaling promotes their proliferation. Related to Figure 2.

(**A**) Expression of phospho-Smad2/3 proteins in HSPCs from WT and *Fancd2*$^{-/-}$ mice as detected by flow cytometric analysis. (**B**) Quantification of mean fluorescence intensity (MFI) of phospho-Smad2/3 staining by flow cytometric analysis shown in (A) in HSPCs from WT and *Fancd2*$^{-/-}$ mice. (**C**) *Fancd2*$^{-/-}$ HSPCs are more sensitive to TGF-β1 than WT HSPCs. Sorted Lin^{-} cells from WT or Fancd2$^{-/-}$ mice were exposed to TGF-β1.After 5 days in culture, LSK cells were analyzed by flow cytometry and quantified. Data are shown after normalizing to untreated WT group. (**D**) Representative immunoblots of the lysates from mouse fibroblast cells showing reduced phosphorylation of Smad2 by treatment with TGF-β inhibitors (1D11 and SD208). (**E**) 1D11 and SD208 rescues inhibitory effect of TGF-β1 on bone marrow Lin^{-} cell growth. Sorted Lin^{-} cells from WT and *Fancd2*$^{-/-}$ mice were exposed to TGF-β1 (1ng/mL) with or without 1D11 (10μg/mL) or SD208 (10μM), and viable cells were counted after 5 days in culture. Data shown are after normalizing to untreated WT group. (**F**) Clonogenic assay of WT and *Fancd2*$^{-/-}$ HSPCs treated with TGF-β1, 1D11 or SD208. Equal number of sorted LSK cells from WT and *Fancd2*$^{-/-}$ mice were cultured in methycellulose medium containing TGF-β1 (1ng/mL), 1D11 (10μg/mL), or SD208 (10μM) in triplicate. Hematopoietic colonies were counted after 7-10 days in culture. (**G**) Schematic of the experimental design for lentivirus shRNA transduction of Lin^{-} bone marrow (BM) cells from WT and *Fancd2*$^{-/-}$ mice. (**H**) Representative immunoblots of the lysates from murine 3T3 cells showing knockdown efficiency of shRNA targeting mouse *Smad3*. (**I**) Representative FACS plots of LSK cells analyzed by flow cytometry for GFP expression after 5 days *in vitro* culture in stem cell culture medium. GFP^{+} or GFP^{-} cells gated for LSK population are shown. (**J**) Representative FACS plots showing percentage of transduced donor-derived cells in the peripheral blood samples of recipient mice at 4 and 16 weeks after bone marrow transplantation. The average percentages in each group were shown. (**K**) GM6914 (FA-A) cells exhibit higher TGFβ activity, compared to the FANCA corrected cells. GM6914 cells were transfected with TGFβ1-responsive luciferase promoter containing plasmid along with FANCA plasmid or empty vector and after 48 hrs. Luciferase activity was measured. (**L**) Binding of FANCD2 to SMAD1 promoter (region -2108 to -1950 bp) upon DNA damage as detected by ChIP assay using anti-FANCD2 antibody in corrected GM6914 (+FANCA) but not GM6914 (+Vector) fibroblast cells. Cells were exposed to MMC (1μM) for 8 hrs before using them in ChIP assays with an anti-FANCD2 antibody or IgG control antibody, followed by real-time PCR. ChIP data are represented as enrichment fold of FANCD2 binding to the region after normalization with IgG. Error bars represent mean ± s.e.m.

Figure S3

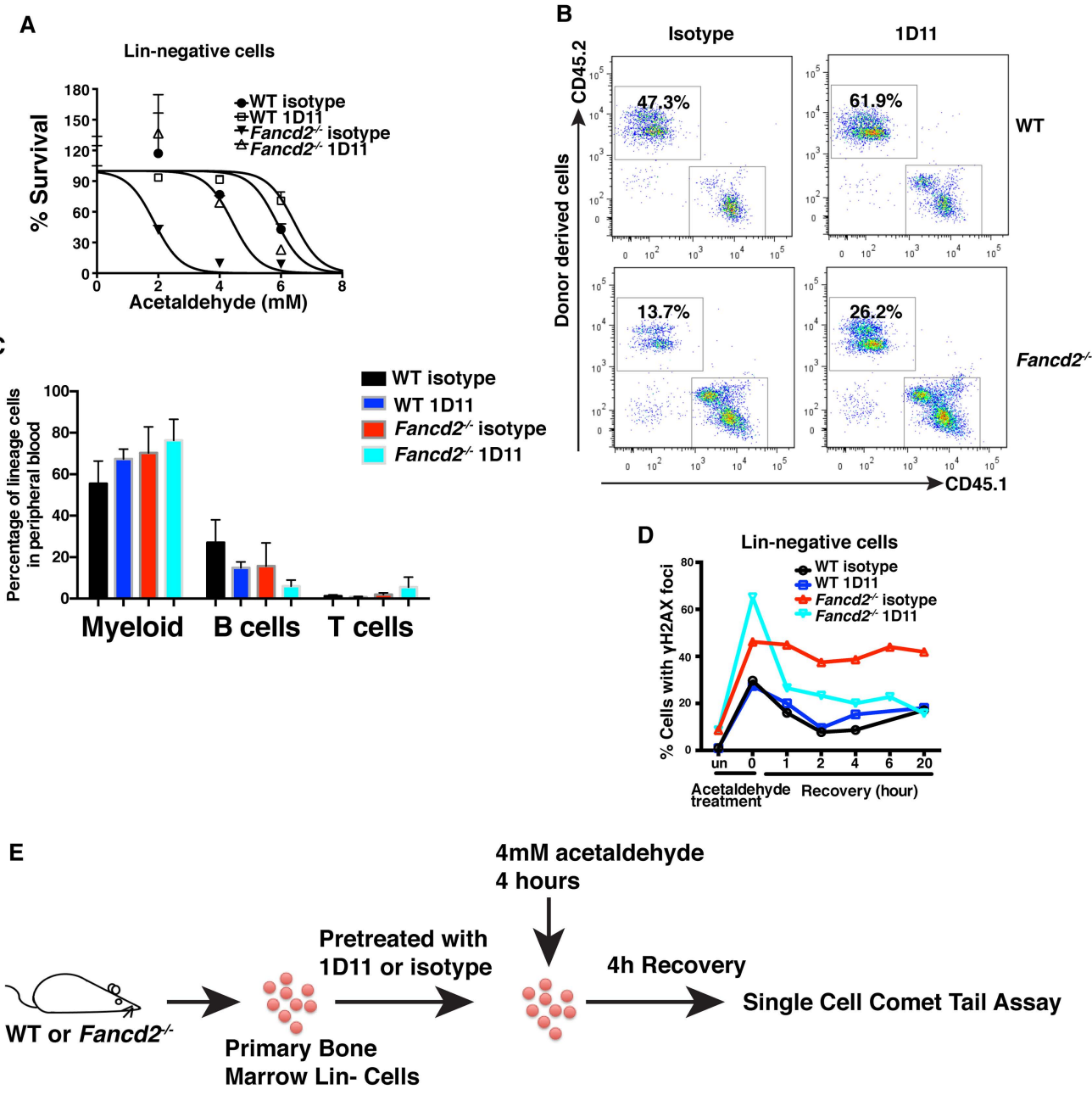

A
Lin-negative cells
% Survival
Acetaldehyde (mM)
WT isotype
WT 1D11
Fancd2-/- isotype
Fancd2-/- 1D11
B
Isotype
1D11
CD45.2
CD45.1
Donor derived cells
47.3%
61.9%
13.7%
26.2%
WT
Fancd2-/-
C
Percentage of lineage cells in peripheral blood
Myeloid
B cells
T cells
WT isotype
WT 1D11
Fancd2-/- isotype
Fancd2-/- 1D11
D
Lin-negative cells
% Cells with γH2AX foci
Acetaldehyde treatment
Recovery (hour)
E
4mM acetaldehyde
4 hours
Pretreated with
1D11 or isotype
4h Recovery
Single Cell Comet Tail Assay
WT or Fancd2-/-
Primary Bone
Marrow Lin- Cells

Figure S3. Inhibition of TGF-β Pathway Rescues Acetaldehyde-induced Genotoxicity in HSPCs from FA Mice. Related to Figure 3.

(**A**) Acetaldehyde sensitivity of Lin^- cells from bone marrow of WT or *Fancd2*$^{-/-}$ mice incubated with isotype control or 1D11 antibody (10μg/mL). Cells were exposed to 2-8 mM acetaldehyde for 4 hours and survival was determined by counting live cell numbers after 4 days in culture. (**B**) Representative FACS plots of the peripheral blood samples after staining with antibodies against CD45.1 or CD45.2 are shown. Lin^- cells from WT or *Fancd2*$^{-/-}$ murine bone marrow were exposed to acetaldehyde for 3 hrs and then cultured with isotype or 1D11 antibody before transplantation into lethally irradiated recipients. The peripheral blood from recipients was analyzed for donor cell engraftment at 4 weeks after transplantation by staining with CD45.1 and CD45.2 antibodies. The percentage of donor-derived $CD45.2^+$ cells or recipient-derived $CD45.1^+$ cells in peripheral blood of recipients are shown in FACS plots. The average percentage of $CD45.2^+$ cells are shown in the plot. (**C**) Lineage distribution of donor-derived cells (CD45.2) in peripheral blood of recipients as detected after staining the blood samples with antibodies against lineage markers in the transplantation experiments described in (B). (n=5 mice per group). (**D**) DNA repair kinetics in WT and *Fancd2*$^{-/-}$ Lin^- immature cells as shown by the percentage of cells with γH2AX foci. Lin^- cells were exposed to 4mM acetaldehyde for 4 hrs and allowed to recover in presence of isotype or 1D11 antibody. The cells were analyzed for γH2AX foci and 30-100 cells were counted in each time point. (**E**) Schematic of acetaldehyde and ID11/isotype antibody treatments *in vitro*. Lineage-negative cells from bone marrow of WT and *Fancd2*$^{-/-}$ mice were pretreated with 1D11 or isotype for 30 min followed by 4h treatment with 4mM acetaldehyde. After washing out, the cells were allowed to recover for 4h in presence of 1D11 or isotype antibody. DNA damage was assessed by a single cell comet tail assay.

Error bars represent mean ± s.e.m.

Figure S4

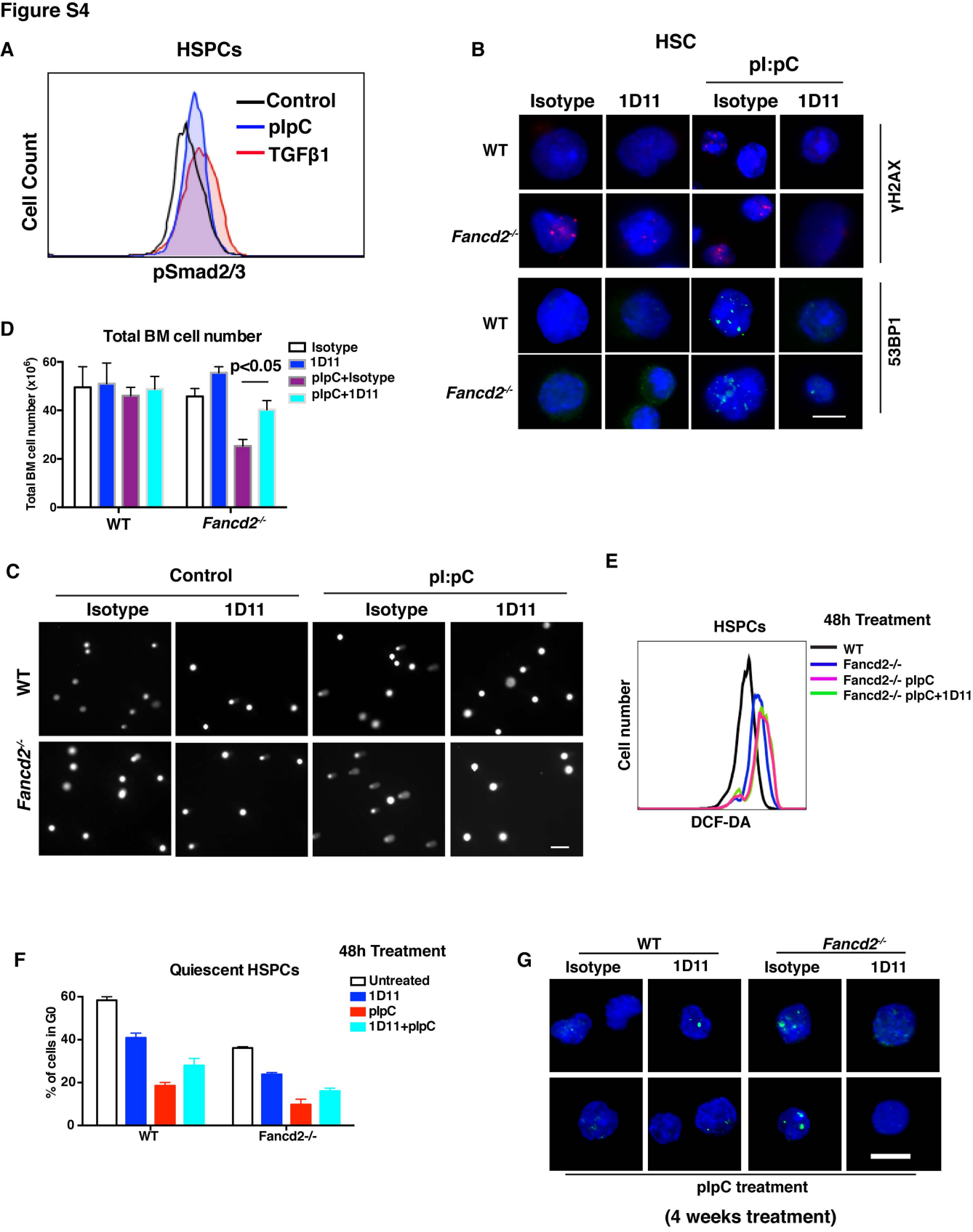

A
HSPCs
Control
pIpC
TGFβ1
Cell Count
pSmad2/3
B
HSC
pI:pC
Isotype
1D11
Isotype
1D11
WT
Fancd2-/-
γH2AX
WT
Fancd2-/-
53BP1
D
Total BM cell number
Isotype
1D11
pIpC+Isotype
pIpC+1D11
p<0.05
Total BM cell number (x10^6)
60
40
20
0
WT
Fancd2-/-
C
Control
pI:pC
Isotype
1D11
Isotype
1D11
WT
Fancd2-/-
E
HSPCs
48h Treatment
WT
Fancd2-/-
Fancd2-/- pIpC
Fancd2-/- pIpC+1D11
Cell number
DCF-DA
F
Quiescent HSPCs
48h Treatment
Untreated
1D11
pIpC
1D11+pIpC
% of cells in G0
60
40
20
0
WT
Fancd2-/-
G
WT
Fancd2-/-
Isotype
1D11
Isotype
1D11
pIpC treatment
(4 weeks treatment)

Figure S4. Inhibition of TGF-β Pathway Promotes DNA Repair in HSPCs from pI:pC-treated FA Mice. Related to Figure 4.

(A) Physiological stress induced by pI:pC activates TGF-β pathway in HSPCs. Phospho-Smad2/3 levels in HSPCs of pI:pC- or TGFβ1-treated mice were analyzed by flow cytometry. **(B)** Representative immunofluorescence staining showing γH2AX and 53BP1 foci in HSCs from WT and *Fancd2*$^{-/-}$ mice treated with pI:pC (5mg/kg) plus 1D11 or isotype antibody (10mg/kg) for 48h. (Scale bar: 20μm). **(C)** Representative images of alkaline comets of HSCs from WT and *Fancd2*$^{-/-}$ mice are shown. (Scale bar: 50μm). **(D)** Total BM cell numbers in WT and *Fancd2*$^{-/-}$ mice treated with pI:pC plus 1D11 or isotype antibody. **(E)** DCF-DA staining showing that inhibition of TGF-β pathway does not reduce pI:pC-induced reactive oxygen species (ROS) levels in *Fancd2*$^{-/-}$ HSPCs. Mice were treated with pI:pC plus 1D11 or isotype antibody and after 48 hrs, HSPCs were analyzed. **(F)** Inhibition of TGF-β pathway partially prevents pI:pC-induced exit from quiescence of HSCs. (n=3 mice per group). **(G)** Representative immunofluorescence staining showing γH2AX foci in HSPCs from WT and *Fancd2*$^{-/-}$ mice treated with pI:pC plus 1D11 for four weeks. (Scale bar: 20μm).

Error bars represent s.e.m.

Figure S5

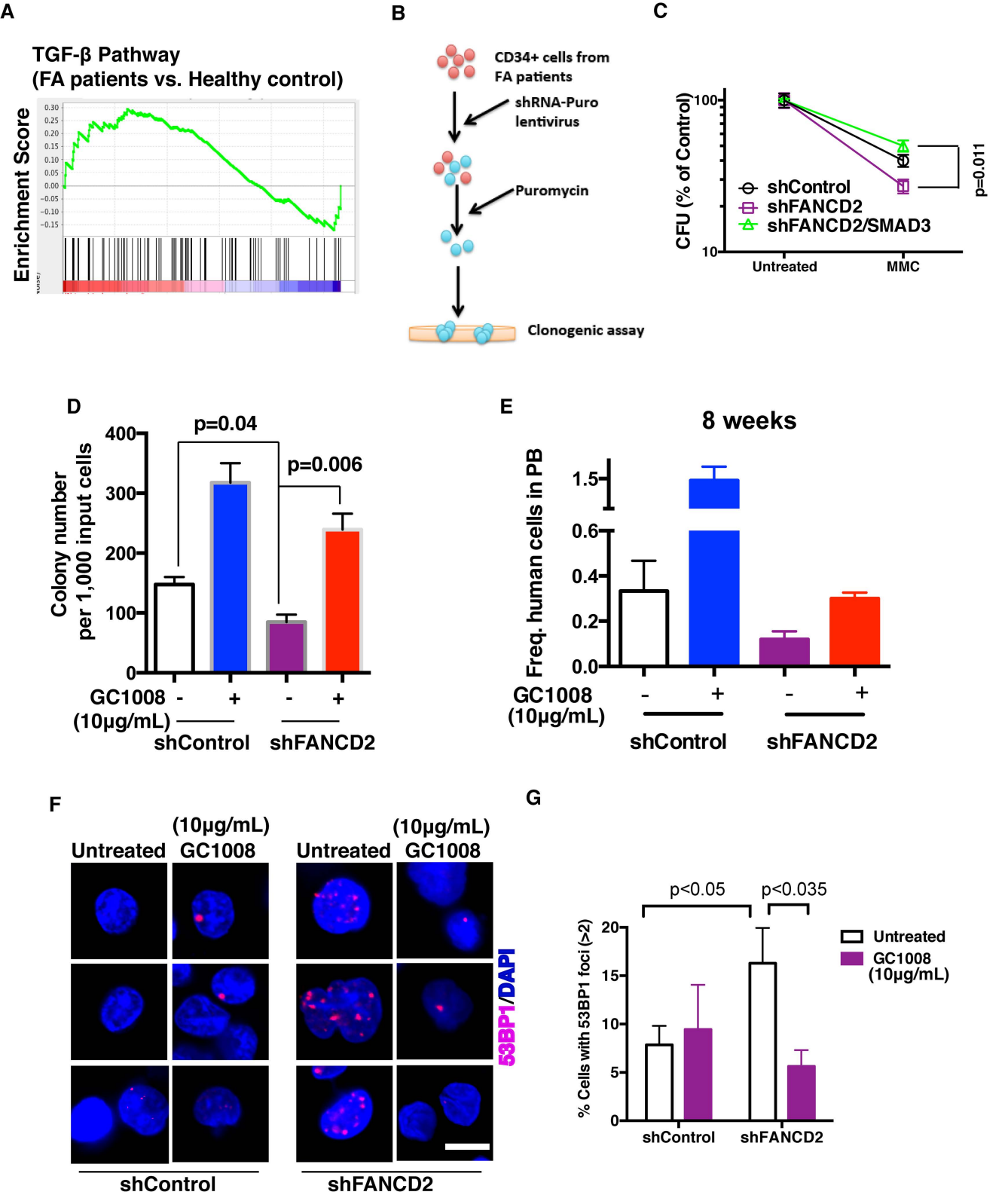

A
TGF-β Pathway
(FA patients vs. Healthy control)
Enrichment Score
B
CD34+ cells from
FA patients
shRNA-Puro
lentivirus
Puromycin
Clonogenic assay
C
CFU (% of Control)
100
10
shControl
shFANCD2
shFANCD2/SMAD3
p=0.011
Untreated
MMC
D
p=0.04
p=0.006
Colony number
per 1,000 input cells
400
300
200
100
0
GC1008
(10μg/mL)
-
+
-
+
shControl
shFANCD2
E
8 weeks
Freq. human cells in PB
1.5
0.6
0.4
0.2
0.0
GC1008
(10μg/mL)
-
+
-
+
shControl
shFANCD2
F
(10μg/mL)
Untreated
GC1008
Untreated
(10μg/mL)
GC1008
53BP1/DAPI
shControl
shFANCD2
G
p<0.05
p<0.035
% Cells with 53BP1 foci (>2)
20
15
10
5
0
Untreated
GC1008
(10μg/mL)
shControl
shFANCD2

Figure S5. **Inhibition of TGF-β Pathway Rescues Impaired Function of HSPCs from Patients with FA. Related to Figure 5.**

(**A**) Gene set enrichment analysis (GSEA) displays the expression profiling of TGF-β pathway genes in bone marrow cells from FA patients and healthy control. (Dataset: GSE16334). (**B**) Schematic of the clonogenic assay of primary $CD34^+$ cells from FA patients. (**C**) Clonogenic survival of FA-like $CD34^+$ cells exposed to MMC. Cells were cultured in methylcellulose medium post MMC treatment (2h at 100ng/mL) and hematopoietic colonies were counted after 10 days in culture. Colony numbers in each MMC treated group were normalized to untreated control of each group respectively. (**D**) Colony forming assay of FA-like $CD34^+$ cells. Human cord blood $CD34^+$ cells expressing control shRNA or shFANCD2 were treated with GC1008 (10μg/mL). for clonogenic assay in triplicates. Hematopoietic colonies were counted after 10-14 days in culture. **(E)** *In vivo* xenograft assay. Human cord blood $CD34^+$ cells were transduced with lentivirus encoding shFANCD2 or shControl. After selection with puromycin, cells were transplanted into sub-lethally irradiated NSG mice. Recipient mice were treated with GC1008 at 3 doses per week for 2 weeks. Human cells were analyzed in the peripheral blood by flow cytometry at 8 weeks post transplantation. Data shown are combined from two independent experiments (n= 4-5 recipient mice). (**F, G**) GC1008 treatment promotes DNA repair in FA-like HSPCs. Human cord blood CD34+ cells expressing control shRNA or shFANCD2 were treated with MMC (100 ng/mL) for 2h, and then allowed to recover for 24h. Representative images (**F**) and quantification (**G**) of 53BP1 foci are shown. (Scale bar: 20μm)

Error bars represent mean ± s.e.m

Figure S6

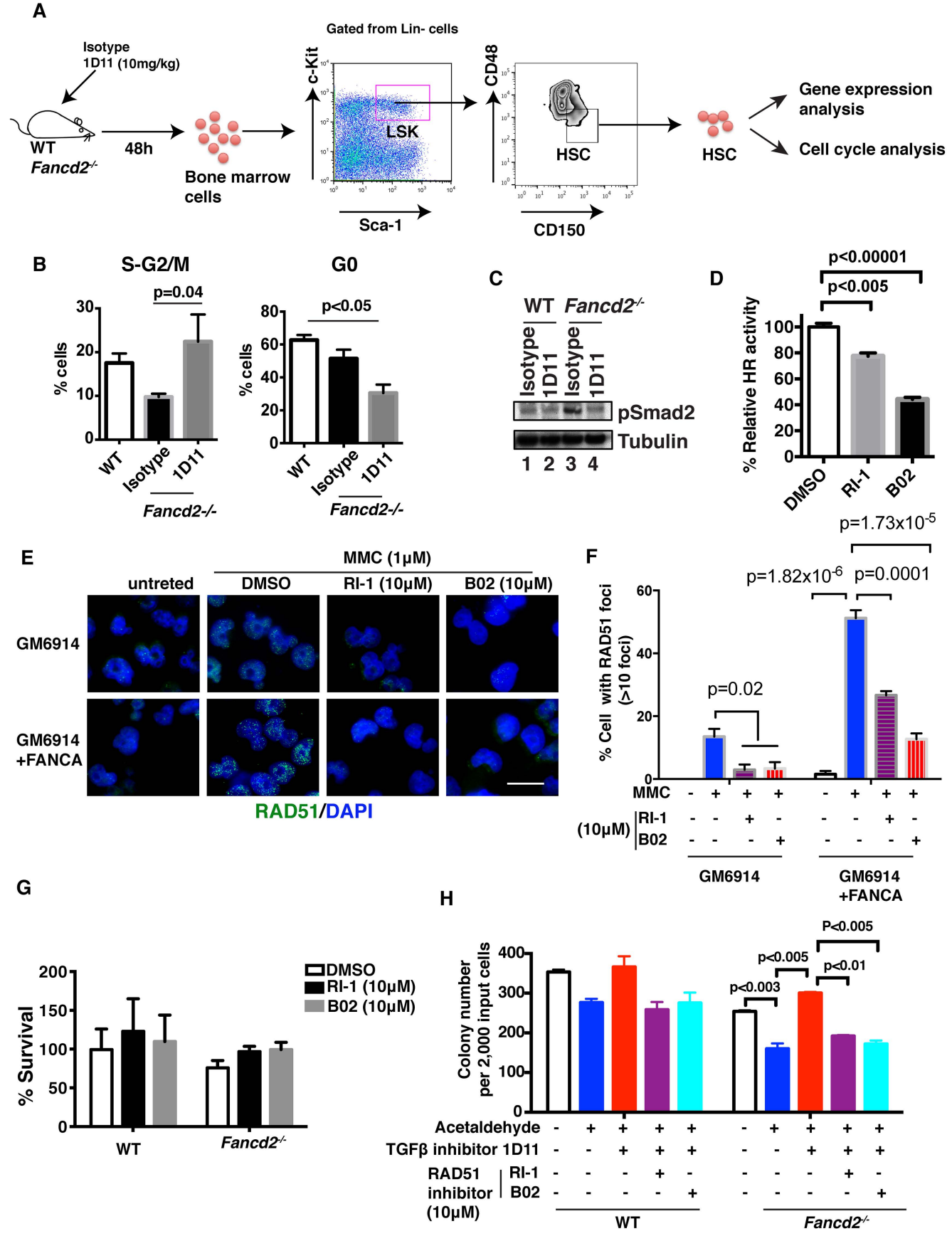

A
Isotype
1D11 (10mg/kg)
WT
Fancd2-/-
48h
Bone marrow cells
Gated from Lin- cells
c-Kit
LSK
Sca-1
CD48
HSC
CD150
HSC
Gene expression analysis
Cell cycle analysis
B
S-G2/M
G0
p=0.04
p<0.05
% cells
WT
Isotype
1D11
Fancd2-/-
C
WT
Fancd2-/-
Isotype
1D11
pSmad2
Tubulin
1 2 3 4
D
p<0.00001
p<0.005
% Relative HR activity
DMSO
RI-1
B02
E
MMC (1μM)
untreted
DMSO
RI-1 (10μM)
B02 (10μM)
GM6914
GM6914 +FANCA
RAD51/DAPI
F
p=1.73x10-5
p=1.82x10-6
p=0.0001
p=0.02
% Cell with RAD51 foci (>10 foci)
MMC
(10μM)
RI-1
B02
GM6914
GM6914 +FANCA
G
% Survival
DMSO
RI-1 (10μM)
B02 (10μM)
WT
Fancd2-/-
H
P<0.005
p<0.005
p<0.01
p<0.003
Colony number per 2,000 input cells
Acetaldehyde
TGFβ inhibitor 1D11
RAD51 inhibitor (10μM)
RI-1
B02
WT
Fancd2-/-

Figure S6. Inhibition of TGF-β Pathway in murine HSPCs Promotes DNA Repair Activity. Related to Figure 6.

(A) Experimental scheme for inhibition of TGF-β Pathway in mice. WT or *Fancd2*$^{-/-}$ mice were treated with isotype or 1D11 antibody (10mg/kg) for 48h, and bone marrow HSCs were sorted for gene expression profile and cell cycle analysis. (**B**) Increased number of *Fancd2*$^{-/-}$ HSCs in S-G2M phase of cell cycle after 1D11 treatment. Cell cycle analysis of HSCs from WT or *Fancd2*$^{-/-}$ mice treated with isotype or 1D11 antibody for 48h is shown. (n=4 mice per group). (**C**) Representative immunoblots showing 1D11 efficiently inhibits the level of p-Smad2 in hematopoietic progenitors from *Fancd2*$^{-/-}$ mice. (**D**) RAD51 inhibitors, RI-1 and B02, significantly decrease HR efficiency. Homologous recombination assay was measured in U2OS cells with DR-GFP reporter after treatment with 10μM RI-1 and 10μM B02. The representative of two independent experiments is presented. (**E, F**) RAD51 inhibitors, RI-1 and B02, efficiently block RAD51 foci formation. GM6914 (FA-A) cells or corrected GM6914+FANCA cells were treated with 1μM MMC and 10μM inhibitors for 6h before immunofluorescence analysis. Representative images (**E**) and quantification (**F**) of RAD51 foci are shown. (Scale bar: 50μm). (**G**) RAD51 inhibitors do not show cytotoxicity in HSPCs. HSPCs from WT or *Fancd2*$^{-/-}$ mice were treated with B02 (10μM) or RI-1 (10μM) *in vitro* for five days and survival was determined. **(H)** Colony forming assay showed that RAD51 inhibitors block the protective function of 1D11 after genotoxic stress in *Fancd2*$^{-/-}$ HSPCs. HSPCs from WT or *Fancd2*$^{-/-}$ mice were exposed to 1D11 (10μg/mL) and RAD51 inhibitors (10μM) for 30 min followed by exposure to acetaldehyde for 4 hrs. The cells were then washed and cultured in presence of 1D11 and RAD51 inhibitors for 7-9 days, and hematopoietic colonies were counted.

Error bars represent mean ± s.e.m.

Figure S7

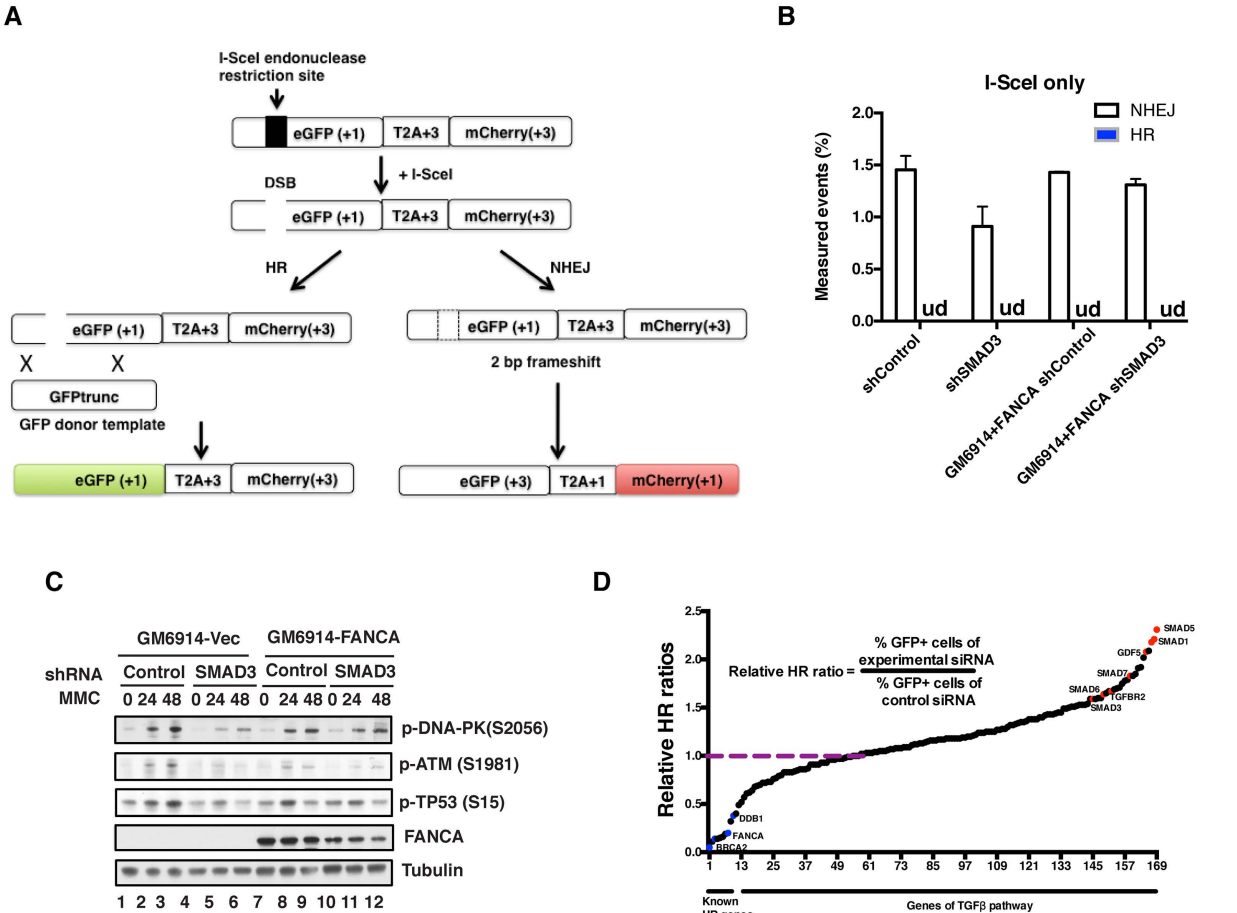

A
I-SceI endonuclease restriction site
eGFP (+1)
T2A+3
mCherry(+3)
DSB
+ I-SceI
HR
NHEJ
X
X
GFPtrunc
GFP donor template
2 bp frameshift
eGFP (+3)
T2A+1
mCherry(+1)
B
I-SceI only
NHEJ
HR
Measured events (%)
2.0
1.5
1.0
0.5
0.0
ud
shControl
shSMAD3
GM6914+FANCA shControl
GM6914+FANCA shSMAD3
C
GM6914-Vec
GM6914-FANCA
shRNA
Control
SMAD3
MMC
0 24 48
p-DNA-PK(S2056)
p-ATM (S1981)
p-TP53 (S15)
FANCA
Tubulin
1 2 3 4 5 6 7 8 9 10 11 12
D
Relative HR ratios
Relative HR ratio =
% GFP+ cells of experimental siRNA
% GFP+ cells of control siRNA
SMAD5
SMAD1
GDF5
SMAD7
SMAD6
TGFBR2
SMAD3
DDB1
FANCA
BRCA2
1 13 25 37 49 61 73 85 97 109 121 133 145 157 169
Known HR genes
Genes of TGFβ pathway

Figure S7. Inhibition of TGF-β Pathway Increases HR Activity and Decreases NHEJ Activity in FA Cells. Related to Figure 7.

(A) Schematic of traffic light reporter assay. Endonuclease I-SceI induces double strand break (DSB) in the restriction site. If the DSB is repaired by HR using truncated GFP template, the full eGFP gets reconstituted and cells are GFP-positive; if the DSB is repaired by NHEJ, 2bp frameshift leads to *T2A* and *mCherry* sequences in frame, and cells are mCherry-positive. (**B**) HR and NHEJ repair analyzed by traffic light reporter (TLR) system. GM6914 (FA-A cells) or FANCA corrected GM6914 cells with shControl or shSMAD3 were infected with lentivirus encoding TLR-BFP reporter and were then infected with and I-SceI only encoding lentivirus to generate DNA breakpoints**.** HR and NHEJ repair events (GFP or mCherry positive cells) were quantified by flow cytometry. (**C**) Immunoblots with the indicated antibodies of the lysates from GM6914 (FA-A) cells or FANCA-corrected GM6914 cells with shRNA-mediated knockdown of SMAD3. Cells were exposed to 1μM MMC for 8h and allowed to recover for 24 and 48 hours. (**D**) Analysis of siRNA screening data showing that siRNA mediated knockdown of the majority of the TGF-β pathway genes enhances HR efficiency. [siRNA screening database was used from Adamson et al., A genome-wide homologous recombination screen identifies the RNA-binding protein RBMX as a component of the DNA-damage response. *Nature Cell Biology*, 14: 318-328 (2012)].

Error bars represent mean ± s.e.m.

Supplemental Table

Table S1 Primer Sets Used in This Study, Related to Figure 5 and 6.

Supplemental Experimental Procedures

Genome-Wide shRNA Screen and Analysis of the Data

The library consists of six viral pools each containing approximately 13,000 different MSCV-PM retroviral shRNA particles targeting human genes. For each pool, three replicates of at least 2.0×10^7 GM6914 cells were incubated with an equivalent number of retroviral colony-forming units in media containing polybrene (8μg/ml) (Sigma-Aldrich, St. Louis, MO), for a 1000-fold representation of each shRNA sequence at a multiplicity-of-infection (MOI) of ~0.7. Cells were exposed to MMC (19 nM) for 7 days after puromycin (2μg/mL) selection, and surviving cells were washed and cultured for 2 additional weeks. Genomic DNA was extracted, and shRNA hairpins were amplified by PCR and identified by sequencing on illumine HiSeq2000. Briefly, an equal amount of input genomic DNA for each sample was used. For each sample, we performed 8 separate 100 μL reactions, and then combined the resulting amplicons.

Primer sequences to recover half-hairpin shRNA in the first PCR reaction were:
JH353F 5'– TAGTGAAGCCACAGATGTA –3'
HHR2L 5'– ATGTATCAAAGAGATAGCAAGGTATTCAG –3'
Two more PCR reactions were performed to attach Illumina adaptors and barcodes. Primer sequences were as follows:
IndexSeqPrimer(ISP)-shRNAloop:
5'– GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtagtgaagccacagatgta –3'
P7-IndexingPrimer:
5'-CAAGCAGAAGACGGCATACGAGAT[xxxxxxx]GTGACTGGAGTTCAGAC
GTGT-3'
P5-HHR2L: 5'– AATGATACGGCGACCACCGAatgtatcaaagagatagcaaggtattcag –3'
Resulting amplicons were gel extracted, quantified, mixed and identified by sequencing using Illumina HiSeq2000.

To align the sequences with the reference shRNA sequence library, bowtie was used with the following parameters:
bowtie -p 2 --best --nomaqround --norc --trim3 28 -k 1 -n 0 -v 2 -a <path to the base-name of the reference files> $i <output filename>
To collate and count the hairpins, grep and awk was used. To normalize the values, the R package DESeq was used to perform TMM normalization. To compare the abundances of shRNA integrants pre and post MMC treatment, the log2 fold change of the mean of the replicates was determined for each hairpin. To rank genes, RIGER was used using the pre-scored option.

Hematopoietic Stem/progenitor Cell Culture and Flow Cytometry Analysis

The procedures of enrichment and cell staining for isolating HSPCs ($Lin^-cKit^+Sca\text{-}1^+$) and HSCs ($Lin^-cKit^+Sca\text{-}1^+CD48^-CD150^+$) from mice bone marrow were performed as described (Zhang et al., 2012). Briefly, bone marrow cells were incubated with biotin-labeled lineage antibody cocktail containing a mixture of antibodies against CD3, CD11b, CD19, B220, Gr-1 and Ter119, and Lin^- immature cells were first enriched using mouse hematopoietic progenitor cell isolation kit (StemCell Technology, 19856). After washing, cells were stained with the fluorochrome-labeled secondary antibody (APC-eFluor 780-conjugated Streptavidin, eBioscience, 47-4317-82) for recognizing biotin and PE-c-Kit (eBioscience, 12-1172-83), APC-Sca-1 (eBioscience, 17-5981-83), PE-Cy7-CD48 (eBioscience, 25-0481-80), FITC-CD150 (eBioscience, 11-1501-82) antibodies. Cell cycle analysis of HSCs was performed by staining cells with antibodies in combination with Hoechst 33342, followed by flow cytometric analysis. For Ki67 staining, cells were fixed and permeabilized before staining with PerCP-Cy5.5-conjugated anti-Ki67 antibody (eBioscience, 46-5698-82). For phospho-Smad2/3 staining, sorted HSPCs were fixed with 1.5% paraformaldehyde for 10 min at room temperature, and permealized with ice-cold methanol for 30 min at 4°C cells. Cells were stained with phospho-Smad2/3 antibody [PE-CF594 mouse anti-Smad2 (pS465/pS467)/Smad3 (pS423/pS425), BD Bioscience, 562697] for 60 min at room temperature. ROS levels were measured by staining with 10μM DCF-DA (Abcam, 113851) for 30 min at 37°C. Cells were analyzed using BD Fortessa X-20 instrument,

or sorted with BD FACSAria II SORP at DFCI Flow Cytometry Core Facility, and data were analyzed by Flowjo software.

For mouse hematopoietic stem/progenitor cell culture, Lin^- cells were cultured *in vitro* in StemSpan SFEM media (Stem Cell Technologies) with 10ng/mL SCF, 20ng/mL IGF-2, 20ng/mL TPO, 10ng/mL heparin, and 10ng/mL α-FGF. For human cord blood cells, $CD34^+$ cells were isolated using CD34 microbead kit (Miltenyi Biotec). Human $CD34^+$ cells were cultured in StemSpan SFEM media with 100ng/mL hSCF, 100ng/mL FLT3 ligand, 10ng/mL TPO, 10ng/mL IL-6. Cells were treated with 10μg/mL 1D11, 10μg/mL GC1008 or 10 μM SD208.

***In Vitro* Colony-forming Assay**

Mouse cells were cultured in methycellulose medium (Methocult GF M3434; Stem Cell Technologies). Human FA $CD34^+$ cells or FA-like cells (shFANCD2 transduced human cord blood $CD34^+$ cells) were transduced with lentivirus encoding shSMAD3. After selection by puromycin or sorting by FACS, 500-1000 cells were cultured in methylcellulose medium (Methocult GF H4435; Stem Cell Technologies). Colonies were counted under microscope after 10-14 days.

Cell Culture and Western Blotting

Human 293T, U2OS, and FA cells including GM6914, and PD20, and GM6914 reconstituted with FANCA (GM6914+FANCA cells), were cultured in Dulbecco's Modified Eagle's Medium (Life Technologies) supplemented with 10% fetal calf serum (FBS). EUFA316, human FA lymphoblast cells and EUFA316 reconstituted with FANCG (EUFA319+FANCG cells) were grown in RPMI1640 (Life Technologies) supplemented with 10% fetal calf serum. Whole cell extracts for western blotting were prepared by lysing cells in radioimmunoprecipitation assay buffer (50 mmol/L Tris, pH 7.3, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.5% Na-deoxycholate, and 0.1% SDS) with complete protease inhibitors, NaVO4, and NaF.

Antibodies and Reagents

The following antibodies were used for western blotting or immunofluorescence: anti-SMAD3 (Cell Signaling,), anti-p53 (Cell Signaling, 2524), anti-phospho-p53 (Ser15) (Abcam, ab1431), anti-p21 (Santa Cruz, sc-397), anti-RAD51 (Santa Cruz, sc-8449), anti-phospho-DNA-PK(Ser2056) (Abcam, ab18192), anti-phospho-ATM (Ser1981) (Cell Signaling, 5883), anti-phospho-H2AX (Millipore, 05-636), anti-53BP1 (Novus Biologicals, NB100-904), anti-Tubulin (Abcam, ab6160). SMAD3 inhibitor SIS3 was purchased from EMD Millipore. TGF-β inhibitor SD208 was purchased from Sigma. RAD51 inhibitor B02 was purchased from Sigma (SML0364), and RI-1 from Selleck (S8077). Recombinant human TGF-β1, SCF, TPO, IGF, and bFGF were from ProPeptide, and mouse TGF-β1 was purchased from R&D. Anti-mouse TGFβ antibody 1D11 and anti-human TGFβ antibody GC1008 were kindly provided by Genzyme, and were used for *in vitro* experiments at 10μg/mL and for *in vivo* experiments at 10mg/kg.

shRNA

Lentiviral shRNAs in vectors pLKO.1 were obtained from RNAi Core facility of Dana Farber Cancer Institute. Sequences of *SMAD3* and *TP53* shRNAs were as follows: sh*SMAD3#1*: 5'- CTGTGTGAGTTCGCCTTCAAT-3': sh*SMAD3*#2: 5'-CCCAGCACATAATAACTTGGA-3'. sh*TP53*: 5'- CGGCGCACAGAGGAAGAGAAT-3'. shRNA-*FANCD2: 5'*- CGACTCATTGTCAGTCAACTA-3'. sh*Smad3*: 5'-GCACACAATAACTTGGACCTA-3'.

Lentivirus Production and Transduction.

To produce lentivirus, HEK293T cells were seeded at ~50% confluence 24 hours before transfection. Transfection was performed using LTX and plus reagent (Life Technologies). Virus was harvested 48 hours post transfection, and filtered through a 0.45μm low protein binding membrane (Millipore). To transduce human FA cells an MOI of about 0.5 was used. To transduce bone marrow cells from WT and *Fancd2*$^{-/-}$ mice, Lin^- cells were isolated using EasySep^{TM} stem cell enrichment kit (Stem Cell Technologies) and transduced with lentivirus at MOI of 8-10.

TGF-β Pathway Activity Using Luciferase Reporter Assay

293T cells were transiently transfected with a TGF-β responsive luciferase promoter (CAGA-luc) plasmid (kindly provided by H.Y. Lin, Massachusetts General Hospital) along with FANCD2 or control vector. Cells were harvested at 48h after transfection and luciferase activity was determined using the Dual-Lucifease Reporter Assay system (Promega).

Traffic Light Reporter (TLR) Assay

Genome engineering experiments were performed as previously described (Certo et al., 2011). Briefly, single copy of TLR cell lines including *FANCA*$^{-/-}$ fibroblast cells (GM6914) and FANCA corrected GM6914 with or without shSMAD3 were generated by transducing cells with TLR-BFP reporter lentivirus, typically yielding ~5% transduction based on fluorescence. Two days after transduction, transduced cells (BFP^{+}) were sorted by FACS. To generate double strand break, cells were seeded at 2x10^{5} cells per well in 6-well plate 24h before transduction, and cells were transduced with lentivirus containing *I-SceI* alone or *I-SceI* plus GFP donor template. For SD208 treatment, cells were treated with 10μM SD208 after 3-4h post-transduction. All transductions were carried out in the presence of 8 μg/mL polybrene. Twenty-four hours after transduction, medium was changed. Genome engineering events were analyzed by flow cytometry at 72 hours after transduction. NHEJ is represented by mCherry fluorescence, and HR by GFP fluorescence.

Drug Sensitivity Assays

For survival assays, cells were seeded at a density of 1x10^{3} cells per well in 96-well plates. After 3-6 days of culture in indicated concentrations of MMC or post exposure of acetaldehyde, viability was assessed using CellTiterGlo reagent (Promega). In order to assess clonogenicity, cells were seeded at a low density (500-1000 cells per well) in 6-well plates and allowed to form colonies. The cells were then fixed in methanol/20% acetic acid and stained with 1% crystal violet. Colonies were counted after crystal violet staining.

Immunofluorescence

Cells were grown on coverslips for 24 hours before treating with MMC. Cells were fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature, washed three times with PBS, followed by extraction with 0.3% Triton X-100 for 10min on ice. The incubation with the primary antibody (anti-RAD51) was done at 37^{o}C, followed by incubation with secondary antibody [alexa Fluor 488 goat anti-rabbit IgG (A11034, Life Technologies)].

Immunofluorescence staining using sorted HSPCs and HSCs from WT and *Fancd2*$^{-/-}$ mice was performed as previously described (Flach et al., 2014). Briefly, cells were pipetted onto Poly-L-lysine precoated slides, and incubated for 10min at 37 ^{0} C. After fixing with 4% (w/v) paraformaldehyde for 10 min at room temperature, cells were treated with 0.15% Triton X-100 for 2 min at room temperature. Cells were incubated with primary antibody anti-γH2AX or anti-53BP1 for 2 hour at 37^{o}C, followed by incubation with secondary antibody [alexa Fluor 568 goat anti-mouse IgG (A11031, Life Technologies)].

Comet Tail Assay

Alkaline comet tail assay was performed with Trevigen Comet Assay kit according to the manufacturer's instructions. Briefly, cells were seeded onto Trevigen HT CometSlides after embedding in LMAgarose. The immobilized cells were lysed in lysis solution overnight at 4^{o}C, followed by incubation in freshly prepared alkaline unwinding solution for 1h at 4^{o}C. Electrophoresis was performed in alkaline condition. Cells were stained with SYBR Gold, and imaged. Data was analyzed using OpenComet.

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was performed as described previously (Park et al., 2013). Briefly, 10x10^{6} cells were treated with MMC (1μM) for 8 hours. Cells were chemically crosslinked with 1% formaldehyde for 15 min at room temperature. Cells were rinsed twice with 1x PBS and harvested in Farnham lysis buffer (5mM PIPES pH8.0, 85mM KCl, 0.5% NP-40, and protease inhibitor cocktail). After washing, cells were resuspended in sonication buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail), and sonicated with10×30 seconds pulses, 5 min in total, 18-21 Watts of power. After sonication, 5% samples were used as input. Sonicated samples were further divided in half, and incubated overnight with 100 μL of DynaI Protein G magnetic beads that had been preincubated with anti-FANCD2 (Novus Biologicals, NB100-316) or IgG control antibody. Beads were washed 5x with LiCl wash buffer (100mM Tris pH 7.5, 500mM LiCl, 1% NP-40, 1% sodium deoxycholate), and 1x TE buffer containing 50mM NaCl. Bound complexes were eluted from the beads by heating at 65^{o}C for 1 hour (vortexing every 5min). Crosslinking was reversed by incubating samples at 65^{o}C for overnight. DNA was purified and then analyzed by real-time PCR using SMAD1 promoter primers (primer #1: 5'-AAGGCAGGAGAATTGCTTGA-3', 5'-CCTTCACCTTCTGCCATGAT-3'; primer#2: 5'-CAAGGGAGGGTTTCAACAG-3', 5'-TGAGCACTTACTGGTCAATTCG-3').

Real-time RT-PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen, CA). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Real time PCR reactions were done using Vii A 7 PCR machine. 20μL reaction system was composed of 10μL SYBR Green, 2.5μL 10μM primer mixture, 10ng cDNA and nuclease-free water. All the experiments were performed in triplicates. β-actin was used as the internal control. The primer sequences are shown in Table S1.

Supplemental References:

Flach, J., Bakker, S.T., Mohrin, M., Conroy, P.C., Pietras, E.M., Reynaud, D., Alvarez, S., Diolaiti, M.E., Ugarte, F., Forsberg, E.C*., et al.* (2014). Replication stress is a potent driver of functional decline in ageing haematopoietic stem cells. Nature *512*, 198-202.