Stem Cell Reports, Volume *4*

Supplemental Information

Oxymetholone Therapy of Fanconi Anemia Suppresses Osteopontin Transcription and Induces Hematopoietic Stem Cell Cycling

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Supplemental Data

Supplementary figure legends:

Figure S1: OXM's bioactivity, related to Figure 2

A). Representative kidney pictures for OXM- or placebo-treated mice. The mice under OXM treatment had larger kidney sizes than their placebo-treated controls. B). OXM-treated mice showed significantly higher kidney weights but maintained similar body weights. Data are pooled results from multiple mice (n = 9 for each group). C). *Oat* gene expression levels in the kidneys of OXM-treated mice were 75% lower than those in placebo-treated gender-matched controls. Data are pooled results from multiple mice (n = 4 for each group).

Figure S2: OXM's effect on the proliferation of LIN^+ bone marrow cells, related to Figure 4

Representative cell cycle profiles on LIN^+ cells from OXM-treated mice and their gender-matched placebo-treated littermate controls. For comparison purposes, the profiles were from the same mice used for representative KSL cell cycle profiles shown in Figure 4A. The denoted percentage for each gate was from an individual experiment.

Figure S3: OXM's effect on the proliferation of $LIN^-c\text{-}KIT^+SCA\text{-}1^-$ progenitor cells, related to Figure 4

A). Statistical quantification of the cell cycle analysis on $LIN^-c\text{-}KIT^+SCA\text{-}1^-$ cells in OXM-treated versus placebo-treated mice. Data represent the mean values from multiple mice (n = 9 for either OXM or placebo group of *Fancd2*$^{-/-}$ mice, n = 14 for *Fancd2*$^{+/+}$

placebo group, and n = 10 for *Fancd2*$^{+/+}$ OXM group). B). Representative cell cycle profiles on LIN^{-}c-KIT^{+}SCA-1^{-} cells from OXM-treated mice and their gender-matched placebo-treated littermate controls. For comparison purposes, the profiles were from the same mice used for representative KSL cell cycle profiles shown in Figure 4A. The denoted percentage for each gate was from an individual experiment.

Figure S4: AR target site in intron 3 of *Spp1* gene, related to Figure 5

A). Sequence of AR target site identified in a previous report (Massie et al., 2011). Note that the authors used human genome assembly version hg18. But the sequence remains the same with latest assembly version hg38. B). Bioinformatics analysis with UCSC PhyloP basewise conservation tool. The AR target site in *Spp1* gene intron 3 was highly conserved across different species. The green arrow indicates this intronic sequence. C). Representative pictures for immunohistochemistry staining of bone sections with anti-Spp1 antibody. Femoral bone sections were stained with anti-osteopontin antibody (R&D Systems, Cat# AF808, 1:100 dilution) and shown in dark brown color. DAPI counterstaining was shown in blue. Original magnification: ×400.

Figure S5: EPO's effects on hematopoiesis, related to Figure 5

A). CBC tests of EPO-treated mice and placebo-treated controls. Data are pooled results from multiple mice (n = 6 for each group). B). Serum EPO levels in OXM-treated mice and placebo-treated controls. Data are pooled results from multiple mice (n = 8 for either OXM or placebo group of *Fancd2*$^{+/+}$ mice, n = 7 for either OXM or placebo group of *Fancd2*$^{-/-}$ mice) and presented as mean ± SEM. NS denotes not significant.

Figure S6: *Spp1* gene expression level in SPKSL cells, related to Table 2

Spp1 gene expression level was from a search result in a previously published gene expression database of SPKSL cells (side population KSL cells, a highly enriched HSC population) (Chambers et al., 2007).

Supplementary figures

Figure S1

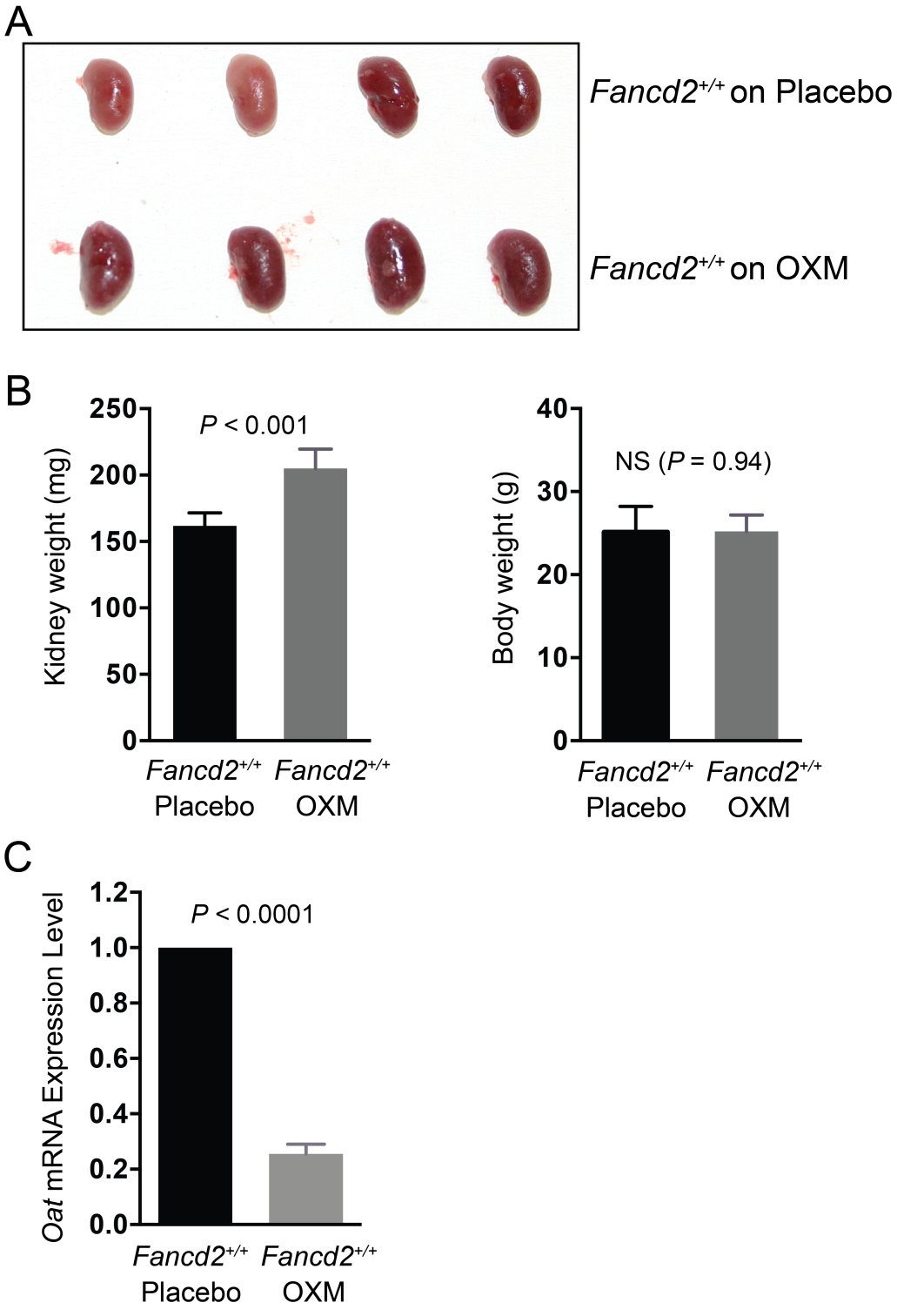
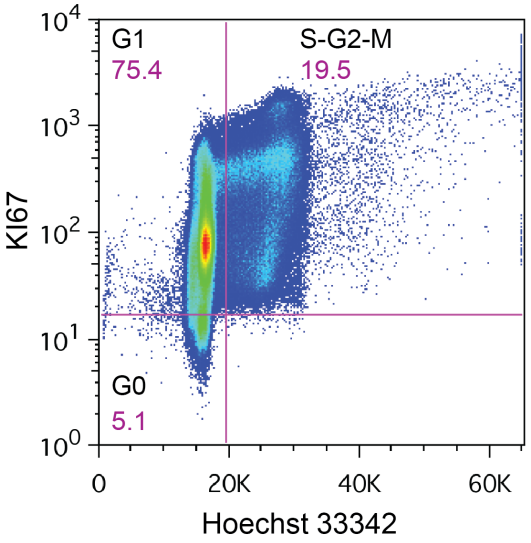
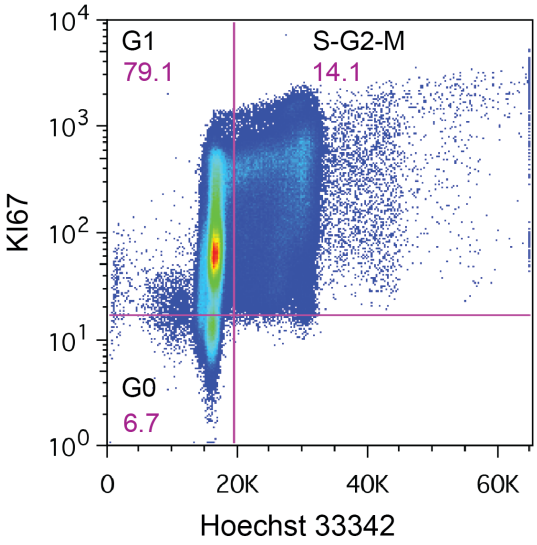


Figure S2

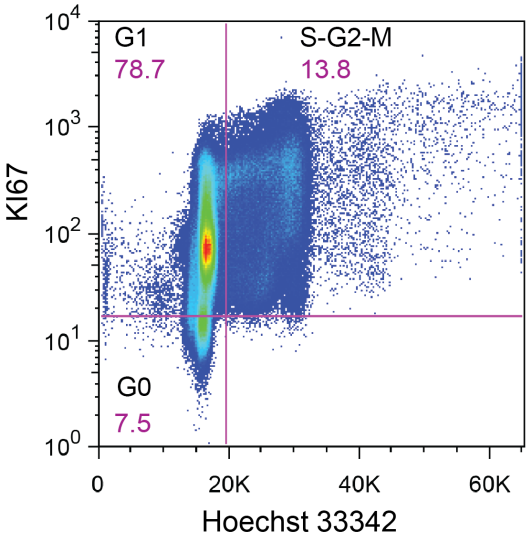
Placebo-treated *Fancd2*$^{+/+}$ LIN^{+} cells:



OXM-treated *Fancd2*$^{+/+}$ LIN^{+} cells:



Placebo-treated *Fancd2*$^{-/-}$ LIN^{+} cells:



OXM-treated *Fancd2*$^{-/-}$ LIN^{+} cells:

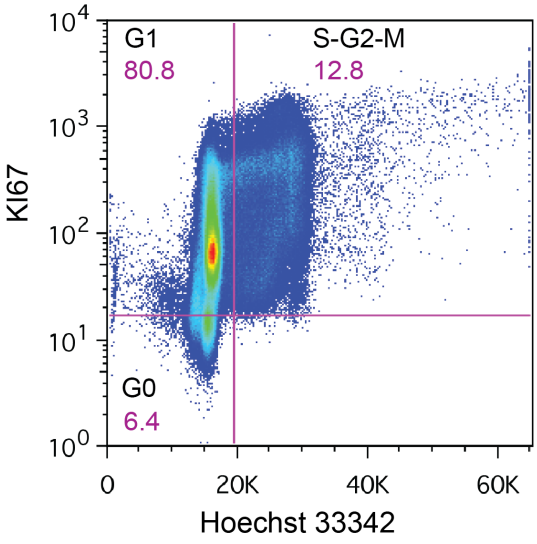


Figure S3

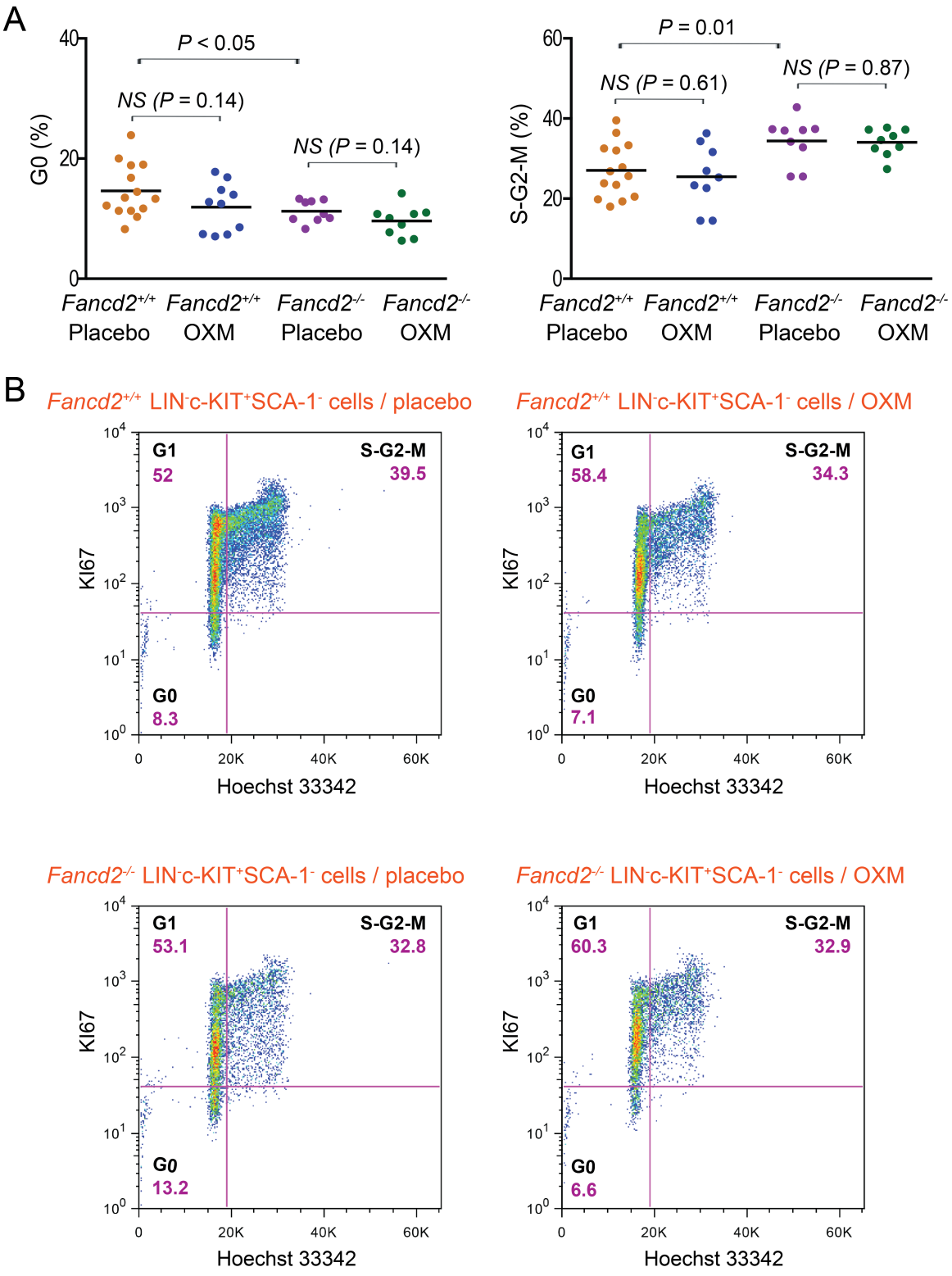
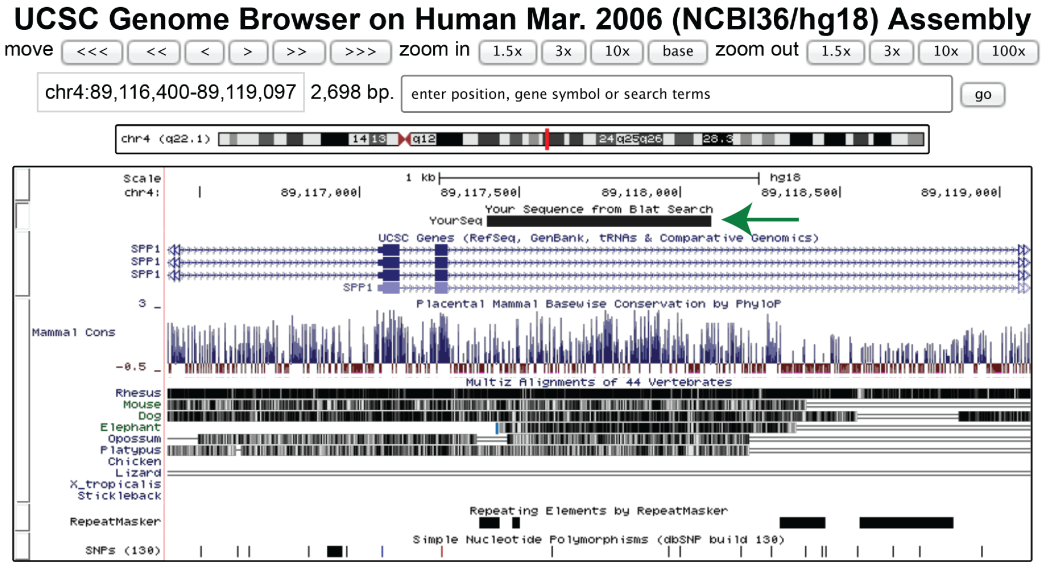

A
G0 (%)
P < 0.05
NS (P = 0.14)
NS (P = 0.14)
Fancd2+/+ Placebo
Fancd2+/+ OXM
Fancd2-/- Placebo
Fancd2-/- OXM
S-G2-M (%)
P = 0.01
NS (P = 0.61)
NS (P = 0.87)
B
Fancd2+/+ LIN-c-KIT+SCA-1- cells / placebo
G1 52
S-G2-M 39.5
G0 8.3
Fancd2+/+ LIN-c-KIT+SCA-1- cells / OXM
G1 58.4
S-G2-M 34.3
G0 7.1
Fancd2-/- LIN-c-KIT+SCA-1- cells / placebo
G1 53.1
S-G2-M 32.8
G0 13.2
Fancd2-/- LIN-c-KIT+SCA-1- cells / OXM
G1 60.3
S-G2-M 32.9
G0 6.6
KI67
Hoechst 33342

Figure S4

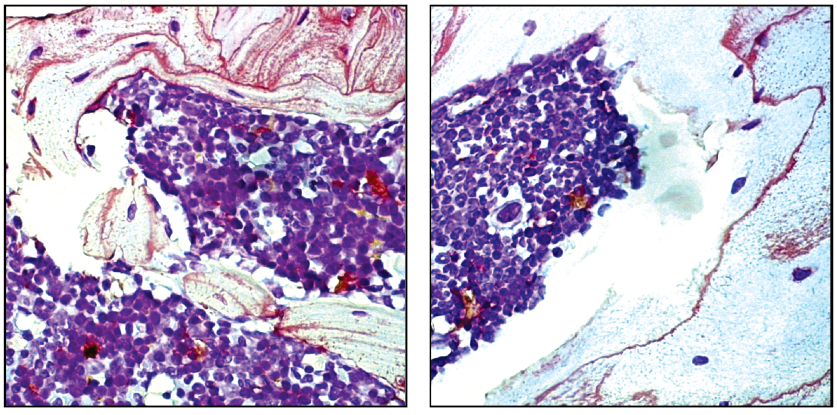
A. AR target site in intron 3 of human *Spp1* gene

TGACTGCCTGCTATGAATCTAGGCCAGTACCAAGCACAGTATAGTTTTTAATAAA
TATAAGTTTATAAAACCAACCCAGATATTTTAAATATAATAATATCTAGGCATGT
ATGATGAGTTATCGCATGTAAGATAAGTTATATGAAGTTGTGTGACTTTTTTTCC
ATTAGTCCACATACTGATCTAAAAGCAGAAAATTCCAGCTTTTGCTTTGTTTAGT
GGATTGCTAAGTTTAAAATTCACATTGGATATTAGTCAGAACTGTTTGTATGACC
ATAATATTCACAATATTGTCTGAGATATTAGCTGAGAAGCCCATTGTGAAAAGAA
AGTCTATGTGTGCTGTTTGTATCTATTGTGATTGTCAGCTGATGTTAGATCACAT
TTTCTAACCAAACATAAGACCAACCAAACTCTTTATTATAATTATTTGACCAGCA
CTAAAGATGTACCTACCCCTCCACAACAGATGAAACTGTGCCAGCCAAACAACAA
ATGGGCATTGTCCCCAGAAGCTTGGACAAAAAGGCACACAGAGTTCAATTCCAGT
TGAACAGAATAAAGGCCAAAATAGAGCTGCCTTGGGGGTCACTGCAATTAGACTG
CTTAATGAAGACATTAAAAGAAGTATTCTGTGTTCGTTTGTGTGTGGAGGGGTGT
GTGTGTCTGTTTTTCAACTGATTTGAAAATACAGGTGT

B



C



Fancd2$^{-/-}$ Placebo *Fancd2*$^{-/-}$ OXM

Figure S5

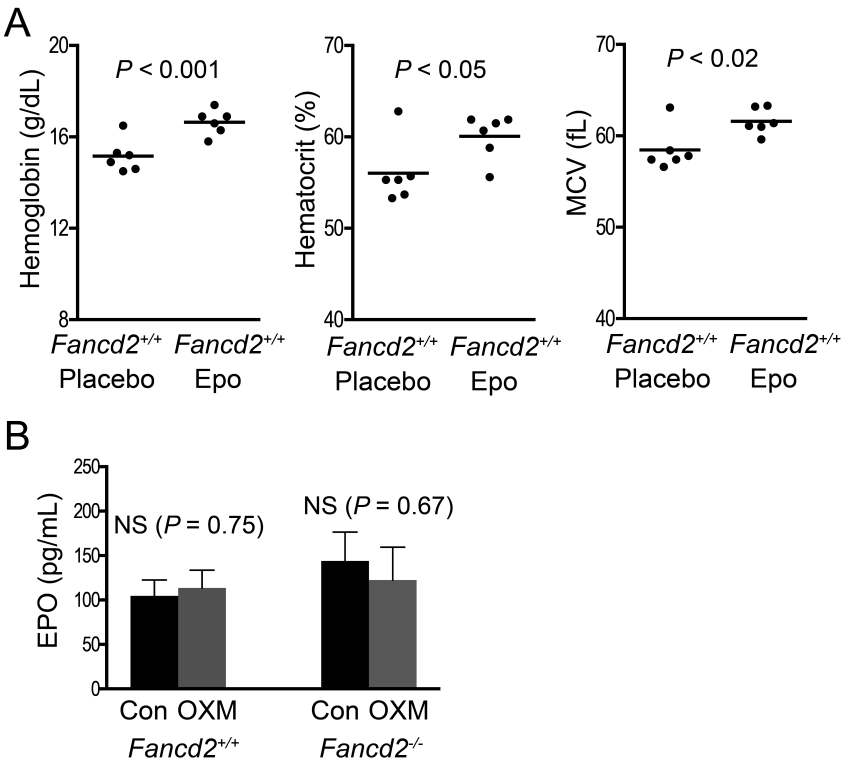
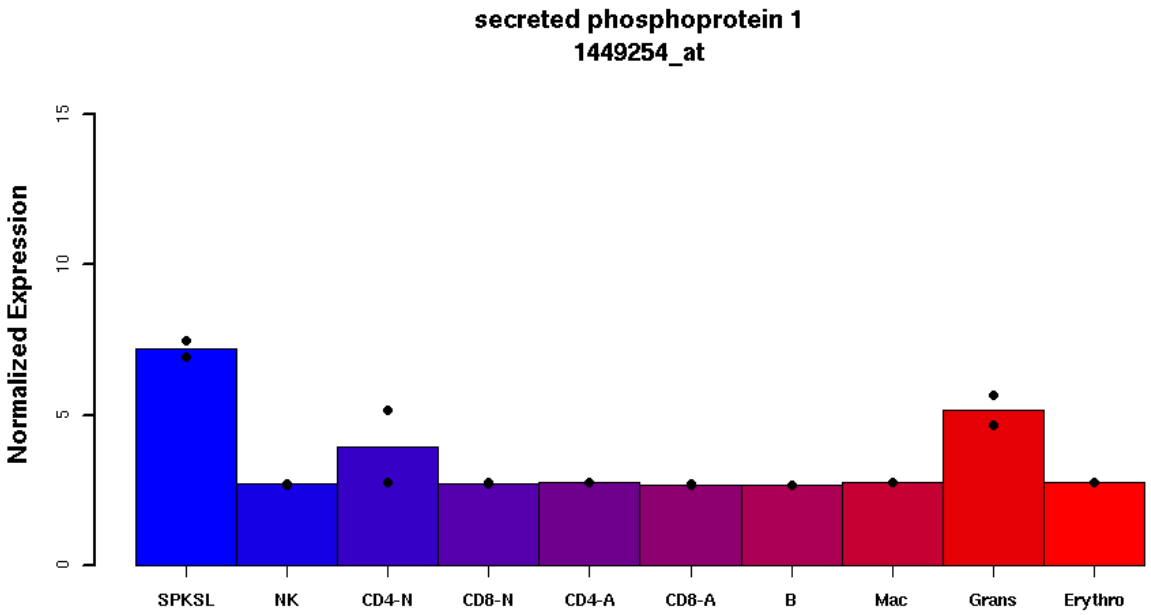

A
Hemoglobin (g/dL)
20
16
12
8
P < 0.001
Fancd2+/+ Placebo
Fancd2+/+ Epo
Hematocrit (%)
70
60
50
40
P < 0.05
Fancd2+/+ Placebo
Fancd2+/+ Epo
MCV (fL)
70
60
50
40
P < 0.02
Fancd2+/+ Placebo
Fancd2+/+ Epo
B
EPO (pg/mL)
250
200
150
100
50
0
NS (P = 0.75)
NS (P = 0.67)
Con OXM
Fancd2+/+
Con OXM
Fancd2-/-

Figure S6:


secreted phosphoprotein 1
1449254_at
Normalized Expression
0
5
10
15
SPKSL
NK
CD4-N
CD8-N
CD4-A
CD8-A
B
Mac
Grans
Erythro

Supplemental tables

Table S1: Blood counts in young adult *Fancd2*$^{-/-}$ and *Fancd2*$^{+/+}$ mice, related to Figure 1

	Fancd2$^{+/+}$	*Fancd2*$^{-/-}$	*p*
WBCs, x10^3/μL	6.7 ± 0.5	6.8 ± 0.5	0.92
RBCs, x10^6/μL	10.0 ± 0.1	9.9 ± 0.1	0.38
Hemoglobin, g/dL	14.9 ± 0.1	15.1 ± 0.2	0.25
Hematocrit, %	54.1 ± 0.6	53.8 ± 0.5	0.70
MCV, fL	54.1 ± 0.3	55.1 ± 0.4	0.03
MCH, pg	14.9 ± 0.1	15.4 ± 0.1	0.00
MCHC, %	27.6 ± 0.2	27.9 ± 0.2	0.23
Platelets, x10^3/μL	812 ± 32	643 ± 22	0.00

Comprehensive CBC tests were measured for *Fancd2*$^{+/+}$ and *Fancd2*$^{-/-}$ mice (4-6 months old, 23 mice in each group) by IDEXX Laboratories. Data were analyzed with Prism 6.0c software and presented as mean value ± SEM.

WBCs denotes white blood cells; RBCs, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; and MCHC, mean corpuscular hemoglobin concentration.

Table S2: mRNA expression changes in *Fancd2*$^{-/-}$ KSL cells compared to wild-type KSL cells, related to Table 1

– See the Excel file for Table S2

Table S3: mRNA expression changes in wild-type KSL cells compared to wild-type whole bone marrow cells, related to Table 1

– See the Excel file for Table S3

Table S4: Lists of genes in the important pathways altered in *Fancd2*$^{-/-}$ HSPCs as compared to wild-type HSPCs, related to Table 1

Symbol	RefSeq ID	Gene Name	Change	Q Value
Cell cycle regulation:				
Ccna2	NM_009828	cyclin A2	1.6	0.000
Ccnb1	NM_172301	cyclin B1	2.0	0.000
Ccnb2	NM_007630	cyclin B2	1.8	0.000
Ccnd1	NM_007631	cyclin D1	1.7	0.000
Cdk1	NM_007659	cell division cycle 2 homolog A	1.8	0.000
Cdc25c	NM_009860	cell division cycle 25 homolog C	1.9	0.000
Cdca3	NM_013538	cell division cycle-associated 3	1.7	0.000
Cdkn3	NM_028222	Cyclin-Dependent Kinase Inhibitor 3	1.8	0.003
Cks1b	NM_016904	CDC28 protein kinase subunit 1b	1.6	0.000
Cdca5	NM_026410	cell division cycle-associated 5	1.6	0.000
Cdc45	NM_009862	cell division cycle 45 homolog	1.5	0.000
Ube2c	NM_026785	ubiquitin-conjugating enzyme E2C	2.2	0.000
Nek2	NM_010892	NIMA-related expressed	1.8	0.010
Birc5	NM_001012273	survivin	1.8	0.000
Mad2l1	NM_019499	MAD2-like 1	1.5	0.000
Pttg1	NM_013917	pituitary tumor-transforming 1	2.0	0.000
Aurka	NM_011497	serine/threonine protein kinase 6	1.8	0.000
Inflammatory and immune response:				
Cfp	NM_008823	complement factor properdin	1.6	0.000
Ccr5	NM_009917	chemokine (C-C motif) receptor 5	2.4	0.000
Socs2	NM_007706	suppressor of cytokine signaling 2	2.5	0.000
Chga	NM_007693	chromogranin A	3.5	0.000
Ccr1	NM_009912	chemokine (C-C motif) receptor 1	3.4	0.000
Ccr2	NM_009915	chemokine (C-C motif) receptor 2	1.8	0.000
Ifi30	NM_023065	interferon gamma inducible protein 30	1.8	0.004
Lgmn	NM_011175	legumain	1.6	0.011
Sell	NM_011346	L-selectin	1.8	0.000

Table S5: Differential gene expression of telomerase protein subunit in *Fancd2*$^{-/-}$ or wild-type HSPCs in response to OXM administration, related to Table 2

Genotype	Symbol	Gene ID	Fold change	[c]Q value
Fancd2$^{-/-}$	*Tert*	NM_009354	-1.05	0.27
Wild-type	*Tert*	NM_009354	1.05	0.70

Note: [c]Q value cut-off was set at 0.05.

Table S6: mRNA expression changes of critical EPO-inducible or target genes in wild-type basophilic erythroblasts in response to OXM administration, related to Figure 5

Symbol	RefSeq ID	Con1	Con2	Con3	Oxm1	Oxm2	Oxm3	Q value	AvgFC
Cish	NM_009895	19	29	7	32	13	9	1	n/a
Socs1	NM_009896	4	4	2	2	2	1	1	n/a
Socs2	NM_007706	2	2	2	2	1	0	1	n/a
Socs3	NM_007707	13	6	12	11	2	6	1	n/a
Tfrc	NM_011638	67455	64327	61344	69170	64641	62324	1	1.02
Bcl2l1	NM_009743	6599	4948	6058	5929	5765	6129	1	1.01
Cdc25a	NM_007658	1500	1846	1660	1682	1648	1683	1	1.00
Btg3	NM_009770	0	1	2	0	1	0	1	n/a
Ccnd2	NM_009829	26	8	13	11	7	9	1	n/a
Lyl1	NM_008535	4573	4323	3953	4905	4293	4179	1	1.04
Pim3	NM_145478	131	84	54	83	75	81	1	-1.13
Tnfrsf13c	NM_028075	22	24	26	33	24	16	1	n/a

The total reads for each library were normalized to 20 million. Columns Con1 thru Oxm3 showed the normalized reads for each gene in different libraries. Con1, Con2, and Con3 denote library 1, 2, and 3 for placebo-treated mice, respectively, whereas Oxm1, Oxm2, Oxm3 for OXM-treated mice. A Q value lower than 0.05 was considered as significant.

AvgFC denotes average fold change. When both the average OXM read and the average placebo read were below 50, the expression level of the particular gene was deemed as too low to calculate a fold change. Instead, n/a, which denotes not applicable, was filled in the AvgFC column.

Table S7: Comparison of *Spp1* expression levels between KSL and basophilic erythroblast libraries, related to Table 2

Libary	Average normalized tag count for *Spp1*
OXM-treated KSL cells	146
Placebo-treated KSL cells	1529
OXM-treated basophilic erythroblasts	1.5
Placebo-treated basophilic erythroblasts	1.2

The total reads for each individual library were normalized to 20 million first. The tag count for *Spp1* gene shown here was the average of all six libraries in each condition. For any particular gene, an average tag greater than 50 was considered as true signal.

Table S8: Primers used for qPCR amplification, related to Figure 3

Name	Sequence	Note
MG3157	5' GTGGCGCGGCGACTTCCAGT 3'	For *ROSA26* transgenic allele
MG3158	5' TCCGCGTGCAGCAGATGGCG 3'	
MG1711	5' GAGCTGCCTGATACGGATGCTG 3'	For *Fancc*$^{-/-}$ allele
MG1791	5' GGGCTGCTAAAGCGCATGCTC 3'	
MG3153	5' CACGGTGTGGTGGGCCCAGGT 3'	For *Fancd2*$^{-/-}$ allele
MG3154	5' GGGAGCCCTTGCATGACAATTCTGCT 3'	
MG1711	5' GAGCTGCCTGATACGGATGCTG 3'	For *Fancc*$^{+/+}$ allele
MG1712	5' GAGAAATGGCTCAGTGGTTAAGAG 3'	
OatF	5' GCTGCCCTCTGACGTTGTGAC 3'	For *Oat* RT-PCR
OatR	5' TCGAAGTCGCAGGCACACCT 3'	
TfrcF	5' GGCGCTTCCTAGTACTCCCTTGT 3'	For *Tfrc* RT-PCR
TfrcR	5' TGCCGAGCAAGGCTAAACCG 3'	
OpnF	5' CCTCCCTCCCGGTGAAAGTG 3'	For *Spp1* RT-PCR
OpnR	5' GAGATGGGTCAGGCACCAGC 3'	
GapF	5' ATGGTGAAGGTCGGTGTGAACG 3'	For *Gapdh* RT-PCR
GapR	5' GTCAATGAAGGGGTCGTTGATGGCA 3'	