Mutant p53 enhances leukemia-initiating cell self-renewal to promote leukemia development

Supplemental Methods

Mice

FLT3-ITD knock-in mice were obtained from the Jackson Laboratory (Lee et al., 2007). The humanized mutant *p53* knock-in mice ($p53^{R248W/+}$) have been backcrossed to the *C57BL6* background for at least 8 generations (Chen et al., 2018). Wild type C57BL/6 ($CD45.2^+$), B6.SJL ($CD45.1^+$) and F1 mice ($CD45.2^+$ $CD45.1^+$) mice were obtained from an on-site core breeding colony. All mice were maintained in the Indiana University Animal Facility according to IACUC-approved protocols.

Flow Cytometry

Flow cytometry analysis of hematopoietic stem and progenitor cells was performed as described previously (Liu et al., 2009). Murine hematopoietic stem and progenitor cells were identified and evaluated by flow cytometry using a single cell suspension of bone marrow mononuclear cells (BMMCs). Hematopoietic stem and progenitors are purified based upon the expression of surface markers. Bone marrow (BM) cells were obtained from femurs by flushing cells out of the bone using a syringe and phosphate-buffered saline (PBS) with 2mM EDTA. Red blood cells (RBCs) were lysed by RBC lysis buffer (eBioscience) prior to staining. Experiments were performed on FACS LSR IV cytometers (BD Biosciences) and analyzed by using the FlowJo Version 9.3.3 software (TreeStar).

Serial Replating Assays

Clonogenic progenitors were determined in methylcellulose medium (MethoCult GF M3434, StemCell Technologies) with cytokines (SCF, TPO, EPO, IL-3 and GM-CSF) using 2 x 10^4 BMMCs per well (6-well plate). Colonies were scored after 7 days of the initial culture, and all cells were collected and washed twice in phosphate-buffered saline. Subsequently cells were cultured at 2 x10^4 per well in the same medium. Colony scoring and replating were repeated every 7 days for at least two times, or until no colonies were observed in the cultures.

Transplantation

For the competitive repopulation assays, we injected 5 x 10^5 BM cells from *p53*$^{+/+}$, *p53*$^{R248W/+}$, *FLT3*$^{ITD/+}$ and *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ mice (CD45.2$^+$) plus 5 x 10^5 competitor BM cells (CD45.1$^+$) into lethally irradiated (11Gy) F1 mice (CD45.1$^+$CD45.2$^+$). Peripheral blood was obtained by tail vein bleeding every 4-week after transplantation, RBC lysed, and the PB mononuclear cells stained with anti-CD45.2 FITC and anti-CD45.1 PE, and analyzed by flow cytometry. 16 weeks following transplantation, bone marrow cells from recipient mice were analyzed to evaluate donor chimerism in bone marrows. For secondary transplantation, 3 x 10^6 BM cells from mice reconstituted with *p53*$^{+/+}$, *p53*$^{R248W/+}$, *FLT3*$^{ITD/+}$ and *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ BM cells were injected into lethally irradiated F1 mice (CD45.1$^+$CD45.2$^+$).

Western Blot Analysis

Bone marrow mononuclear cells from *p53*$^{+/+}$, *p53*$^{R248W/+}$, *FLT3*$^{ITD/+}$ and *p53*$^{R248W/+}$*FLT3*$^{ITD/+}$ mice were differentiated into macrophage progenitors with 10ng/ml M-CSF for 7-10 days. Total cell lysates were collected, run on SDS-PAGE gels, and transferred to PVDF membrane. Blots were probed with indicated primary antibody [p-FAK(Cell Sig. #3283), T-FAK(BD #610087), p-STAT5(Cell Sig. #9351), T-STAT5(Cell Sig. #9363), p-AKT(Cell Sig. #9271), T-AKT(Cell Sig. #4691), p-ERK(Cell Sig. #9101), T-ERK(Cell Sig. #9102), FLT3 (Cell Signaling. #3462S), GAPDH(Santa Cruz #sc-32233), or Vinculin(sc-73614)] and incubated with secondary antibody, and imaged.

Tissue Specimens

All mice tissue samples were collected following a detailed LARC approved lab animal protocol, and were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee and adhered to all standards set forth in the ARVO Statement for the Use of Animals in Research.

Tissue Processing

In the histology study, all mice tissues were fixed in 10% neutral buffered formalin at 4°C for 24 hours following tissue processing, and then embedded in paraffin. Five-micrometer sections were stained for routine H&E.

Pathology Scoring

The **lymphomas** were sub classified into the following groups based on the St. Jude's pathology classification of mouse lymphomas (Ward JM et al., 2012; Frith CH et al., 1993): 1: Diffuse Large B Cell Lymphoma involving multiple organs, 2: Thymic Lymphoma (usually associated with thymic and around the heart), and 3: Follicular Lymphoma, seen as a diffuse proliferation of lymphocytes in the white pulp area in the spleen.

Myeloid proliferations include a wide range of disease, those similar to human disease at one end to subtle increases in the splenic nonlymphoid hematopoietic cells in genetic engineered mice at the other end. These can progress to leukemias. The mouse myeloproliferative disease is broken down into two subclassisfication, one in genetic engineered mice and in naturally occurring mice. This is characterized by the combination of the following: A. Erythrocytosis, leukocytosis of myeloid cells, and/or thrombocytosis/circulating micromegakaryocytes; B. Increased in non-lymphoid hematopoietic cells in the spleen and bone marrow. Chronic myeloid leukemia (CML) in the mouse is a progression of myeloproliferative disease and is characterized by at least 20% immature blast cells with moderate differentiation and neutrophilic in blood, spleen, and or bone marrow (Kogan et al., Blood, 2002).

The **leukemia** were classified by the following criteria: 1. Presence of large lymphocytes in the blood vessel lumens and/or vascular channels in the following organs examined. 2. Liver, lung, and kidney (blood vessels) and 3. Bone marrow and spleen (vascular channels).

The **MPN** were classified as follows: 1) myeloid proliferation in the bone marrow, spleen and liver. 2) Myelofibrosis in the bone marrow. This was composed of a proliferative infiltrate of mononuclear cells with features of blast and immature myeloid cells in the bone marrow, liver, and spleen.

All histology slides were hand read by a pathologist and the cause of death was determined. All images obtained were scanned using Aperio Whole Slide Digital Imaging Platform.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6 software (GraphPad software, Inc). All data are presented as mean ± standard error of the mean (SEM). The sample size for each experiment are included in the figure legends. Statistical analyses were performed using unpaired, two-tailed Student's t test where applicable for comparison between two groups, and a One-way ANOVA test or Two-way ANOVA was used for experiments involving more than two groups. Survival curves were tested using Geham-Breslow-Wilcoxon test and those P-values were then tested using 'analyze stack of P values' to check significance. Statistical significance was defined as *p< 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant.

Supplementary Figure Legends

Figure S1.

(**a**) Recipient mice repopulated with $FLT3^{ITD/ITD}$ and $p53^{R248W/+}FLT3^{ITD/ITD}$ bone marrow cells show significantly reduced survival compared to that of the $p53^{+/+}$ cells (n=6, $p53^{+/+}$; n=4, $FLT3^{ITD/ITD}$; and n=6, $p53^{R248W/+}FLT3^{ITD/ITD}$ mice, **p<0.01). (**b**) Disease spectrums in $FLT3^{ITD/ITD}$ and $p53^{R248W/+}FLT3^{ITD/ITD}$ whole bone marrow transplant recipients were determined by pathological analysis of bone marrow, spleen, liver, and peripheral blood (n=4, $FLT3^{ITD/ITD}$; n=6, $p53^{R248W/+}FLT3^{ITD/ITD}$ mice). (**c**) Peripheral white blood cell (WBC) counts in $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$ and $p53^{R248W/+}FLT3^{ITD/+}$ mice. Mean values (±SEM) are shown (n=6). (**d**) Bone marrow cell numbers of young $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$

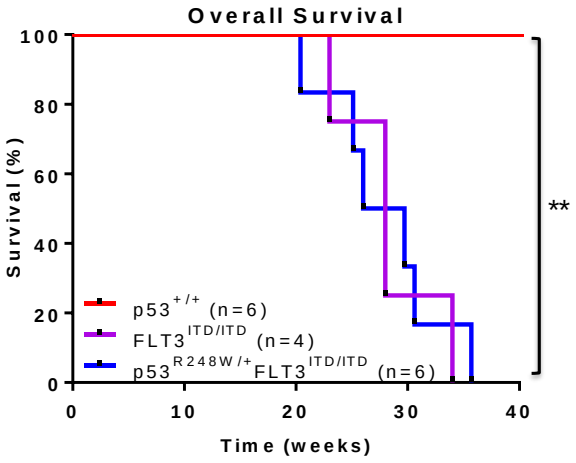
and $p53^{R248W/+}FLT3^{ITD/+}$ mice. Mean values (±SEM) are shown (n=6). (**e**) Spleen weight of young $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$ and $p53^{R248W/+}FLT3^{ITD/+}$ mice. Mean values (±SEM) are shown (n=8).

Figure S2. (**a**) Frequency of LT-HSCs ($CD48^{-}CD150^{+}$LSKs), ST-HSCs ($CD48^{-}CD150^{-}$LSKs), MPPs ($CD48^{+}CD150^{-}$LSKs) and LSKs in the BM of young $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$ and $p53^{R248W/+}FLT3^{ITD/+}$ mice. Mean values (±SEM) are shown (n=8, *p<0.05, **p<0.01, ***p<0.001). (**b**) The frequency of myeloid progenitor cells ($Lin^{-}Kit^{+}$), CMPs ($Lin^{-}Sca1^{-}Kit^{+}FcγRII/III^{low}CD34^{high}$), GMPs ($Lin^{-}Sca1^{-}Kit^{+}FcγRII/III^{high}CD34^{high}$), and MEPs ($Lin^{-}Sca1^{-}Kit^{+}FcγRII/III^{low}CD34^{low}$) in the bone marrow of young $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$ and $p53^{R248W/+}FLT3^{ITD/+}$ mice. Mean values (±SEM) are shown (n=8, *p<0.05, **p<0.01, ****p<0.0001. (**c**) The frequency of common lymphoid progenitors (CLPs) in the bone marrow of young $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$ and $p53^{R248W/+}FLT3^{ITD/+}$ mice. Mean values (±SEM) are shown (n=5). (**d**) Cell cycle status of LSK cells from young $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$ and $p53^{R248W/+}FLT3^{ITD/+}$ mice was determined by flow cytometry analysis. Mean values (±SEM) are shown (n=4, *p<0.05). (**e**) Peripheral white blood cell counts in recipient mice at 16 weeks following bone marrow transplantation. Mean values (±SEM) shown (n=7). (**f**) The frequency of donor-derived LSKs, LT-HSCs, ST-HSCs, and MPPs in the bone marrow of primary recipient mice 16 weeks following transplantation. Mean values (±SEM) shown (n=7, *p<0.05, ***p<0.001).

Figure S3. (**a**) The frequency of donor-derived GMPs, CMPs, and MEPs in the bone marrow of primary recipient mice 16 weeks following transplantation. Mean values (±SEM) shown (n=7, **p<0.01). (**b**) Spleen weight of recipient mice at 16 weeks following bone marrow transplantation. Mean values (±SEM) shown (n=7). (**c**) Western blot analysis of FLT3 in $p53^{+/+}$, $p53^{R248W/+}$, $FLT3^{ITD/+}$ and $p53^{R248W/+}FLT3^{ITD/+}$ mononuclear cells differentiated into macrophage progenitors. Loading controls GAPDH and Vinculin are also shown. (**d**) ERK inhibitor treatment decreased the replating potential of $p53^{R248W/+}$, $FLT3^{ITD/+}$ and $p53^{R248W/+}FLT3^{ITD/+}$ bone marrow cells. Mean values (±SD) are shown (n=3, *p<0.05, **p<0.01).

Figure S1

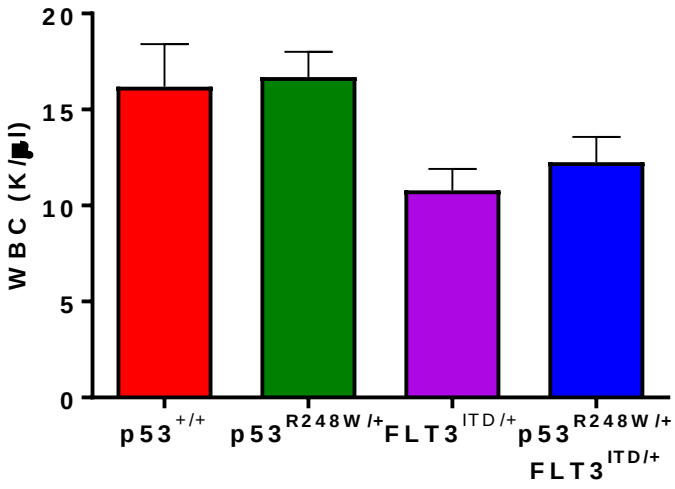
a



b

Disease Type	$FLT3^{ITD/ITD}$ (n=4)	$p53^{R248W/+}FLT3^{ITD/ITD}$ (n=6)
MPN and Lymphoma	4/4 (100%)	4/6 (67%)
CML and Lymphoma	0	2/6 (33%)

c



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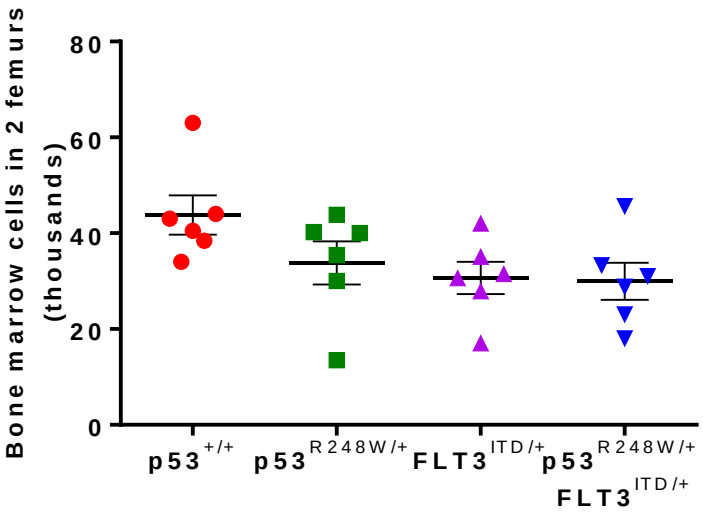

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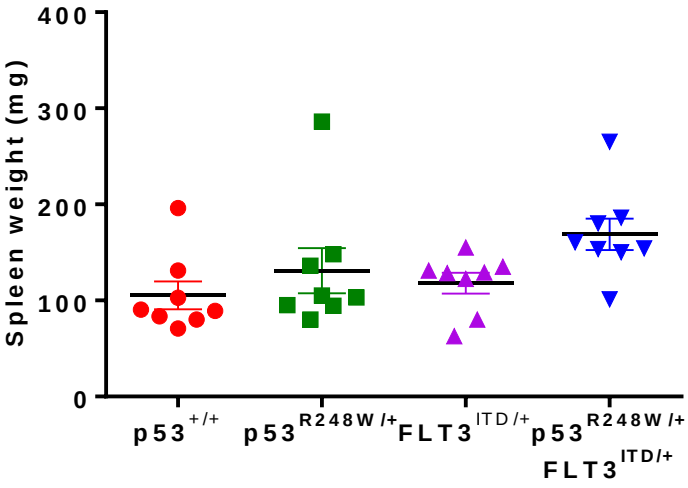


Figure S2

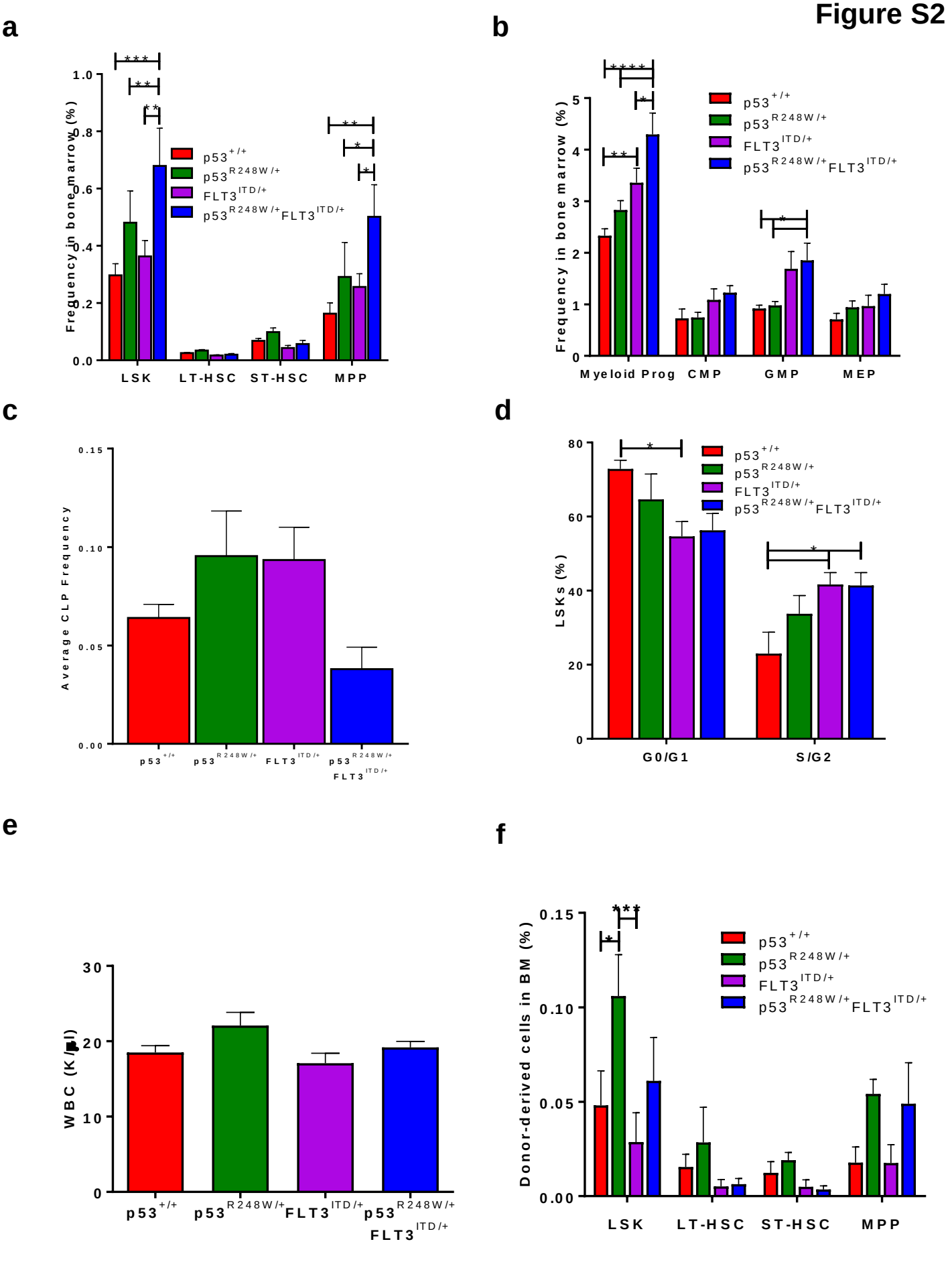
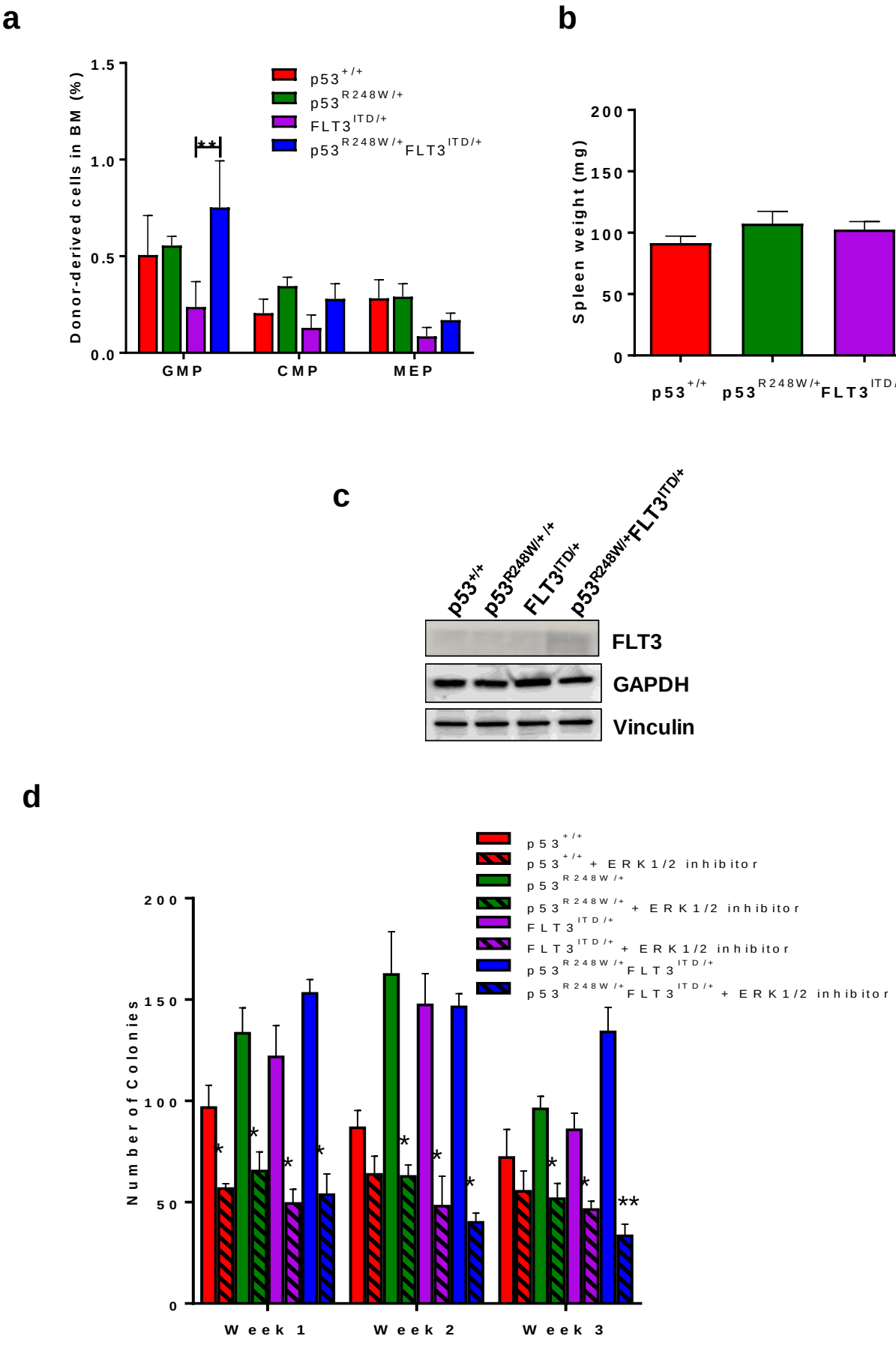

a
Frequency in bone marrow (%)
1.0
0.8
0.6
0.4
0.2
0.0
***
**
**
**
*
*
p53+/+
p53R248W/+
FLT3ITD/+
p53R248W/+FLT3ITD/+
LSK
LT-HSC
ST-HSC
MPP
b
Frequency in bone marrow (%)
5
4
3
2
1
0
****
*
**
*
p53+/+
p53R248W/+
FLT3ITD/+
p53R248W/+FLT3ITD/+
Myeloid Prog
CMP
GMP
MEP
c
Average CLP Frequency
0.15
0.10
0.05
0.00
p53+/+
p53R248W/+
FLT3ITD/+
p53R248W/+FLT3ITD/+
d
LSKs (%)
80
60
40
20
0
*
*
p53+/+
p53R248W/+
FLT3ITD/+
p53R248W/+FLT3ITD/+
G0/G1
S/G2
e
WBC (K/μl)
30
20
10
0
p53+/+
p53R248W/+
FLT3ITD/+
p53R248W/+FLT3ITD/+
f
Donor-derived cells in BM (%)
0.15
0.10
0.05
0.00
***
*
p53+/+
p53R248W/+
FLT3ITD/+
p53R248W/+FLT3ITD/+
LSK
LT-HSC
ST-HSC
MPP

Figure S3


a
Donor-derived cells in BM (%)
1.5
1.0
0.5
0.0
**
p53+/+
p53R248W/+
FLT3ITD/+
p53R248W/+FLT3ITD/+
GMP
CMP
MEP
b
Spleen weight (mg)
200
150
100
50
0
p53+/+
p53R248W/+
FLT3ITD/+
p53R248W/+ FLT3ITD/+
c
p53+/+
p53R248W/+/+
FLT3ITD/+
p53R248W/+FLT3ITD/+
FLT3
GAPDH
Vinculin
d
p53+/+
p53+/+ + ERK1/2 inhibitor
p53R248W/+
p53R248W/+ + ERK1/2 inhibitor
FLT3ITD/+
FLT3ITD/+ + ERK1/2 inhibitor
p53R248W/+ FLT3ITD/+
p53R248W/+ FLT3ITD/+ + ERK1/2 inhibitor
Number of Colonies
200
150
100
50
0
Week 1
Week 2
Week 3