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## Supplemental Information

# Cell-Cycle-Specific Function of p53 in Fanconi Anemia Hematopoietic Stem and Progenitor Cell Proliferation

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## Inventory of Supplemental Information

## Supplemental Experimental Procedures

### 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-C-KIT (BD Biosciences, # 560185), PE-cy7-anti-SCA-1 (BD Biosciences, #558162), Pacific blue-anti-CD150 (BioLegend, #115924), FITC-anti-CD48 (BioLegend, #103403), FITC-anti-CD34 (BD Biosciences, #560238), PE-anti-CD45.1 (BioLegend #110707), APC-anti-CD45.2 (BioLegend, # 109814), FITC-anti-Mac1 (BD Bioscience, #557396), PerCP-Cy5.5-anti-Gr1 (BD Bioscience # 552093), APC-anti-Ki67(BD Biosciences, #558615), and APC-anti-BrdU (BD Biosciences, #557892) antibodies. The lineage antibody cocktail included the following biotin-conjugated anti-mouse antibodies: Mac1, Gr-1, Ter119, CD3e, and B220 (BD Biosciences, # 559971). The secondary reagents used included streptavidin-PerCP-Cy5.5 (BD Biosciences, # 551419). Initially, for LSK ($Lineage^{-}SCA\text{-}1^{+}C\text{-}KIT^{+}$) staining, cells were stained by using biotin-conjugated anti-lineage antibody cocktail followed by staining with a secondary Percp-cy5.5-anti Streptavidin antibody (BD Biosciences, # 551419), PE-cy7-anti-SCA-1 antibody (BD Biosciences, #558162), and APC-cy7-anti-C-KIT antibody (BD Biosciences, #560185). To access Long-term HSC subpopulation, cells were stained with LSK antibodies in addition to CD150-pacific blue (BioLegend, #115924), CD48-FITC (BioLegend, #103403). 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 the cell sorting, lineage negative cells were enriched using lineage depletion reagents (StemCell Technologies) according to the

manufacturer's instruction. The LSK population or HSCs ($Lin^-C\text{-}KIT^+SCA\text{-}1^+CD34^-$) were acquired by using the FACSAria II sorter (BD Biosciences).

**In Vivo Bromodeoxyuridine Incorporation Assay**

Mice received an intraperitoneal injection of BrdU (Sigma-Aldrich; 1mg/6g mouse weight) 24 hours prior to sacrifice. BM cells were harvested and stained with biotin-conjugated anti-lineage antibody cocktail followed by staining with a secondary Percp-cy5.5-anti Streptavidin (BD Biosciences, # 551419), PE-cy7-anti-SCA-1 (BD Biosciences, #558162), APC-cy7-anti-C-KIT (BD Bioscience, #560185), and FITC-anti-CD34 (BD Biosciences, #560238) antibodies, and then fixed and stained with APC-anti-BrdU antibody using the Cytofix/Cytoperm Kit (BD Biosciences, #557892), according to the manufacturer's instructions. Analysis was performed on a FACS LSRII (BD Biosciences).

**Cell-cycle and Apoptosis Analysis**

To analyze the cell cycle status of the HSC subsets, bone marrow cells were initially stained with antibodies against $Lin^+$ cells, C-KIT, SCA-1, CD150 and CD48 described above. After incubation with these cell surface antibodies, the cells underwent fixation and permeabilization with transcription factor buffer set (BD Biosciences, #562725) according to the manufacturer's instruction. After fixation, cells were incubated with APC-anti-Ki67 (BD Biosciences, #558615), washed and stained with PI. Cells were analyzed by flow cytometry. For the apoptosis detection, bone marrow cells were stained with the antibodies for the HSC surface markers, and then stained with APC-Annexin V (BD Biosciences, #550474) and PI. Annexin V-positive populations were determined as apoptotic cells using the FACS LSR II (BD Biosciences).

**BM Transplantation**

For the competitive repopulation assay, 50 LSK $CD150^{+}CD48^{+}$ cells from *WT, p53*$^{-/-}$*, Fanca*$^{-/-}$ and *p53*$^{-/-}$*Fanca*$^{-/-}$ mice ($CD45.2^{+}$) plus $4\times10^{5}$ recipient WBMCs ($CD45.1^{+}$) were transplanted into lethally (11 Gy) irradiated BoyJ mice ($CD45.1^{+}$). Five mice were transplanted for each genotyping group. Blood samples were collected from the recipients every 4 weeks after BMT and the donor cells were determined. The donor-type blood chimerism was determined by staining peripheral blood samples with APC-anti-CD45.2 (BioLegend, #109814). The percentage of donor-derived myeloid cells was determined by co-staining with FITC-anti-Mac1 (BD Bioscience, #557396) and PerCP-Cy5.5-anti-Gr1 (BD Bioscience # 552093) and analyzed on a FACS Canto instrument (BD Biosciences).

**RNA Isolation, Quantitative PCR**

Total RNA from SLAM cells isolated from *WT, p53*$^{-/-}$*, Fanca*$^{-/-}$*, p53*$^{515C/515C}$*, p53*$^{515C/515C}$ *Fanca*$^{-/-}$ and *p53*$^{-/-}$*Fanca*$^{-/-}$ mice was prepared with RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's procedure. Reverse transcription was performed with random hexamers and Superscript II RT (Invitrogen, Grand Island, NY) 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 PCR using the following primers: *PumaF- gcggcggagacaagaaga, PumaR-agtcccatgaagagattgtacatgac; NoxaF-tcgcaaaagagcaggatgag, NoxaR- cactttgtct ccaatcctccg; p21F-caggcaccatgtccaatcct, p21R-gagacaacggcacactttgct; Ccng1F-gcgaa gcatcttgggtgtgt, Ccng1R-tcctttcctcttcagtcgcttt; p15*$^{INKb}$*F-agatcccaacgccctgaaccg, p15*$^{INKb}$*R-tgctcttcagccaagtctacc; p16*$^{INK4a}$*F-cgaactctttcggtcgtaccc, p16*$^{INK4a}$*R-ttgagcagaagagctgctacg; p19*$^{ARF}$*F-cggaatcctggaccaggtg, p19*$^{ARF}$*R -accagcgtgtccaggaagc; p21*$^{Cip1/WAF1}$*F-ggcccggaacatctcagg, p21*$^{Cip1/WAF1}$*F-*

*aaatctgtcaggctggtctgc; GapdhF-tcaccaccatggagaaggc, GapdhR-gctaagcagttggtggtgca.* Samples were normalized to the level of *GAPDH* mRNA, and the relative expression levels were determined by the standard curve method.

**Colony Forming Unit Assay**

For the in vitro colony forming unit (CFU) assay, 1000 sorted LSK cells were seeded in MethoCult GF M3434 (STEMCELL Technologies) according to the manufacturer's recommendations. Colonies were visualized and counted at day 7. The experiment was performed in triplicate for each sample.

**Competitive Repopulating Unit (CRU) Assays**

Serial dilutions of donor SLAM cells harvested from *WT, p53$^{-/-}$, Fanca$^{-/-}$, p53$^{515C/515C}$, p53$^{515C/515C}$Fanca$^{-/-}$* and *p53$^{-/-}$Fanca$^{-/-}$* mice were injected into BoyJ mice (CD45.1). The CRU frequencies were then calculated from the proportions of negative mice (<1% donor engraft) with L-calc software (Stemcell Technologies), which uses Poisson statistics.

**Immunoblotting and Immunofluorescent Analyses**

LSK cells sorted from each genotyping (WT, p53$^{-/-}$, Fanca$^{-/-}$, and P53$^{-/-}$Fanca$^{-/-}$ ) or BM cells from healthy donors and FA patients with AML were washed with ice-old PBS, and resuspended in ice-cold lysis buffer containing 50mM Tris-HCL (pH7.4), 0.1% NP40, and 1M NaCl supplemented with protease and phosphatase inhibitors (10 µg/ml of aprotinin, 25 µg/ml of leupeptin, 10 µg/ml of pepstatin A, 2mM PMSF, 0.1M NaP2O4, 25mM NaF, and 2mM sodium orthovanadate) for 30 minutes on ice. Cell debris was removed from the lysate by centrifuging them at 16873g for 30 minutes at 4ºC. Protein lysate was resolved on SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblots

were then incubated with primary antibodies for total p53 (Clone PAb240; Calbiochem, #OP29), p53-S15 (Abcam, #ab1431), and β-actin (Sigma-Aldrich, #A2228) for 12-16 hours at 4ºC. Signals were visualized by incubation with anti-mouse or rabbit secondary antibody (Jackson ImmunoResearch, #115-035-003 or #111-035-003), followed by ECL chemiluminescence (Amersham Biosciences). Quantification of Western blot was conducted with ImageJ software (NIH) by measuring the density of each single band.

For immunofluorescent analysis, sorted $CD34^{-}$LSK cells were cytospun on slides, fixed by 4% paraformaldehyde, permeabilized with blocking solution (1XPBS / 0.25% Triton X-100 / 5% BSA), and subsequently processed for p53 (Clone PAb240; Calbiochem, #OP29) primary antibody and Alexa 488-conjugated secondary antibodies (Jackson ImmunoResearch, #115-545-003). DNA was stained by using DAPI.