Supplemental materials for

**LNK (SH2B3) Inhibition Expands Healthy and Fanconi Anemia Human Hematopoietic Stem and Progenitor Cells**

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Supplemental Methods

Lentiviral constructs and vector production. miR30-based shRNAs targeting human LNK (#1-CAAAGATGATGTCTGTCCG, #2-TGAACCTCTGGGTGGGTCG, #3-TTAACATATCCTGTAAAGC) were cloned into the pCL20.MSCV.miR30.PGK.mCherry lentiviral vector. The pTRIP-MND-GFP lentiviral vectors containing shRNAs targeting FANCD2 were described previously. shRNAs targeting Luciferase were used as controls in both vectors. All lentiviral vectors were produced and titered in 293T cells cultured in DMEM supplemented with 10% FBS, penicillin/streptomycin and L-glutamine. 293T cells were transfected using FuGENE 6 (Promega) in Opti-MEM (Thermo Fisher Scientific) with the transfer plasmid (pCL20 or pTRIP) alongside the 3rd generation lentiviral packaging and envelope plasmids. Viral supernatant was collected after 48hrs. Viral titer was calculated by transducing 293T cells with limiting dilutions of viral supernatant and analyzing percent of cells transduced by mCherry (pCL20) or GFP (pTRIP) expression on a BD LSR Fortessa.

Flow cytometry. For flow cytometric analysis of the peripheral blood (PB), red blood cells were first lysed. Cells from PB, BM and spleen, were first incubated with human and mouse Fc Blocks (BD Biosciences, #564220, 553142) for 10 minutes followed by staining with anti-mouse CD45 FITC or PE-Cy7 (#553080, 560578) and anti-human CD45 PE-Cy7 or BV711 (#557748, 564357) antibodies, along with and cell-type specific antibodies as follows: Myeloid and lymphoid lineages in PB, BM and spleen were stained with anti-human CD3 APC-Cy7 (#557832), CD33 PE (#561816) and CD19 APC (#561742); Erythroid cells and platelets in BM were stained with anti-human CD61 PerCP-Cy5.5 (#564173), CD41a PE (#555467) and CD235a APC (#551336); BM and spleen CD34+ HSPCs were stained with anti-human CD34 APC (#555824), CD90 PE (#555596), CD45RA PE-Cy7 (#560675) all from BD Biosciences and CD38 APC-Cy7 (Biolegend, #303533). DAPI was used in all flow studies to determine live/dead cells. All data for analysis was collected on the BD LSR Fortessa, and analyzed using FlowJo v10. All sorts for mCherry+ and GFP+mCherry+ cells were performed on a BD FACSAnia Fusion.

Colony forming cell assays. CD34+ HSPCs were sorted 48hrs post-transduction for mCherry positivity. Sorted
mCherry$^+$ HSPCs were either directly plated onto semi-solid methylcellulose media (Methocult H4230, Stemcell Technologies) supplemented with 5 U/mL EPO, 10 ng/mL IL-3, 5 ng/mL SCF, 5 ng/mL GM-CSF, or subjected to an extended in vitro culture for 5 additional days before plating in methylcellulose media. For FA-like HSPC CFC survival assays, GFP$^+$mCherry$^+$ HSPCs were sorted 48hrs post-second transduction. They were either directly plated onto semi-solid methylcellulose plates with a graded concentrations of mitomycin C (MMC) or hydroxyurea (HU), or treated with different doses of acetaldehyde (Ace) for 4hr before plating. All colonies were enumerated 12–14 days after plating. Pictures of CFC plates were taken on an Evos FL Auto microscope.

**CD34$^+$ HSPC Western blot analysis.** CD34$^+$ HSPCs were starved in cytokine-free media and then stimulated with GM-CSF for 10 min. Cells were lysed in a buffer containing 1% NP 40, 150 mM NaCl, 10 mM Tris-Cl and protease inhibitor cocktail (Roche) on ice. Protein lysates were clarified via centrifugation and the supernatants were resuspended in LDS reagent (Invitrogen) and heated at 90°C. Lysates were resolved on a Tris-Glycine polyacrylamide gel (National Diagnostics) and transferred to a nitrocellulose membrane (Bio-Rad). The following antibodies were used in Western blots: anti-LNK/SH2B3 (#sc-393709, 1:500), GAPDH (#sc-365062, 1:1000) and STAT5 (#sc-835, 1:1000), from Santa Cruz Biotechnology. pJAK2 (#3776, 1:1000), JAK2 (#3230, 1:1000), pSTAT5 (#9351, 1:1000), pAKT (#4051, 1:1000), AKT (#9102, 1:1000), pERK1/2 (#9106, 1:2000), ERK1/2 (#9102, 1:1000) were from Cell Signaling Technology, Inc. Secondary antibodies conjugated to horseradish peroxidase were used and detected with a digital enhanced chemiluminescence substrate (ECL) solution on a KwikQuant Imager (Kindle Biosciences). Intensity of the western blot bands were quantified in ImageJ$^{46}$ and normalized to GAPDH level.

**TF-1 cell culture and transduction.** TF-1 cells stably expressing MPL were established as previously reported$^{46}$. TF-1 cells were cultured in RPMI supplemented with 10% FBS, penicillin/streptomycin, L-glutamine and 2 ng/mL GM-CSF. To establish stably transduced TF-1 cells, $10^6$ TF-1 cells were resuspended in 1 mL viral supernatant supplemented with 5mg/mL polybrene and transduced by spinoculation at 1200xg at 37°C for 90 min.
in a 12-well plate.

**TF-1 Cell Western blot analysis.** TF-1 cells were lysed in a lysis buffer containing 1% NP 40, 150 mM NaCl, 10 mM Tris-Cl and protease inhibitor cocktail (Roche) on ice. Protein lysates were clarified via centrifugation and the supernatants were resuspended in LDS reagent (Invitrogen) and heated at 90°C. Lysates were resolved on a Tris-Glycine polyacrylamide gel (National Diagnostics) and transferred to a nitrocellulose membrane (Bio-Rad). The following antibodies were used in Western blots: anti-LNK/SH2B3 (Santa Cruz Biotechnology #sc-393709, 1:500) and Tubulin (Cell Signaling Technology #2128S, 1:1000). Secondary antibodies conjugated to horseradish peroxidase were used and detected with a digital enhanced chemiluminescence substrate (ECL) solution on a KwikQuant Imager (Kindle Biosciences).

**Real-Time quantitative PCR.** RNA was extracted from TF-1 cells using a RNeasy mini kit (Qiagen) and RNA concentration was measured using a nanodrop spectrophotometer. RT-PCR or reverse transcriptase PCR was performed by reacting 500 ng of RNA with qScript reverse transcriptase and master mix (Quantabio). 10 ng of each of the cDNAs were seeded in triplicates in a 384-well plate (10 µl / well) with SYBR Green (Applied Biosystems, #A25741) and the following pairs of primers: LNK (Forward: 5’-AGGACCGGACAGACATCATC-3’, Reverse: 5’-GCATCTCTGCTTCTGTGCTC-3’) and GAPDH (Forward: 5’- CCACCCATGGCAAATTTCC-3’, Reverse: 5’- TGGGATTTCCATTGATGACAAG-3’). Quantities of cDNA were measured and analyzed with the ViiA 7 Real-Time PCR System (ThermoFisher).

**TF-1 cell proliferation assay.** MTT assays were used to examine the growth of cell lines. TF-1 cells transduced with shRNAs targeting LNK or Luc were cultured in a 96-well plate (10k cells/100uL per well) in a graded concentration of cytokines for 72 hours. A final concentration of 0.5 mg/mL 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; M6494, Invitrogen) was added for 4 hours at 37°C, followed by termination of the reaction with stopping buffer. The absorbance was read by a spectrophotometer at 570 nm wavelength.
Supplemental Data Figure 1. Lentiviral miR30-based shRNA vectors efficiently target LNK in TF-1 cells. TF-1 cells were transduced with lentivirus expressing miR30-based shRNAs to either Luc or LNK and co-express mCherry as a fluorescent marker. mCherry+ TF-1 cells were sorted 48 hours post-transduction. (A) LNK mRNA transcript levels in mCherry+ cells transduced with shLNK vectors was measured by qRT-PCR relative to non-targeting shLuc control vector. Bars indicate mean values; error bars indicate ±SD. *: p<0.05; **: p<0.01; ***: p<0.001, as determined by one-way ANOVA followed by Dunnett’s multiple comparisons to shLuc. (B) LNK protein level in mCherry+ TF-1 cells was measured by western blot. (C-D) mCherry+ TF-1 cells were cultured in different concentrations of (C) GM-CSF and (D) TPO for 72 hours and cell number was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) absorbance. (E) Shows MTT growth curves of nontransduced TF-1 cells and TF-1 cells expressing shLuc, indicating shRNA to Luc does not affect TF-1 cell growth. Symbols indicate mean values of technical triplicates; error bars indicate ±SD. *: p<0.05; **: p<0.01; ***: p<0.001, as determined by two-way ANOVA followed by Dunnett’s multiple comparisons to shLuc.
**Supplemental Data Figure 2. Cord blood CD34+ HSPCs efficiently engraft NBSGW mice.** (A) Eight CB units were used for xenotransplantation in Figure 1-3. mCherry+ transduction rates at time of transplant are shown for each CB sample. (B-C) Total human chimerism (hCD45+) of xenotransplanted mice (Figure 1-3) was measured in the PB every 4 weeks for 16 weeks and in the BM and spleen after 16-27 weeks. (B) Total human chimerisms of engrafted mice by HSPCs transduced with individual lentiviral shRNA or nontransduced HSPCs (grey bars) are shown. (C) hCD45+ chimerisms in the engrafted mice of individual CBs are shown. (D) Lineage distribution of hCD45+ cells in the BM of mice transplanted with individual CB samples. (E) Simple linear regression analysis of the % mCherry+ in hCD45+ PB over time as shown in Figure 1B, C. Each symbol represents an individual mouse. Solid lines indicate line of best fit as calculated by simple linear regression analysis. β=Linear regression slope. p= p-value calculated by general linear F-test and indicates whether slope is significantly non-zero. In all relevant panels, each symbol represents an individual mouse; bars indicate mean values; error bars indicate ±SEM. n= 23 shLuc, n= 9 shLNK #1, n= 7 shLNK #2 and n= 8 shLNK #3 transplanted mice. The data are pooled from biological replicates n= 8 CBs for shLuc, 3 CBs for shLNK #1, 2 CBs for shLNK #2, and 3 CBs for shLNK #3. ND= no data due to COVID-19 shutdowns.
Supplemental Data Figure 3. LNK inhibition increases human reconstitution of all hematopoietic lineages as well as HSPCs in the spleen. CB-derived CD34⁺ HSPCs were transduced with lentiviral shRNAs to Luc or LNK, and transplanted into NBSGW mice. After 16-27 weeks, BMs and spleens from the xenotransplanted mice were analyzed for human engraftment, as described in Figure 2-3. (A) Mean % mCherry⁺ within each hematopoietic lineage of the engrafted hCD45⁺ cells in the spleen. (B) Lineage distribution of engrafted hCD45⁺mCherry⁺ cells in the spleen. (C) Mean % mCherry⁺ in the engrafted CD34⁺ compartment in the spleen. In all relevant panels, each symbol represents an individual mouse; bars indicate mean values; error bars indicate ±SEM. *: p<0.05; **: p<0.01; ***: p<0.001; ns: no significant, as determined by two-way ANOVA followed by Dunnett’s multiple comparisons to shLuc. n= 23 shLuc, n= 9 shLNK #1, n= 7 shLNK #2 and n= 8 shLNK #3 transplanted mice. The data are pooled from biological replicates n= 8 CBs for shLuc, 3 CBs for shLNK #1, 2 CBs for shLNK #2, and 3 CBs for shLNK #3.
Supplemental Data Figure 4. LNK depletion enhances engraftment of FA-like HSPCs in xenotransplanted mice. FA-like HSPCs were established and transplanted into NBSGW mice as described in Figure 5. % GFP\textsuperscript{+}mCherry\textsuperscript{+} was tracked in the hCD45\textsuperscript{+} engrafted cells in the PB of transplanted mice every 4 weeks. The graph shows mean % GFP\textsuperscript{+}mCherry\textsuperscript{+} in the hCD45\textsuperscript{+} PB cells over time. Each symbol represents an individual mouse; bars indicate mean values; error bars indicate ±SEM. *: p<0.05; **: p<0.01; ***: p<0.001; ns: not significant as determined by two-way ANOVA followed by Tukey’s multiple comparisons. n= 12 shLuc/shLuc, n= 15 shFANCD2/shLuc, n= 15 shFANCD2/shLNK. The data are pooled from 4 different biological replicate CBs.
Supplemental Data Figure 5. LNK inhibition expands primary patient-derived FA HSPCs. Primary CD34+ HSPCs were isolated from BM aspirates of healthy individuals or FA patients and transduced with lentivirus and transplanted or plated for CFC as described in Figure 6. (A) Representative CFC assay showing myeloid and erythroid lineage distribution of CFUs (mean ±SD) as in Figure 6. *: p<0.05; **: p<0.01; ***: p<0.001; ns: not significant as determined by two-way ANOVA followed by Šídák’s multiple comparisons. (B) Shows CFU numbers of nontransduced CD34+ cells and CD34+ cells expressing shLuc, indicating shRNA to Luc does not affect CD34+ cell growth. CFU numbers (mean±SD) were quantified after 12-14 days and a representative experiment is shown. ns: not significant, as determined by two-tailed students t-test. (C) HSPCs from a FA patient (FA-D1 #600.01) were transduced and transplanted into NBSGW mice as described in Figure 6. Total chimerism (hCD45+) and mCherry+ chimerism (hCD45+mCherry+) in the BM and spleen after 16 weeks is shown as stacked bars (n=1).