Supplemental Tables for

LNK(SH2B3) Regulates IL-7 Receptor Signaling in Normal and Malignant B-Progenitors

Ying Cheng [1,2], Kudakwashe Chikwava[3], Chao Wu [1], Haibing Zhang [1], Anchit Bhagat[1], Dehua Pei[4], John K. Choi [5], and Wei Tong [1,2, 5]

Materials and Methods

Expression, Purification, and Biotinylation of SH2 Domains. The DNA fragment coding for the mouse Lnk SH2 domain (amino acids 328-436) was isolated by the polymerase chain reaction (PCR) from a mouse cDNA library (Clontech) with the following primers: 5'-GGAATTCCATATGGATCACTTCCTATCCTGCTACCCCTG-3' and 5'-GTGCTCGAGGCGGCCGCGACTACCACATAGCCAGAGAGTCGG -3'. The PCR product was digested with restriction endonucleases *Nde*I and *Not*I, and ligated into the corresponding sites of prokaryotic expression vector pET22b-His-ybbR (1). This cloning procedure resulted in the addition of a six-histidine tag and a ybbR tag (DSLEFIASKLA) to the C-terminus of the Lnk SH2 domain. The authenticity of the DNA constructs was confirmed by dideoxy sequencing of the entire coding regions. Expression of the SH2 domain in *E. coli*, purification by Talon affinity chromatography, and biotinylation (enzymatically at the ybbR tag) were performed as previously described (1).

Peptide Library Screening and Hit Identification. A peptide library containing five random positions, AXXpYXXXLNBBRM-resin (where X is norleucine, L-2-aminobutyric acid, or any of the 18 proteinogenic amino acids except for Cys and Met; B = β-alanine), and 10-fold reduced ligand density on the bead surface was synthesized on 90-μm TentaGel S NH_2 resin (0.26 mmol/g) as previously described (2). A total of 180 mg of the peptide library (~500,000

beads/peptides) was screened against the Lnk SH2 domain in several separate experiments. In each screening experiment, 10-50 mg of resin was placed into a micro BioSpin column (0.8 mL, BioRad), washed extensively with methanol, ddH_2O, and HBST buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, and 0.05% Tween 20), and blocked for 1 h with 800 μL of HBST buffer containing 0.1% gelatin. The resin was drained and resuspended in 800 μL of the biotinylated Lnk SH2 domain (200 nM final concentration) in HBST buffer plus 0.1% gelatin. After overnight incubation at 4 ºC with gentle mixing, the resin was drained and re-suspended in 800 μL of SAAP buffer (30 mM Tris, pH 7.6, 1 M NaCl, 10 mM $MgCl_2$, 70 μM $ZnCl_2$, 20 mM potassium phosphate) containing 1 μL of streptavidin-alkaline phosphatase (Prozyme, ~1 mg/mL). After 10 min of gentle mixing at 4 ºC, the resin was rapidly drained and washed with 400 μL of SAAP buffer, 400 μL of HBST buffer, and 400 μL of staining buffer (30 mM Tris, pH 8.5, 100 mM NaCl, 5 mM $MgCl_2$, 20 μM $ZnCl_2$). The resin was then transferred into a 35-mm Petri dish by rinsing with 3 x 300 μL of the staining buffer and 100 μL of 5 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in the staining buffer was added. The mixture was incubated at room temperature with rotary mixing for 45 min, when the staining reaction was quenched by the addition of 1 mL of 1 M HCl. The positive (turquoise colored) beads were viewed under a dissecting microscope and manually isolated with a micropipette. A control experiment with biotinylated maltose-binding protein produced no colored beads under the same conditions. The positive beads were individually sequenced by the PED-MS method as previously described [1].

Reference:

1. Wavreille, A.-S., Garaud, M., Zhang, Y., and Pei, D. Defining SH2 domain and PTP specificity by screening combinatorial peptide libraries. *Methods.* 2007;42(3):207−19.
2. Chen, X., Tan, P. H., Zhang, Y., Pei, D. On-bead screening of combinatorial libraries: Reduction of nonspecific binding by decreasing surface ligand density. *J. Comb. Chem.* 2009;11(4): 604−11.

Supplemental Table S1. Statistical analysis of the Kaplan-Meier curves that show the survival of Tp53/Lnk mice presented in Figure 1A.

A. Sample sizes and median survivals of mice in each genotype.

Genotype	Sample size	Median survival days
p53+/-;LnkWT	51	501
p53+/-;Lnk+/-	74	469
p53+/-;LnkKO	64	397
p53KO;LnkWT	66	176
p53KO;Lnk+/-	42	146
p53KO;LnkKO	74	113

B. Log-rank (Mantel-Cox) Test analysis of the comparisons between two groups.

	Chi square	P value
p53KO;LnkWT vs. p53KO;Lnk+/-	16.81	< 0.0001
p53KO;LnkWT vs. p53KO;LnkKO	68.55	< 0.0001
p53KO;Lnk+/- vs. p53KO;LnkKO	33.87	< 0.0001
p53+/-;LnkWT vs. p53+/;Lnk+/-	0.05002	0.823
p53+/-;LnkWT vs. p53+/;LnkKO	7.34	0.0067
p53+/-;Lnk+/- vs. p53+/;LnkKO	8.107	0.0044

Supplemental Table S2. Statistical analysis of the Kaplan-Meier curves that show the survival of Ink4aArf/Lnk mice presented in Figure 1B.

A. Sample sizes and median survivals of mice in each genotype.

Genotype	Sample size	Median survival days
Ink4a/Arf+/-;LnkWT	12	575
Ink4a/Arf+/-;Lnk+/-	24	441
Ink4a/Arf+/-;LnkKO	40	340
Ink4a/ArfKO;LnkWT	18	211
Ink4a/ArfKO;Lnk+/-	13	230
Ink4a/ArfKO;LnkKO	34	147

B. Log-rank (Mantel-Cox) Test analysis of the comparisons between two groups.

	Chi square	P value
Ink4a/ArfKO;LnkWT vs. Ink4aArfKO;Lnk+/-	1.242	0.265
Ink4a/ArfKO;LnkWT vs. Ink4aArfKO;LnkKO	23.54	<0.0001
Ink4a/ArfKO;Lnk+/- vs. Ink4aArfKO;LnkKO	22.1	< 0.0001
Ink4a/Arf+/-;LnkWT vs. Ink4aArf+/;Lnk+/-	3.755	0.0526
Ink4a/Arf+/-;LnkWT vs. Ink4aArf+/;LnkKO	13.81	0.0002
Ink4a/Arf+/-;Lnk+/- vs. Ink4aArf+/;LnkKO	12.79	0.0003

Supplemental Table S3: Lnk SH2 domain specificity.

Individual sequences selected from peptide library

SG091108D19	A		K	pY	G	T	Abu
SG091108F10	A	Y	D	pY	W	L	Abu
SG091108F8	A	H	W	pY	Y	Q	Abu
SG091108D5	A	D	W	pY	Nle	G	D
SG093008M6	A	I	I	pY	Y	W	E
SG093008L17	A	D	D	pY	Abu	V	F
SG091108E8	A	H	N	pY	E	Nle	F
SG081908N6	A	H	H	pY	F	Q	F
SG091108E2	A	P	A	pY	I	R	F
SG091108E13	A	A	D	pY	W	L	F
SG081908N15	A		D	pY	W	V	F
SG081908O2	A	N	P	pY	Abu	R	I
SG093008K4	A	S	S	pY	L	R	I
SG091108E24	A	D	E	pY	V	H	I
SG091108E23	A	F	D	pY	V	L	I
SG081908N14	A	D	G	pY	V	Nle	I
SG091108E15	A	E	N	pY	V	R	I
SG093008K9	A	N	S	pY	V	R	I
SG091108D18	A	D	W	pY	A	Q	L
SG091108E21	A	F	G	pY	A	Q	L
SG091108D14	A	I	Q	pY	A	R	L
SG093008L6	A	H	H	pY	A	V	L
SG091108E3	A	P	D	pY	Abu	Abu	L
SG093008M2	A	P	L	pY	Abu	H	L
SG091108E11	A	L	G	pY	Abu	I	L
SG081908N11	A	Q	Abu	pY	Abu	K	L
SG081908N3	A	P	D	pY	Abu	L	L
SG081908N4	A	H	G	pY	Abu	L	L
SG091108D21	A	F	G	pY	Abu	N	L
SG091108D23	A	H	T	pY	Abu	R	L
SG091108E4	A	N	D	pY	Abu	R	L
SG093008L1	A	P	Q	pY	Abu	R	L
SG093008K12	A	Abu	Q	pY	Abu	Y	L
SG093008K16	A	Abu	Q	pY	Abu	Y	L
SG093008L21	A	V	Q	pY	D	I	L
SG091108E14	A	H	Nle	pY	E	A	L
SG091108E22	A	P	S	pY	E	A	L
SG093008K10	A	Y	A	pY	E	A	L
SG093008K5	A	I	F	pY	E	K	L
SG091108D2	A	E	F	pY	E	N	L
SG081908N20	A	H	Nle	pY	E	T	L
SG091108D10	A	P	V	pY	E	V	L
SG091108D15	A	D	F	pY	I	A	L
SG093008L18	A	Nle	D	pY	I	D	L
SG091108D22	A	D	S	pY	I	E	L
SG093008L16	A		Y	pY	I	K	L
SG093008M7	A	A	G	pY	I	Nle	L
SG091108F3	A	E	P	pY	I	Q	L

SG091108D17	A	Q	D	pY	I	T	L
SG093008K24	A	N	A	pY	I	T	L
SG093008M3	A	H	E	pY	I	T	L
SG093008L3	A	N	N	pY	I	W	L
SG091108E7	A	N	D	pY	L	K	L
SG091108D7	A	R	E	pY	L	Q	L
SG091108D8	A	W	N	pY	L	Q	L
SG091108F5	A	S	H	pY	N	Y	L
SG081908N9	A	E	G	pY	S	Abu	L
SG093008K17	A	P	F	pY	S	V	L
SG093008L8	A			pY	T	K	L
SG091108D9	A	F	Y	pY	T	Y	L
SG093008K22	A	V	E	pY	V	A	L
SG093008L7	A	F	D	pY	V	D	L
SG081908N8	A	G	Y	pY	V	E	L
SG093008L24	A	L	G	pY	V	F	L
SG081908N21	A	S	A	pY	V	I	L
SG093008L15	A	H	E	pY	V	I	L
SG091108D16	A	E	D	pY	V	L	L
SG091108E17	A	G	H	pY	V	N	L
SG091108D11	A	N	Q	pY	V	Nle	L
SG091108D6	A	P	F	pY	V	Q	L
SG081908N17	A	I	G	pY	V	R	L
SG093008K7	A	L	D	pY	V	V	L
SG093008K19	A	D	Abu	pY	V	V	L
SG093008M4	A	P	N	pY	V	V	L
SG093008L14	A	V	D	pY	V	Y	L
SG081908N2	A			pY	W	H	L
SG093008L5	A	E	N	pY	A	R	Nle
SG093008L10	A	Q	E	pY	Abu	F	Nle
SG091108E5	A	Y	F	pY	D	R	Nle
SG091108F7	A	H	Y	pY	D	V	Nle
SG093008M1	A	Q	W	pY	E	V	Nle
SG093008K23	A	P	Y	pY	I	H	Nle
SG091108D24	A	T	W	pY	Q	F	Nle
SG093008K13	A			pY	V	R	Nle
SG093008K18	A	L	G	pY	V	T	Nle
SG091108D1	A	Y	N	pY	Y	L	Nle
SG091108E20	A	N	G	pY	Y	W	Nle
SG081908N19	A	K	Nle	pY	R	R	P
SG093008K6	A	P	Q	pY	Abu	L	V
SG081908N16	A	P	A	pY	I	G	V
SG091108D20	A	Y	G	pY	V	E	V
SG093008K21	A	H	D	pY	V	Q	V
SG091108F1	A	Abu	Y	pY	I	H	W
SG091108E9	A	D	N	pY	V	T	W
SG091108F6	A	N	T	pY	Y	Y	W
SG081908N12	A	R	L	pY	H	I	Y
SG093008L13	A	R	K	pY	H	R	Y
SG093008L12	A	F	L	pY	I	E	Y

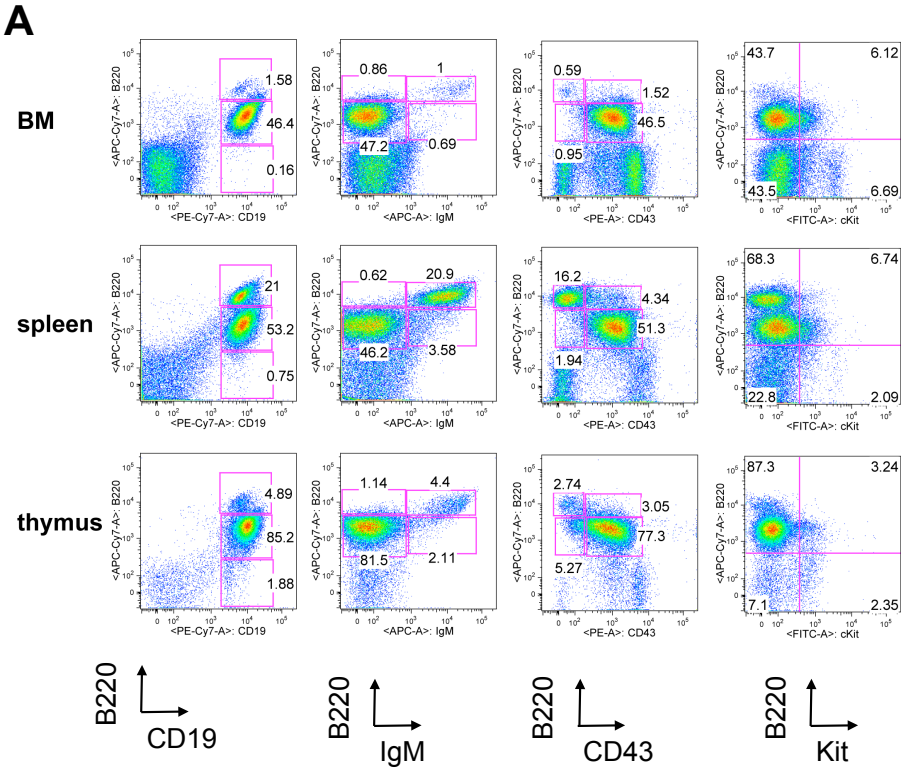
Key: Nle is norleucine (Met replacement) and Abu is 2-aminobutyrate (cysteine replacement)

Supplemental Figures for

LNK(SH2B3) Regulates IL-7 Receptor Signaling in Normal and Malignant B-Progenitors

Ying Cheng [1,2], Kudakwashe Chikwava[3], Chao Wu [1], Haibing Zhang [1], Anchit Bhagat[1], Dehua Pei[4], John K. Choi [5], and Wei Tong [1,2,5]

Figure S1. ***Tp53*$^{-/-}$*Lnk*$^{-/-}$ and *Ink4a/Arf*$^{-/-}$*Lnk*$^{-/-}$ mice develop aggressive and transplantable B-ALL.** Representative flow cytometry analysis of *Tp53*$^{-/-}$*Lnk*$^{-/-}$ **(A)** and *Ink4a/Arf*$^{-/-}$*Lnk*$^{-/-}$ **(B)** mice with B-ALL are shown.


A
BM
spleen
thymus
<APC-Cy7-A>: B220
<PE-Cy7-A>: CD19
<APC-A>: IgM
<PE-A>: CD43
<FITC-A>: cKit
1.58
46.4
0.16
0.86
1
47.2
0.69
0.59
1.52
46.5
0.95
43.7
6.12
43.5
6.69
21
53.2
0.75
0.62
20.9
46.2
3.58
16.2
4.34
51.3
1.94
68.3
6.74
22.8
2.09
4.89
85.2
1.88
1.14
4.4
81.5
2.11
2.74
3.05
77.3
5.27
87.3
3.24
7.1
2.35
B220
CD19
IgM
CD43
Kit

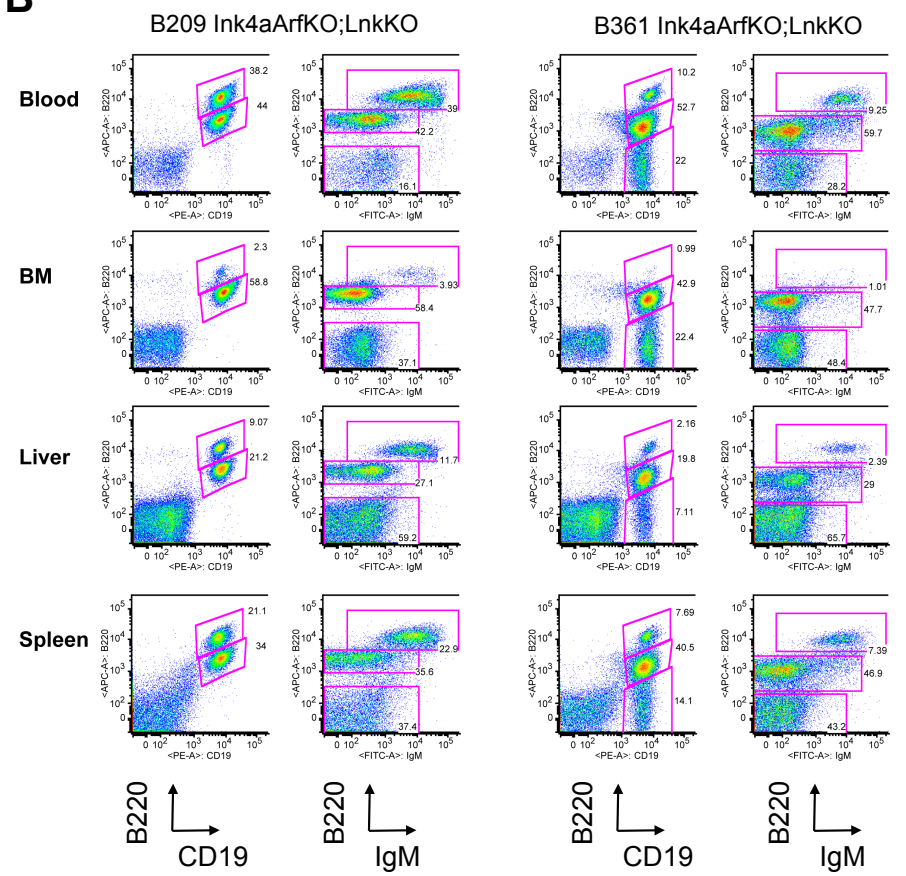

B
B209 Ink4aArfKO;LnkKO
B361 Ink4aArfKO;LnkKO
Blood
BM
Liver
Spleen
<APC-A>: B220
<PE-A>: CD19
<FITC-A>: IgM
38.2
44
39
42.2
16.1
10.2
52.7
22
9.25
59.7
28.2
2.3
58.8
3.93
58.4
37.1
0.99
42.9
22.4
1.01
47.7
48.4
9.07
21.2
11.7
27.1
59.2
2.16
19.8
7.11
2.39
29
65.7
21.1
34
22.9
35.6
37.4
7.69
40.5
14.1
7.39
46.9
43.2
B220
CD19
IgM

Figure S2. LOH analysis of the *Lnk* allele in T-lymphoma cells and cells of various lineages of *Tp53*$^{-/-}$;*Lnk*$^{+/-}$ mice. T-lymphoma and normal myeloid, T- and mature B- cells were purified by flow cytometric sorting, and subjected to PCR analysis of Lnk gene status.

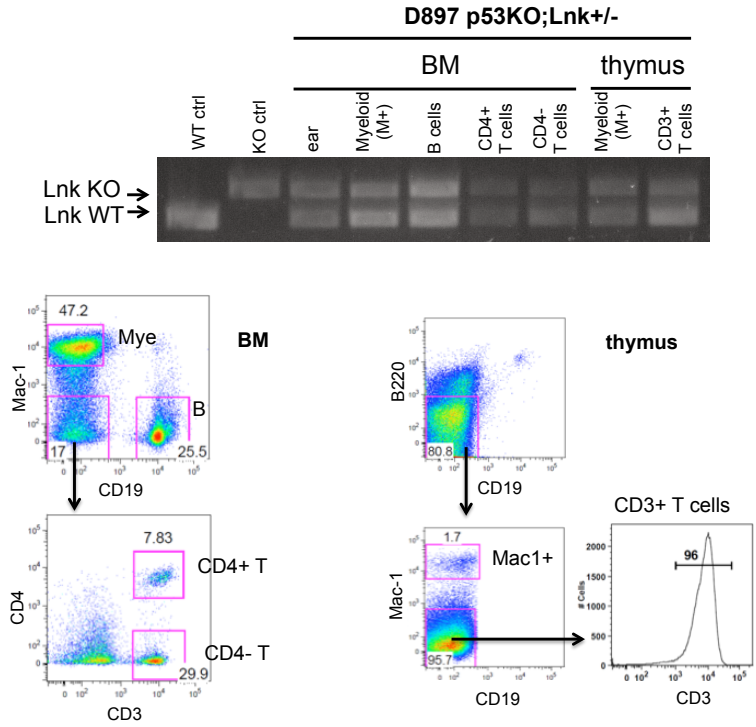


Figure S3: Immunophenotypic analysis of various multipotential progenitor cells and committed B progenitor cells. Representative flow cytometry analysis of hematopoietic stem/progenitor cells (LSK), lymphoid and myeloid-primed progenitors (LMPP), pre-proB progenitors and pro-B cells are shown.

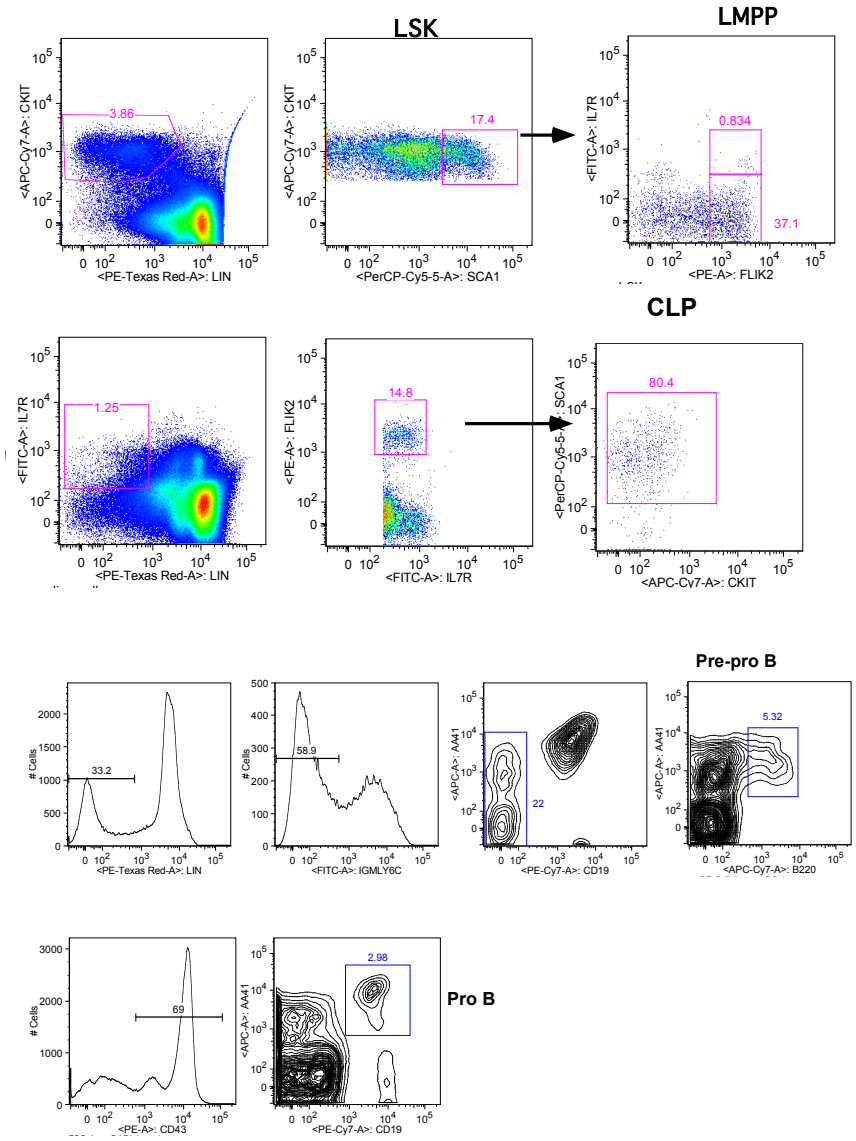


Figure S4: Lnk deficiency enhances IL-7 induced Stat5 activation. (A) BM CD19^{+}B220^{low} cells were sorted from BM for young WT, *Lnk*$^{-/-}$, *Tp53*$^{-/-}$ and preleukemic *Tp53*$^{-/-}$*;Lnk*$^{-/-}$ mice, starved and stimulated with different doses of IL-7 for 10min. Cell lysates were subjected to WB analysis with indicated antibodies. **(B)** Anti-B220 and IgM (mu heavy chain) antibodies were used to stain both fresh BM cells (upper panel) and fixed BM cells (lower panel). In fixed cells, anti-IgM antibodies stain intracellular mu chains (ICμ). Representative flow cytometry plots of fresh and fixed BM samples are shown for young WT, *Lnk*$^{-/-}$, *Tp53*$^{-/-}$, leukemic and leukemic *Tp53*$^{-/-}$*;Lnk*$^{-/-}$ mice.

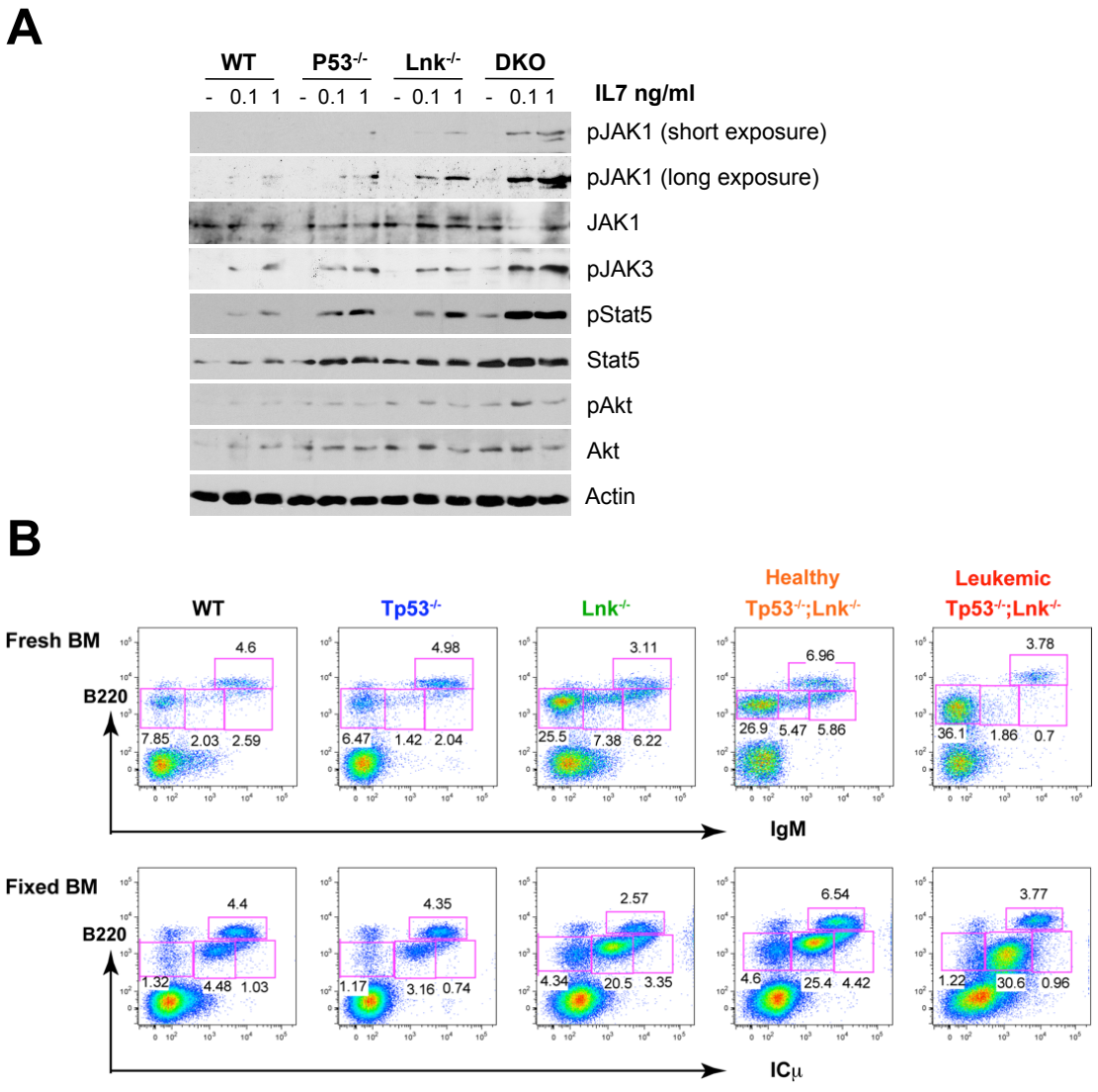


Figure S5: Bar graphs that summarize the frequencies of different amino acids in each position presented in Supplemental Table S3.

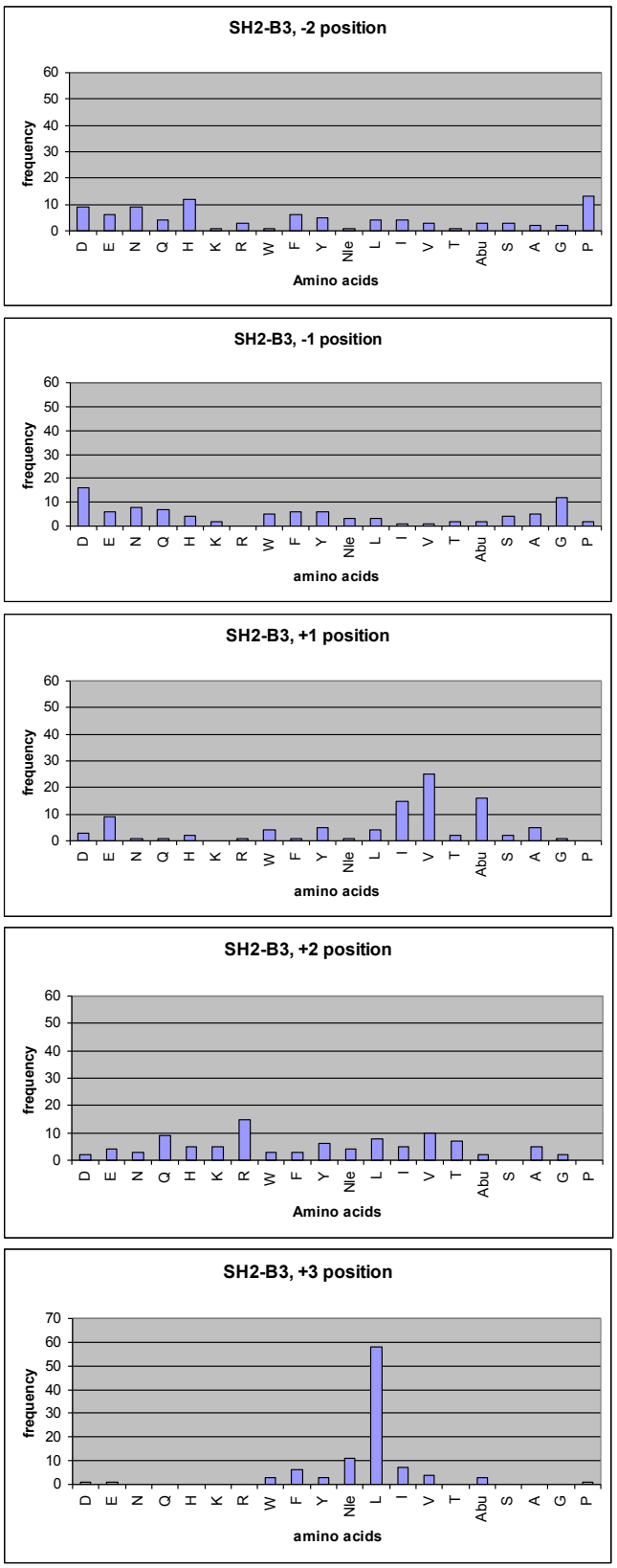
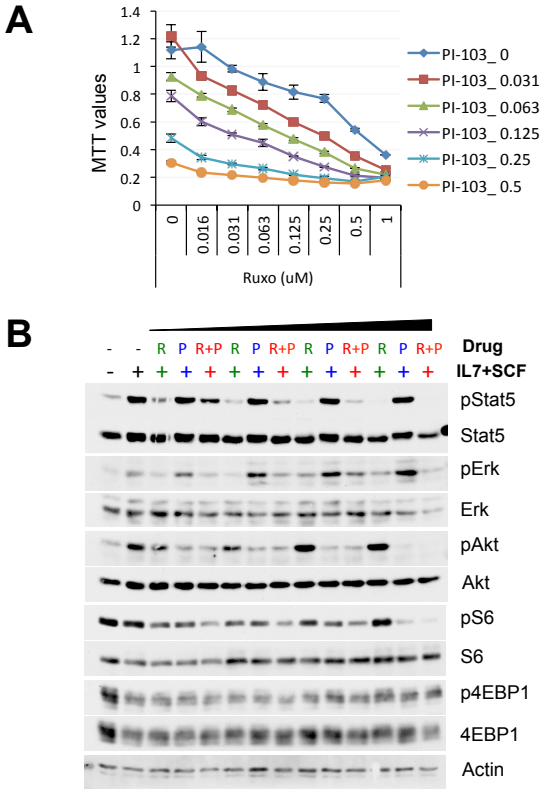


Figure S6: Testing of various *PI3K and ERK inhibitors in affecting Lnk$^{-/-}$p53$^{-/-}$ leukemic cell growth and signaling*. (**A**) Growth response curves for B-ALL cells treated with various concentrations of JAK and pan-PI3K inhibitors. *Lnk$^{-/-}$p53$^{-/-}$* leukemic blasts were cultured in liquid culture supplemented with SCF and IL7 in the presence of different concentrations of inhibitors as indicated. Live cell numbers 3 d of culture were determined by MTT absorbance. (**B**) WB analyses for effector proteins of the Stat5, MEK/MAPK and PI3K/Akt pathways. *Lnk$^{-/-}$p53$^{-/-}$* leukemic blasts were stimulated with SCF and IL-7 for 20 min in the absence or presence of pre-treatment with different concentrations of inhibitors. Cells were lysed and the protein lysates were subjected to WB analysis with indicated antibodies. (**C**) Growth response curves for B-ALL cells treated with various concentrations of JAK inhibitors along with different PI3K isoform-specific inhibitors. (**D**) Growth response curves for B-ALL cells treated with various concentrations of JAK inhibitors along with two different MEK inhibitors. MTT assays were set up as described in (A).


A
MTT values
Ruxo (uM)
PI-103_ 0
PI-103_ 0.031
PI-103_ 0.063
PI-103_ 0.125
PI-103_ 0.25
PI-103_ 0.5
B
Drug
IL7+SCF
pStat5
Stat5
pErk
Erk
pAkt
Akt
pS6
S6
p4EBP1
4EBP1
Actin
C
MTT values
Ruxo (uM)
BYL719_ 0
BYL719_ 0.078
BYL719_ 0.156
BYL719_ 0.313
BYL719_ 0.625
BYL719_ 1.25
BYL719_ 2.5
BYL719_ 5
TGX221_ 0
TGX221_ 0.63
TGX221_ 1.25
TGX221_ 2.5
TGX221_ 5
TGX221_ 10
CAL101_ 0
CAL101_ 0.031
CAL101_ 0.063
CAL101_ 0.125
CAL101_ 0.25
CAL101_ 0.5
CAL101_ 1
AS-605240_ 0
AS-605240_ 0.031
AS-605240_ 0.063
AS-605240_ 0.125
AS-605240_ 0.25
AS-605240_ 0.5
AS-605240_ 1
AS-605240_ 2
D
MTT values
Ruxo (uM)
Selumetinib_ 0
Selumetinib_ 0.004
Selumetinib_ 0.008
Selumetinib_ 0.016
Selumetinib_ 0.031
Selumetinib_ 0.063
Selumetinib_ 0.125
Selumetinib_ 0.25
Trametinib_ 0
Trametinib_ 0.001
Trametinib_ 0.003
Trametinib_ 0.01
Trametinib_ 0.03
Trametinib_ 0.1
Trametinib_ 0.3
Trametinib_ 1