

Supplemental Data

Loss of β -Catenin Impairs the Renewal of Normal and CML Stem Cells In Vivo

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Supplemental Experimental Procedures

Western Blotting

Total bone marrow cells from control or β -catenin knockout mice or sorted GFP+ leukemia cells from mice receiving BCR-ABL transduced wild type or β -catenin knockout cells were lysed in RIPA buffer. 50 mg (Figure 1) or 10-15 mg (Figure 7) of protein extracts were separated on polyacrylamide gel, transferred to PDVF membranes and probed with antibodies specific for the C-terminal of mouse β -catenin or mouse anti- Abl (BD Transduction Laboratories) or anti-phosphotyrosine (clone 4G10, Upstate). The same membrane was re-probed with rabbit anti- β actin antibody (Santa Cruz) as a loading control.

Northern Blotting

Total RNA was isolated from freshly sorted GFP+ tumor cells using the RNeasy Mini Kit (Qiagen, cat. no. 74104). For analysis of β -catenin transcript levels, 5 mg of purified RNA from β -catenin $-/-$ and control tumor cells was run on a denaturing formaldehyde-agarose gel electrophoresis buffered with 1X MOPS (3-N-morpholinopropanesulfonic acid) (20 mM MOPS [pH 7.0-7.5], 5 mM sodium acetate, 1 mM EDTA). RNA was blotted in 10X SSC (1.5 M NaCl, 0.15 M sodium citrate [pH 7.0]) using a Nytran SuPerCharge Turboblotter system (Whatman, cat. no. 10416306). Membrane prehybridization and hybridization were performed in ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion). Hybridization probes were generated by PCR amplification using the oligonucleotide primers 5'-CGG GCA GTA TGC AAT GAC TAG G-3' and 5'-GCA GGA GAT TAT GCA GTG TCG T-3' and subsequently purified using the QIAquick PCR Purification Kit (Qiagen). The amplicons generated were radiolabeled with [α - 32 P]dCTP by random priming. mRNA levels were quantified by Storm 840 PhosphorImager analysis (Amersham Biosciences).

Southern Blotting

Genomic DNA was isolated from spleens and/or bone marrow using the Dneasy Tissue kit (Qiagen, cat. no. 69506). 30 ug of DNA for preleukemic cells and 10 ug of DNA for leukemic cells were digested with EcoRI and was run on a 1 % of agarose gel electrophoresis buffered with 1 X TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). DNA was blotted in 10X SSC (1.5

M NaCl, 0.15 M sodium citrate [pH 7.0]) using a Immobilon- NY+ (Millipore, cat. no. INYC00010). Membrane prehybridization and hybridization were performed in ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion) with 100 ug of salmon sperm DNA (Ambion, cat. no. AM9680). Hybridization probes were generated by PCR amplification using the oligonucleotide primers 5'- ATG GTG AGC AAG GGC GAG GAG CTG -3' and 5'- GTC GAC TCT AGA GTC GCG GCC GCT TTA CTT GTA CA -3' and subsequently purified using the QIAquick PCR Purification Kit (Qiagen). The amplicons generated were radiolabeled with [α -³²P]dCTP by random priming. The blots were scanned by Storm 840 PhosphorImager analysis (Amersham Biosciences).

Viral Production and transduction of bone marrow cells

The MSCV-BCR-ABL-IRES-GFP and control MSCV-IRES-GFP vectors have been described previously (Pear et al., 1998). 293T cells were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin/streptomycin, 2mmol/L L-glutamine, and nonessential amino acids. For transient transfection p210BCR-ABL or the control vector were introduced into 293T cells using Fugene 6 along with gag-pol and VSVG constructs. Viral supernatant was collected for two days and concentrated 100 fold by ultracentrifugation at 19,000 rpm. Control or β -catenin knockout bone marrow cells were harvested, and cells were enriched by auto-MACS (Miltenyi) using anti-c-kit antibody magnetic beads. Enriched cells were then cultured overnight in DMEM plus 15% FCS, 5% WEHI-conditioned medium, 50 ng/ml mouse stem cell factor, 10 ng/ml mouse interleukin-6 and mouse interleukin-3 (R&D Systems) as described (Li et al., 1999; Pear et al., 1998). After pre-stimulation, viable cells were counted and transduced with retroviral stocks in the same medium containing 20% retroviral supernatant, 4mg/ml of polybrene. 48 h later, cells were harvested, washed and $2-5 \times 10^5$ cells were transplanted into lethally irradiated syngeneic recipient mice. Retroviral infection efficiency was confirmed by FACS analysis, and ranged from 10-25% between experiments. Efficiency of transduction of control and knockout cells was equivalent within each experiment. In some experiments, sorted control or β -catenin knockout KLSF cells were used and infected with BCR-ABL exactly the same as when using c-kit enriched cells and equivalent to 400 to 600 BCR-ABL infected KLSF cells were transplanted per mouse.

Real-Time and RT-PCR analysis

RNA was isolated using RNAqueous-Micro (Ambion) and converted to cDNA using Superscript II (Invitrogen). cDNA concentrations were measured with a fluorometer (Turner Designs) using RiboGreen reagent (Molecular Probes). Quantitative real-time PCR was performed using an iCycler (BioRad) by mixing equal amounts of cDNAs, iQ SYBR Green Supermix (BioRad) and gene specific primers. Primer sequences are available upon request. All real time data was normalized to actin.

Primers used to assess integration of lentiviral wnt reporter

Forward 5'- GAGAAAGGTACTAGTCGCCACCAT-3' and reverse 5'- TCACCTTGTAGATGAAGCAGCCGT-3'.

Statistical analysis

Student's t-test was utilized to determine statistical significance. *P* values less than 0.05 were

considered significant.

Supplemental References

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Pear, W. S., Miller, J. P., Xu, L., Pui, J. C., Soffer, B., Quackenbush, R. C., Pendergast, A. M., Bronson, R., Aster, J. C., Scott, M. L., and Baltimore, D. (1998). Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood* 92, 3780-3792.

Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M. M., Birchmeier, W., Tenen, D. G., and Leutz, A. (2006). Hematopoietic stem cell and multilineage defects generated by constitutive β -catenin activation. *Nat Immunol* 7, 1037-1047.

Figure S1. Comparative analysis of cell cycle status of control and β -catenin $-/-$ KLSF cells KLSF cells were sorted, fixed with 70% ethanol, stained with Propidium Iodide and DNA content analyzed by FACS. Top shows representative cell cycle profiles for control and β -catenin knockout cells. Bottom shows average frequency of cells in G1, S or G2/M phases in control (n=3) and β -catenin knockout (n=4) KLSF cells. Error bars show s.e.m.

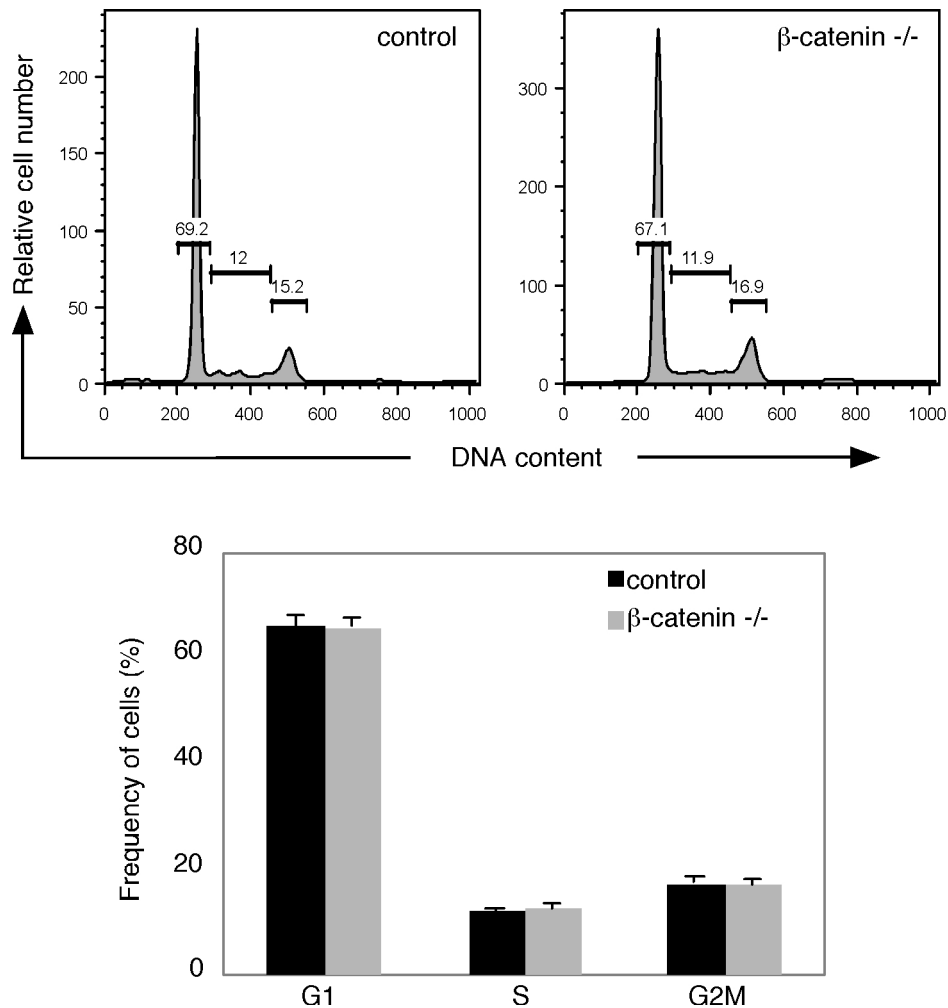


Figure S2. Relative distribution of mature cells, CLP, CMP and GMP in control and β -catenin knockout mice (A)(B) Bone marrow cells from control or β -catenin knockout mice were stained with lineage markers, IL7 receptor, Thy1, c-kit and Sca1 for analysis of CLP. Plots show representative gates for CLP (A) and average frequency over 3 independent experiments (B). (C-E) Bone marrow cells from control or β -catenin knockout mice were stained with lineage markers, IL7 receptor, Thy1, c-kit, Sca1 as well as CD34 and FcR γ II/III. (C) Representative frequencies of CMP, GMP and MEP and average frequencies of CMP (D) and GMP (E) over 3 independent experiments. In (B), (D) and (E) error bars show s.e.m.

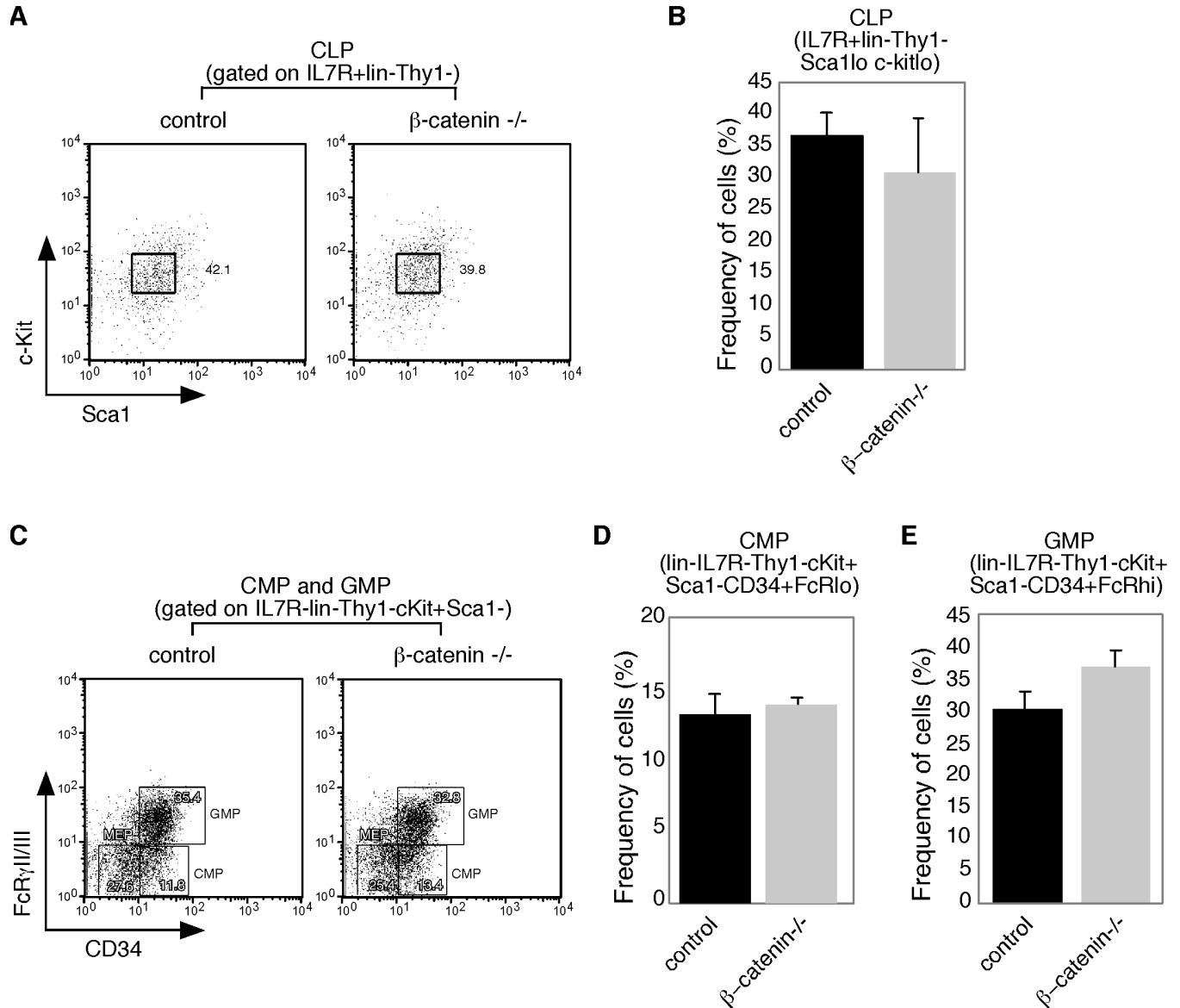


Figure S3. Comparative transplantation efficiency of KLSF cells from β -catenin knockout (loxp/loxp, Cre/+), β -catenin heterozygote (loxp/+, Cre/+) and control (loxp/loxp, +/+) mice. 500 KLSF cells from β -catenin knockout (loxp/loxp, Cre), β -catenin heterozygote (loxp/+, Cre) and control (loxp/loxp) mice were transplanted in the presence of competing bone marrow cells into lethally irradiated congenic recipients and donor derived chimerism monitored for 16 weeks. Graph shows average chimerism of twelve mice in each group from two independent experiments. Error bars show s.e.m.

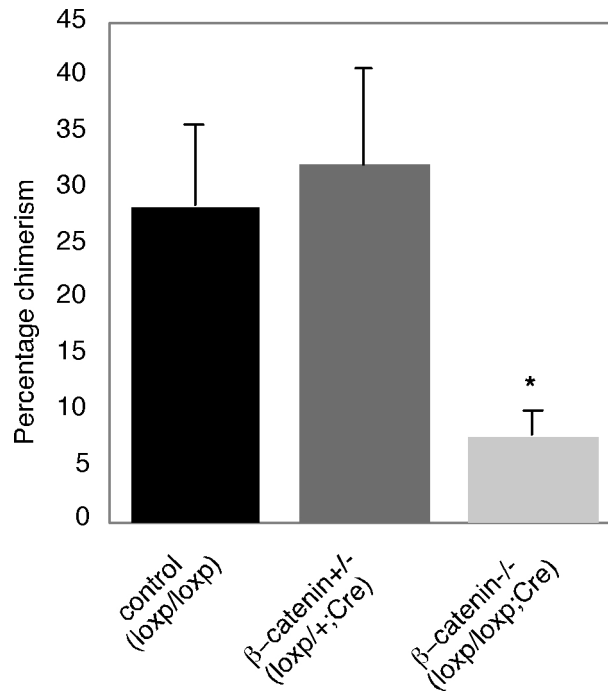


Figure S4. Primary and secondary limiting-dilution competitive repopulation assay

(A) Limiting-dilution competitive repopulation analysis of control and β -catenin knockout mice. Eight mice were transplanted at each dose (10, 20, 40 and 50 cells/recipient for a total of 64 recipients for both genotypes). Peripheral blood cells of the all surviving recipients were analyzed 10 weeks after transplantation and chimerism for individual recipient mice at each dose is shown. (B) Secondary limiting-dilution competitive repopulation analysis of control and β -catenin knockout mice was carried out using the 50 cells/recipient group of primary transplanted mice at 21 weeks. Whole bone marrow cells were isolated from primary donors and transplanted into 5-7 recipients at each dose (10,000, 40,000, 80,000 and 160,000 cells/recipient for a total of 48 recipients). Peripheral blood cells of the secondary recipients were analyzed 10 weeks after transplantation and chimerism for individual recipient mice at each dose is shown.

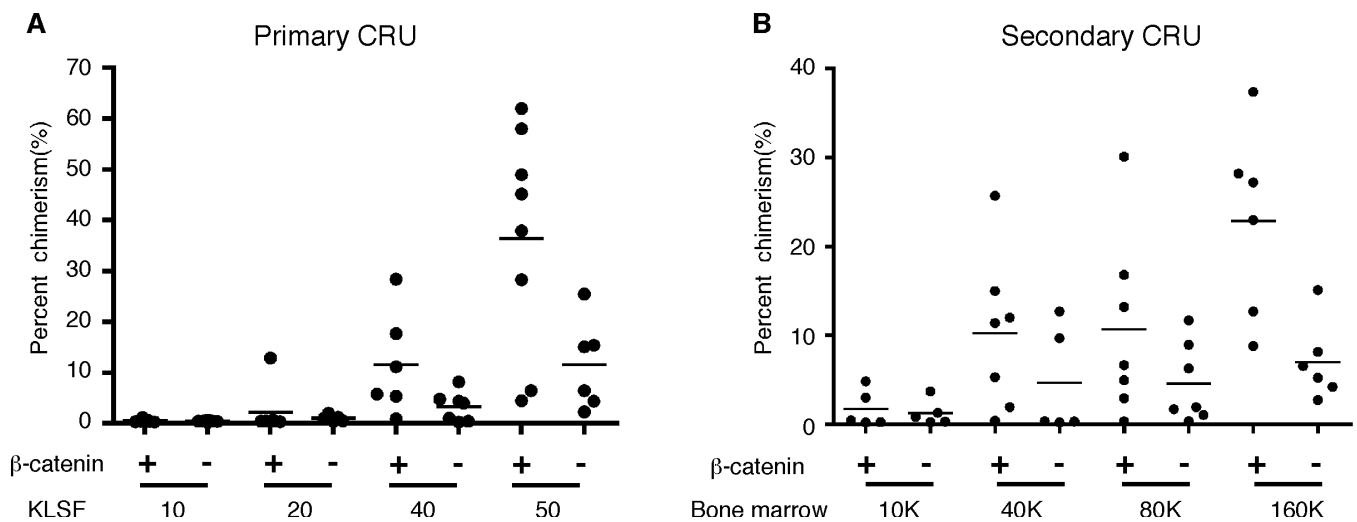


Figure S5. Relative engraftment ability of BCR-ABL transduced control and β -catenin $^{-/-}$ cells C-kit enriched bone marrow cells from control or β -catenin $^{-/-}$ mice were transduced with BCR-ABL-IRES-GFP virus and transplanted into allelically mismatched irradiated recipients (n = 6 per group). Peripheral blood cells were analyzed ten days post transplant for frequency of donor derived GFP $^{+}$ cells. Error bars show s.e.m.

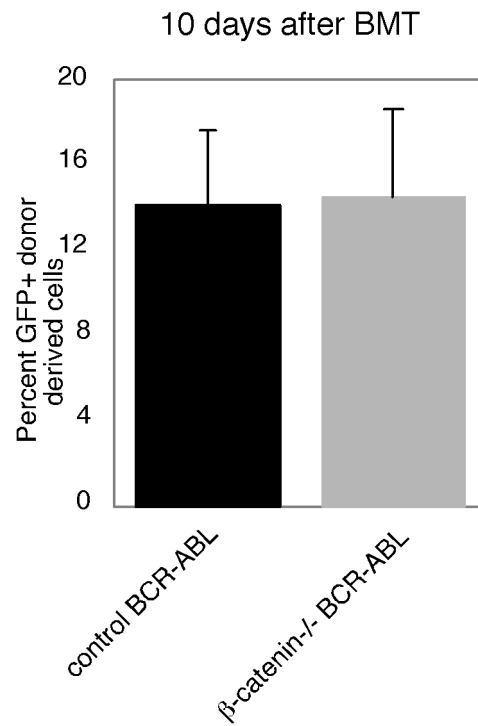


Figure S6. Retroviral integration analysis of BCR-ABL infected control and β -catenin/- cells from preleukemic and leukemic mice (A) Cells from spleens and matched bone marrow of mice transplanted with BCR-ABL transduced control or β -catenin knockout cells were sorted 3 weeks after transplant and genomic DNA isolated. Subsequently, the DNA was digested with EcoRI, electrophoresed, blotted and hybridized with ^{32}P labeled probe for GFP. (B) Cells from spleens of mice with control or β -catenin knockout CML were collected and genomic DNA isolated. Subsequently, the DNA was digested with EcoRI, electrophoresed, blotted and subsequently hybridized with ^{32}P labeled probe for GFP. Bands indicate unique insertion sites.

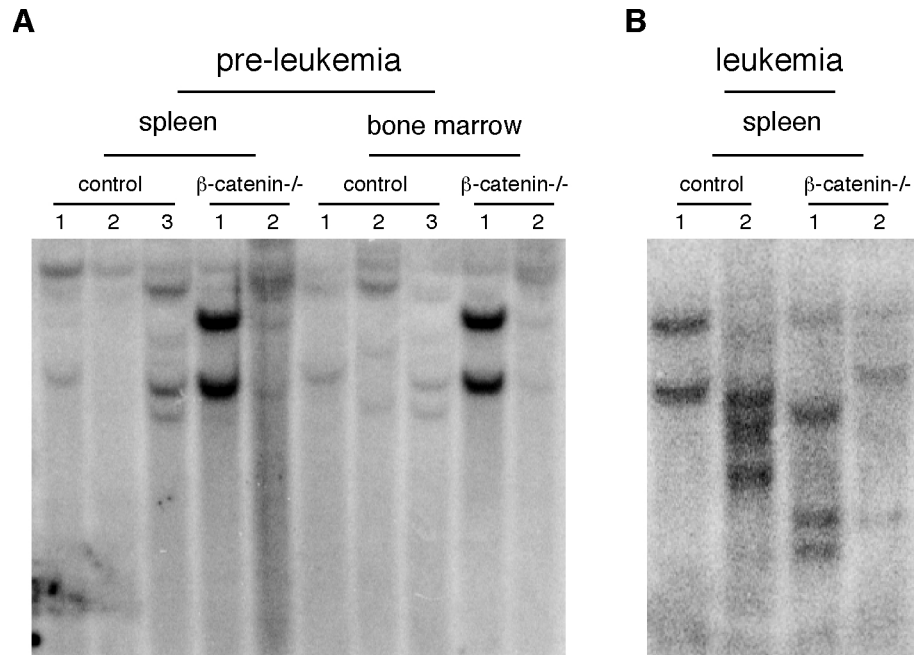


Figure S7. Characterization of ALL induced by BCR-ABL transduction of β -catenin knockout cells (A) Phenotype of primary ALL in bone marrow using BCR-ABL transduced β -catenin knockout cells as determined by FACS analysis. (B) Survival curve of mice serially transplanted with β -catenin knockout ALL. A total of 2 million mixed bone marrow and spleen (1:1) cells were transplanted into each recipient. Data shown is the combination of two independent experiments in which 100% of transplanted mice (9/9) developed B-ALL. (C) Phenotype of secondary leukemias in bone marrow derived from primary ALL.

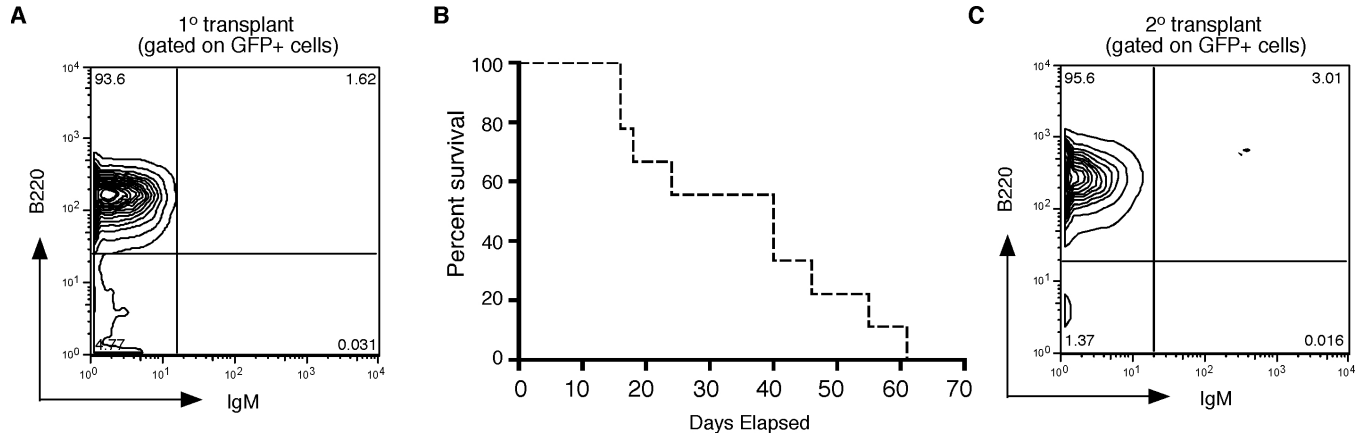
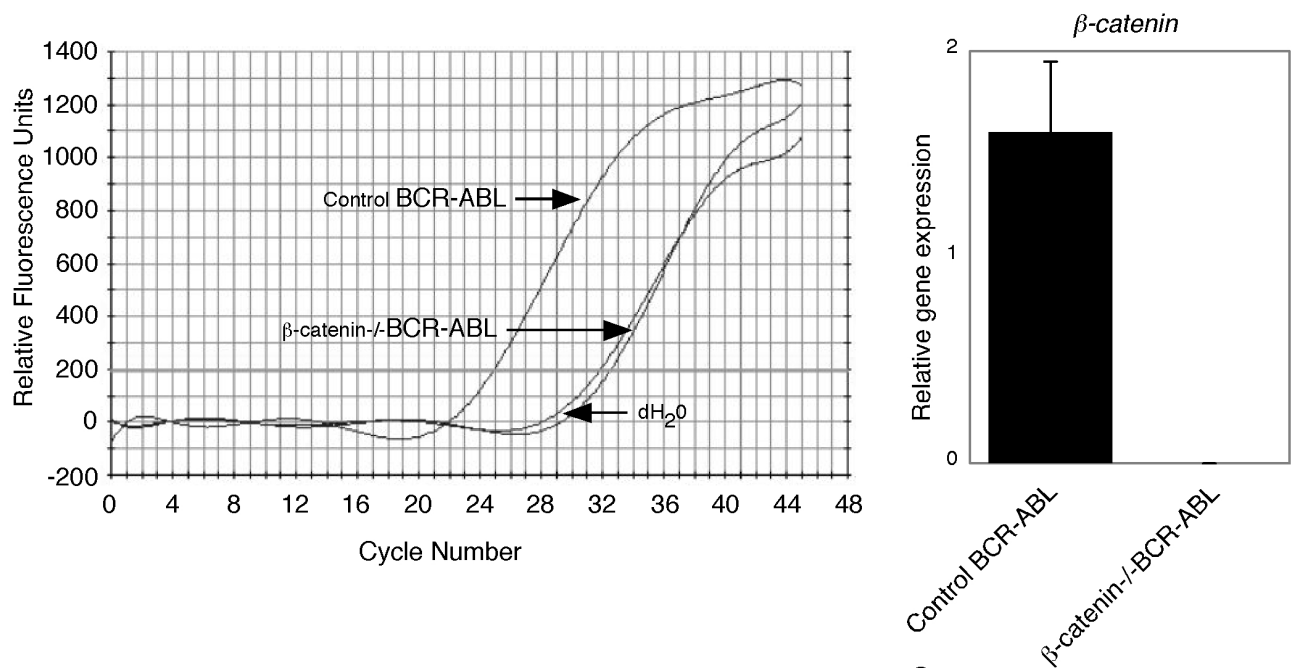
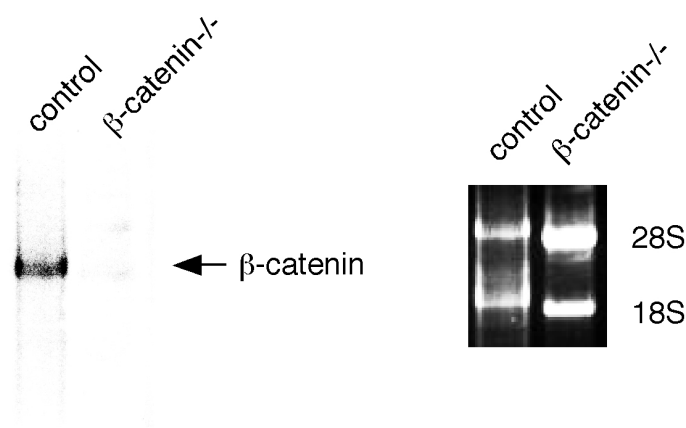


Figure S8. Analysis of β -catenin deletion in β -catenin $-/-$ leukemias (A) Real-time PCR analysis shows levels of β -catenin in ALL derived from β -catenin knockout mice as compared to ALL or CML from control mice. Left, representative amplification curve; Right, Average of relative gene expression level. Data is representative of 3 independent experiments. Error bars show s.e.m. (B) Northern blot analysis of β -catenin expression in control and β -catenin $-/-$ leukemias. 5 μ g of RNA from control or β -catenin $-/-$ leukemias were loaded and hybridized with a 32 P labeled β -catenin probe (left). 28S and 18S bands are shown as loading control (right). (C) Western blot analysis of expression of β -catenin protein in control and β -catenin $-/-$ leukemia cells. 10 to 15 μ g of protein lysate from control and β -catenin $-/-$ leukemia cells were electrophoresed, transferred onto PVDF membranes and blotted with an anti- β -catenin antibody (n=3).

A



B



C

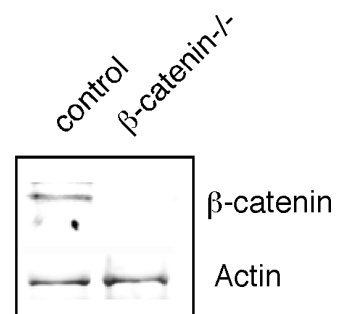


Figure S9. Target cells for transformation by BCR-ABL are equivalent in control and β -catenin $^{-/-}$ bone marrow. (A-C) c-kit enriched cells from control or β -catenin $^{-/-}$ mice were analyzed by flow cytometry to determine the relative frequency of lineage $^{-}$, KLSF $^{+}$ and KLSF $^{-}$ populations. Results are representative of three independent experiments **(D)** c-kit enriched control or β -catenin knockout cells were infected with BCR-ABL for 48 hours, GFP $^{+}$ cells re-sorted and subsequently cultured for 7 days. Frequency of myeloid (Mac1 $^{+}$) and B lineage (B220 $^{+}$) cells were analyzed by flow cytometry. Results are representative of three independent experiments. In **(A-D)**, error bars show s.e.m. **(E)** Survival curve of mice transplanted with BCR-ABL transduced KLSF cells. Data shows two independent experiments carried out using an estimated 400-600 infected KLSF cells (based on infection efficiency). 8 of 8 mice receiving BCR-ABL transduced control cells developed leukemia, among them 7 were CML, while only 1 of 8 mice receiving BCR-ABL transduced β -catenin knockout cells developed leukemia (CML).

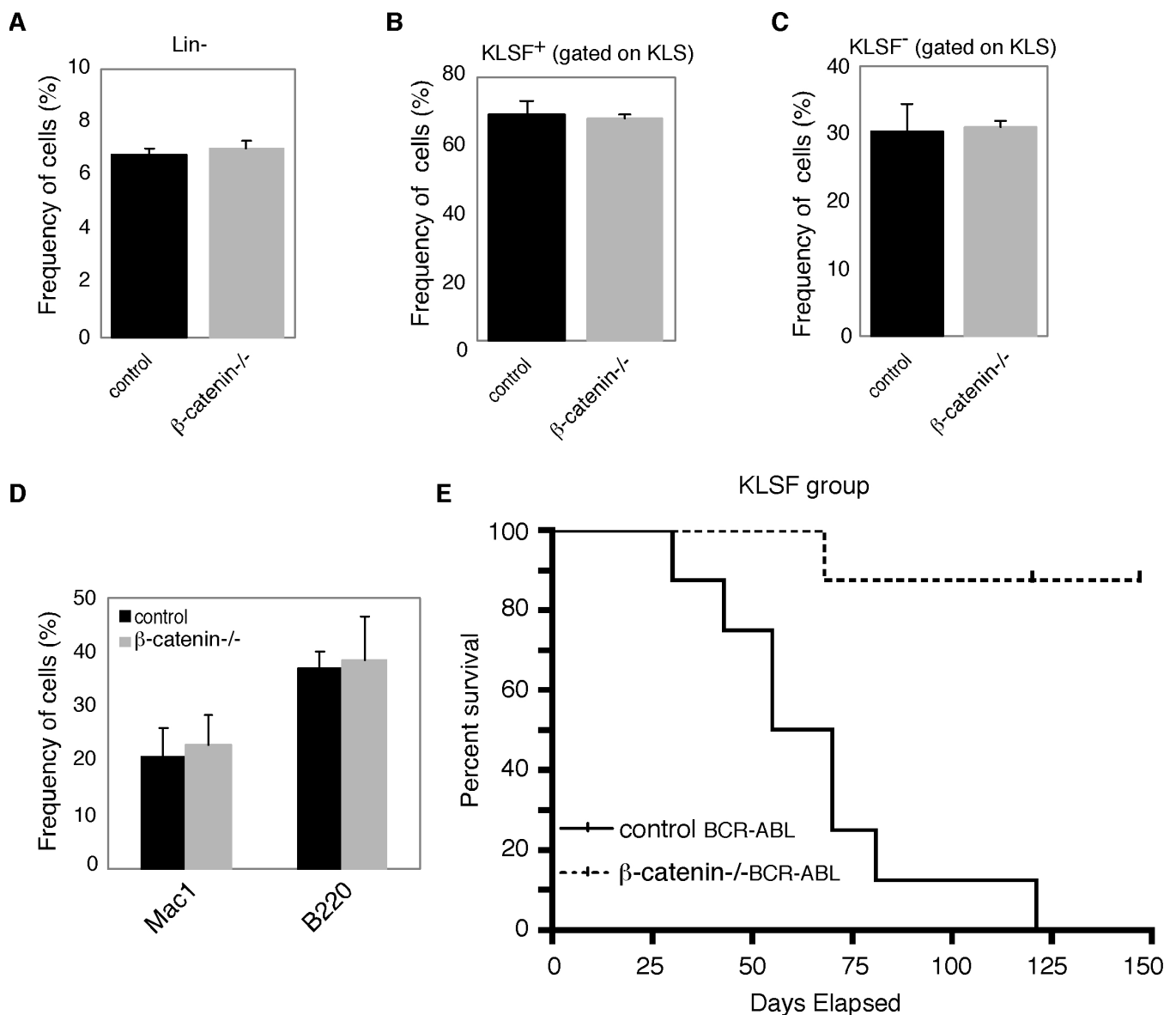


Figure S10. Ectopic expression of activated β -catenin complements the defect in CML formation in BCR-ABL transduced β -catenin knockout cells (A) Control and β -catenin $^{-/-}$ HSCs were infected with BCR-ABL YFP in the presence of vector GFP or β -catenin GFP. Subsequently 10,000 sorted control BCR-ABL $^{+}$ constitutively active (CA) β -catenin $^{+}$, control BCR-ABL $^{+}$ vector $^{+}$, β -catenin $^{-/-}$ BCR-ABL $^{+}$ CA β -catenin $^{+}$, and β -catenin $^{-/-}$ BCR-ABL $^{+}$ vector $^{+}$ cells were transplanted into 6-7 lethally irradiated recipients per cohort over 2 independent experiments, and their survival tracked over time. (B) Representative example of BCR-ABL and β -catenin expression (left panel) and B220 and Mac-1 expression (right panels) in leukemias derived from control or β -catenin knockout cells infected with BCR-ABL YFP and ectopic β -catenin GFP. (C) GFP $^{+}$ CML cells derived from control BCR-ABL $^{+}$ vector $^{+}$, control BCR-ABL $^{+}$ CA β -catenin $^{+}$, β -catenin $^{-/-}$ BCR-ABL $^{+}$ vector $^{+}$ and β -catenin $^{-/-}$ BCR-ABL $^{+}$ CA β -catenin $^{+}$ transplanted recipients were sorted and real time PCR analysis carried out to determine differential expression of β -catenin. It should be noted that the rescue was successful only if the ectopic β -catenin levels were mid/low; however high levels of β -catenin not only did not rescue the defect (at least upto the time point analyzed) but also appeared to impair the leukemia formation of control cells. This observation may help explain the data observed with transgenic mice overexpressing high levels of β -catenin in which following an expansion of a phenotypic HSC population there was a loss of differentiated hematopoietic cells (Kirstetter et al., 2006; Scheller et al., 2006). Thus it is possible that the level of β -catenin is critical to determining its' impact on a cell, and that high levels of β -catenin can be detrimental to the growth or survival of cells while low/mid levels can promote growth and support oncogenesis. Error bars show s.e.m.

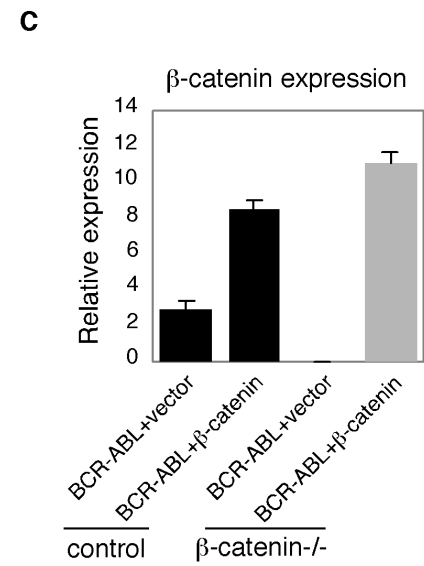
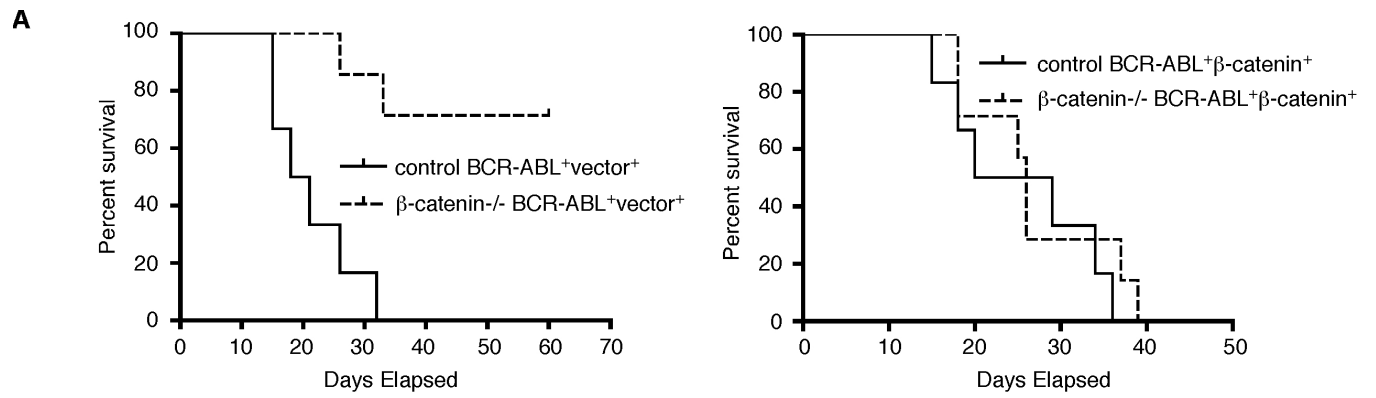


Figure S11. BATRED functions as a Wnt/ β -catenin reporter and turns on DsRED upon introduction of activated β -catenin. (A-C) 293T cells were transfected with BATRED and MSCV-IRES-GFP vector control or (D-F) BATRED and MSCV-CA β -catenin-IRES-GFP. (A, D) GFP expression. (B, E) DsRED expression. (C, F): merged image of GFP and DsRED expression. When BATRED was co-transfected with MSCV-IRES-GFP, weak expression of DsRED was observed in 16% of GFP expressing cells (540 cells counted). When BATRED was co-transfected with MSCV-CA β -catenin-IRES-GFP, 84% of GFP expressing cells were also expressing DsRED (677 cells counted). Scale bar represents 10 μ m.

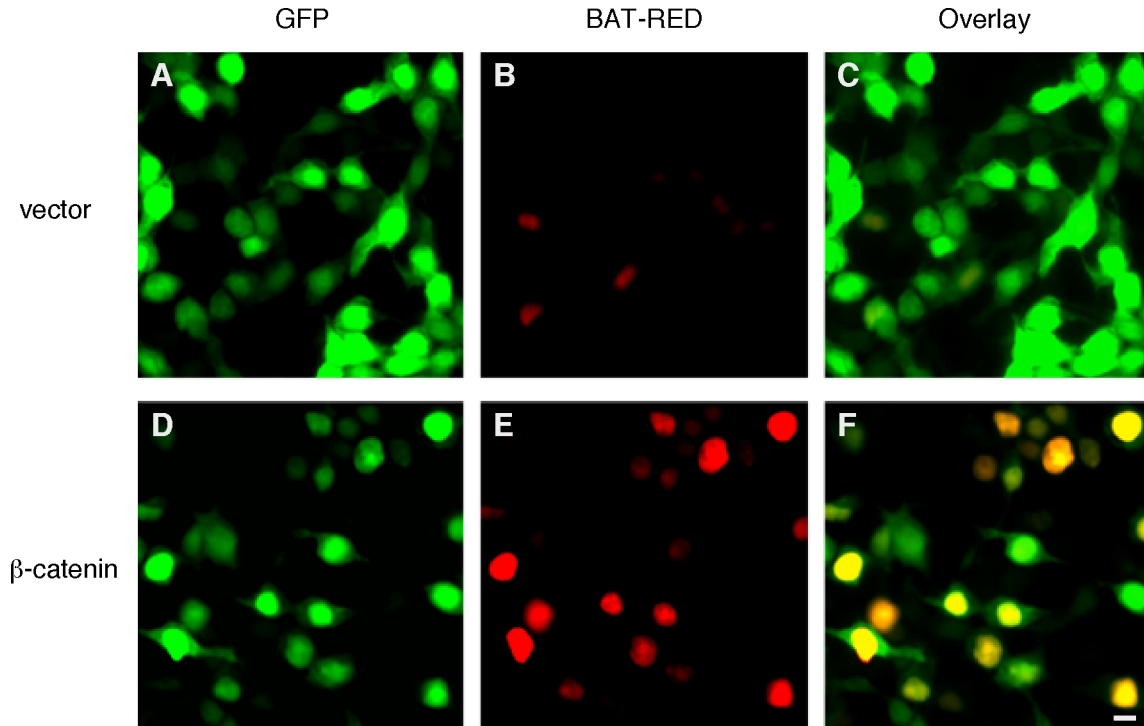


Figure S12. Expression of CEBP α , Id1 and Pax5. Real-time RT-PCR analysis of expression of CEBP α , Id1 and Pax5 in sorted BCR-ABL infected control or β -catenin knockout c-kit enriched cells. Data from 5 independent groups of samples are shown as fold induction in β -catenin $^{-/-}$ cells relative to control controls following normalization to actin expression. Note that no difference between BCR-ABL transduced control and β -catenin knockout cells. Error bars show s.e.m.

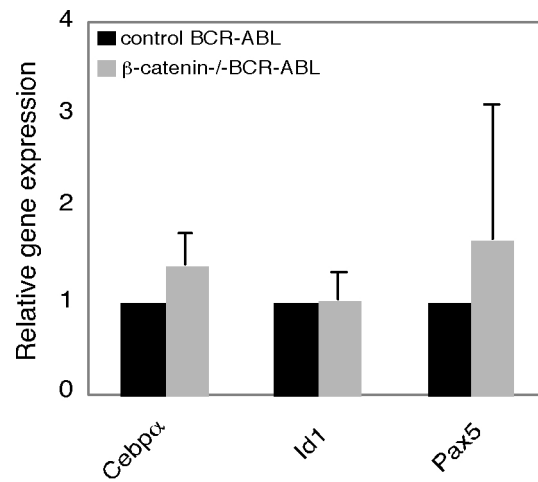


Figure S13. Phenotype of leukemias derived from BCR-ABL transduced B220+IgM- cell population. (A) Survival curve of mice receiving an estimated 30,000 infected BCR-ABL transduced B220+IgM- B cell progenitors. One out of 5 receiving BCR-ABL transfected β -catenin knockout cells and 2 out of 5 receiving control cells developed B cell leukemia **(B)** Phenotype of leukemias as determined by FACS.

