www.sciencetranslationalmedicine.org/cgi/content/full/6/238/238ra71/DC1

Supplementary Materials for

Hematopoietic Stem Cell Origin of *BRAF*V600E Mutations in Hairy Cell Leukemia

Stephen S. Chung, Eunhee Kim, Jae H. Park, Young Rock Chung, Piro Lito, Julie Teruya-Feldstein, Wenhuo Hu, Wendy Beguelin, Sebastien Monette, Cihangir Duy, Raajit Rampal, Leon Telis, Minal Patel, Min Kyung Kim, Kety Huberman, Nancy Bouvier, Michael F. Berger, Ari M. Melnick, Neal Rosen, Martin S. Tallman, Christopher Y. Park,* Omar Abdel-Wahab*

*Corresponding author. E-mail: parkc@mskcc.org (C.Y.P.); abdelwao@mskcc.org (O.A.-W.)

Published 28 May 2014, *Sci. Transl. Med.* **6**, 238ra71 (2014)
DOI: 10.1126/scitranslmed.3008004

The PDF file includes:

Supplementary Materials:

Supplementary Materials and Methods:

Antibodies and FACS analysis of human and murine cells.

All FACS antibodies (for both human and mouse studies) were purchased from BD-Pharmingen or eBioscience. For human studies, BM mononuclear cells were stained with a cocktail of PECy5-conjugated lineage antibodies including CD3 (HIT3a), CD4 (RPA-T4), CD8 (RPA-T8), CD14 (TuK4), GPA (HIR2), and CD56 (B159). CD10 APCCy7 (HI10a) and CD19 PerCPCy5.5 (CC2C6) were excluded from the lineage cocktail and stained with different fluorochromes to assess B-lymphoid progenitor frequencies. CD34 Pacific Blue (581), CD38 PECy7 (HIT2), CD90 PE (5E10), and CD45RA Alexa700 (HI100) were used to sort CD34+CD38-CD90+CD45RA- HSCs, as well as CD34+CD38+ progenitors, excluding the above mentioned lineage markers including CD10 and CD19. CD123 APC (6H6) was used along with CD45RA Alexa700 to fractionate CD34+CD38+ cells into myeloid progenitor subsets. Hairy cells were sorted using CD11c Biotin (Clone 3.9), CD103 FITC (B-Ly7), and CD19 PerCPCy5.5 (CC2C6) (with Streptavidin Qdot secondary). Peripheral blood mononuclear cells were stained with CD19 PerCPCy5.5 (HIB19), CD11c Biotin (Clone 3.9), CD103 FITC (B-Ly7), CD25 PE (BC96), CD3 PECy5 7(SK7), and CD14 Pacific Blue (TuK4) to assess recovery of normal myeloid and T-cell populations. Hairy cell leukemic cells were sorted based on expression of CD19, CD11c, CD103, and CD25.

For mouse studies, BM or spleen mononuclear cells were stained with a lineage cocktail comprised of antibodies targeting CD4, CD8, B220, NK1.1, Gr-1, CD11b, Ter119, and IL-7Rα (all conjugated to APCCy7). Cells were also stained with antibodies against c-Kit PE, Sca-1 APC, FcγRII/III Pacific Blue, and CD34 FITC. Cell populations were analyzed using a FACS-Fortessa (Becton Dickinson) and sorted with a FACSAria II instrument (Becton Dickinson). We used the following antibodies: c-Kit PE (2B8), Sca-1 APC (D7), Mac-1/CD11b APCCy7

(M1/70), Gr-1 APCCy7 (RB6-8C5), NK1.1 APCCy7 (PK136), Ter-119 APCCy7, IL7-Rα FITC (A7R34), CD34 FITC (RAM34), FcγRII/III Pacific Blue (2.4G2), CD4 APCCy7 (RM4-5), CD4 APCCy7 (H129.19), CD8 APCCy7 (53-6.7), CD45.1 FITC (A20), CD45.2 Pacific Blue (104), CD150 PE (9D1), CD48 FITC (HM48-1), Flk2 PECy7 (A2F10), B220 APCCy7 (RA3-6B2), BP-1 FITC (6C3), CD24 FITC (30-F1), CD43 PE (eBioR2/60), IgD PECy7 (11-26), IgM Pacific Blue (eB121-15F9).

The cell surface immunophenotype definitions used for murine HSPC analyses are listed below:

Population	Marker
LSK	Lineage-negative Sca1+ c-KIT+
LT-HSC	Lineage-negative Sca1+ c-KIT+ CD150+ CD48-
ST-HSC	Lineage-negative Sca1+ c-KIT+ CD150+ CD48+
MPP	Lineage-negative Sca1+ c-KIT+ CD150- CD48+
LMPP	Lineage-negative Sca1+ c-KIT+ Flk2+
CLP	Lineage-negative Sca1 intermediate+ c-KIT intermediate + IL7Ra+
MP	Lineage-negative Sca1- c-KIT+
CMP	Lineage-negative Sca1- c-KIT+ CD16/32- CD34+
GMP	Lineage-negative Sca1- c-KIT+ CD16/32+ CD34+
MEP	Lineage-negative Sca1- c-KIT+ CD16/32- CD34-

For Western Blot analysis, the following antibodies were used: phospho-MEK1/2 (Ser217/221), phospho-ERK1/2 (Thr202/Tyr204), total MEK1/2, total ERK1/2, cyclin D1 (all Cell Signaling Technologies), and alpha-tubulin (Sigma Aldrich).

Animals, in vivo studies, and in vivo drug treatment of mice

For transplantation experiments, freshly dissected femurs and tibias were isolated from *BRaf*V600E CD45.2+ or *Mx1-cre BRaf*V600E CD45.2+ mice. BM was flushed with a 3 cc insulin syringe into PBS. The BM was spun at 0.5 x *g* by centrifugation at 4^0C, and red blood cells were lysed in ammonium chloride-potassium bicarbonate lysis buffer for 5 min. After centrifugation, cells were resuspended in PBS, passed through a cell strainer, and counted. For competitive transplantation experiments, 0.5 x 10^6 total BM cells of *BRaf*V600E CD45.2+ or

*Mx1-cre BRaf*V600E CD45.2+ mice were mixed with 0.5 x 10^6 wild-type CD45.1+ support BM and transplanted via tail-vein injection into lethally irradiated (two times 450 cGy) CD45.1+ host mice. Chimerism was measured by FACS in peripheral blood every 4 weeks up to 16-weeks post-transplantation. Additionally, for each peripheral blood analysis, whole blood cell counts were measured on a blood analyzer, and blood smears were scored. Chimerism in the peripheral blood, BM and spleen was evaluated at 16 weeks via animal sacrifice and subsequent FACS analysis.

For competitive transplantation experiments performed with *Cre-ER^T* mice, CD45.2 *Cre-ER^T BRaf* wildtype or Cre-*ER^T BRaf*V600E BM cells were transplanted into CD45.1 congenic recipients as described above. In these experiments, however, Cre-mediated excision of the lox-stop-lox cassette was performed in recipient mice 2 weeks after transplantation with intraperitoneal injection of 5 mg tamoxifen every other day x 3 days.

For noncompetitive transplantation experiments, 1.0 x 10^6 total BM cells of *BRaf*V600E CD45.2+ or *Mx1-cre BRaf*V600E CD45.2+ mice were transplanted via tail-vein injection into lethally irradiated (two times 450 cGy) CD45.1+ host mice.

For PLX4720 drug studies, all animals were dosed with vehicle (5% DMSO, 1% methylcellulose) or PLX4720 suspended in vehicle for 10 consecutive days at a dose of 50 mg twice daily by oral gavage.

Murine peripheral blood analyses

Blood was collected by submandibular bleeding using EDTA-containing tubes (Fisher). Automated peripheral blood counts were obtained using a HemaVet 950 (Drew Scientific) according to standard manufacturer's instruction. Differential blood counts were confirmed on blood smears stained using Wright-Giemsa staining and visualized using a Zeiss Axio Observer A1 microscope (Zeiss).

In vitro colony-forming assays.

For human studies, sorted LN- CD34+CD38+CD90+CD45RA- HSCs or total LN-

CD19- CD10- CD38+CD34+ myeloid progenitors were seeded at a density of 1,000 cells/ml for HSCs and 4,500 cells/ml for myeloid progenitors each in technical triplicates in methylcellulose medium supplemented with cytokines promoting myeloid and erythroid differentiation (MethoCult H4435; STEM CELL Technologies). Colony number and lineage were scored after 14 days.

For murine studies, whole BM cells from *BRaf*V600E and littermate *Mx1-cre* *BRaf*V600E mice were seeded at a density of 10,000 cells/replicate into myeloid and erythroid cytokine-supplemented methylcellulose medium (Methocult, M3434; STEMCELL Technologies) and 40,000 cells/replicate in rhIL-7 cytokine-supplemented methylcellulose medium (Methocult, M3630; STEMCELL Technologies). Colonies propagated in culture were scored at day 7. Representative colonies were isolated from the plate for cytospins. Remaining cells were resuspended, counted, and a portion was taken for replating (20,000 cells/replicate) for a total of 7 platings.

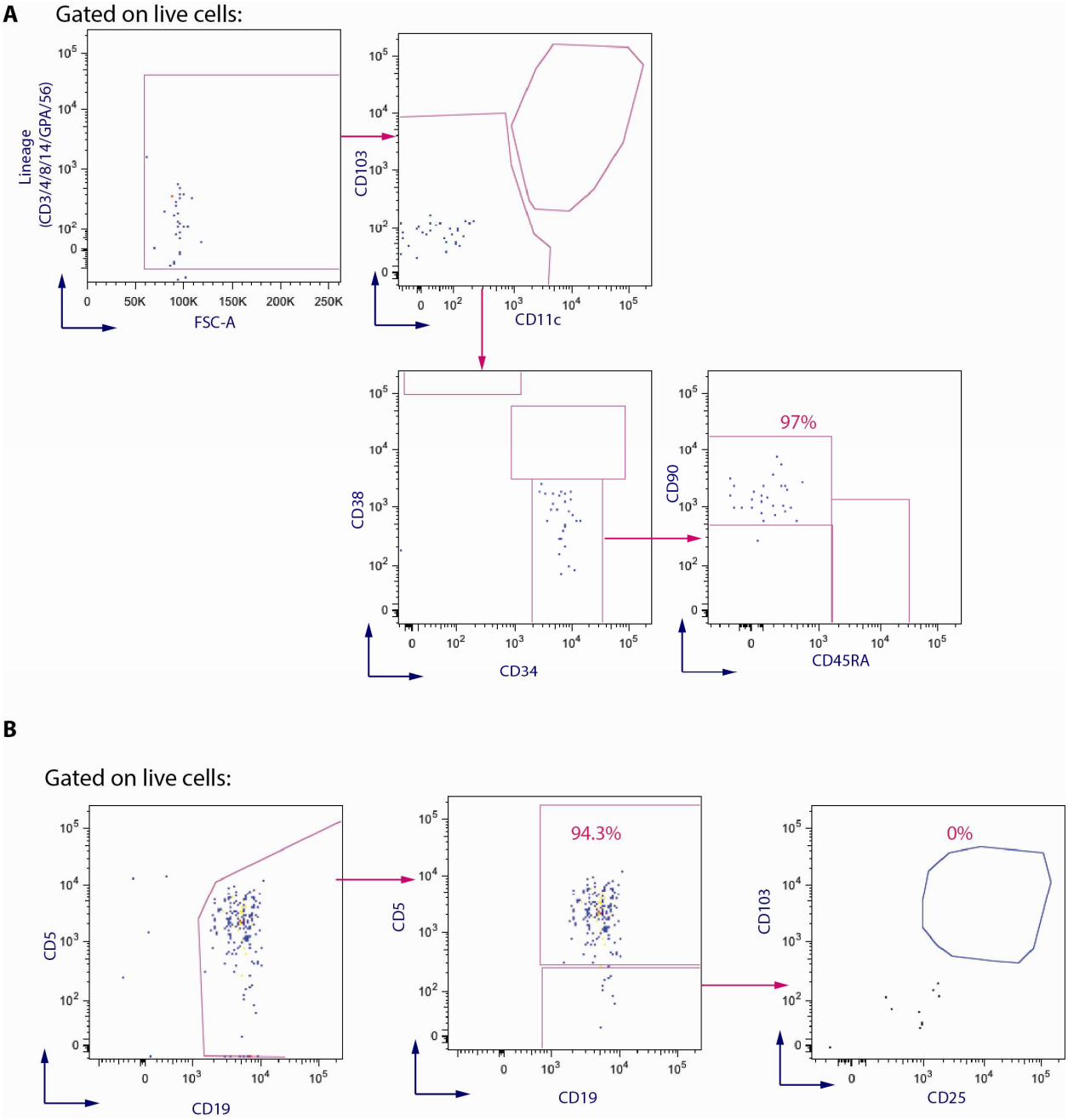

Fig. S1. HSPC abnormalities in HCL and the presence of *BRAF*V600E in HSCs. (**A**) Demonstration of sort purity of HSCs shown in **Fig. 1A** and used for genetic analysis in **Fig. 1C**. Cells were sorted as described in **Fig. 1A** and then each cell population was resorted to ensure lack of contamination prior to genetic analysis of the *BRAF*V600E mutation. (**B**) Sort purity for peripheral blood separation of CLL cells versus HCL cells in an individual with both HCL and CLL as shown in **Fig. 1E**.

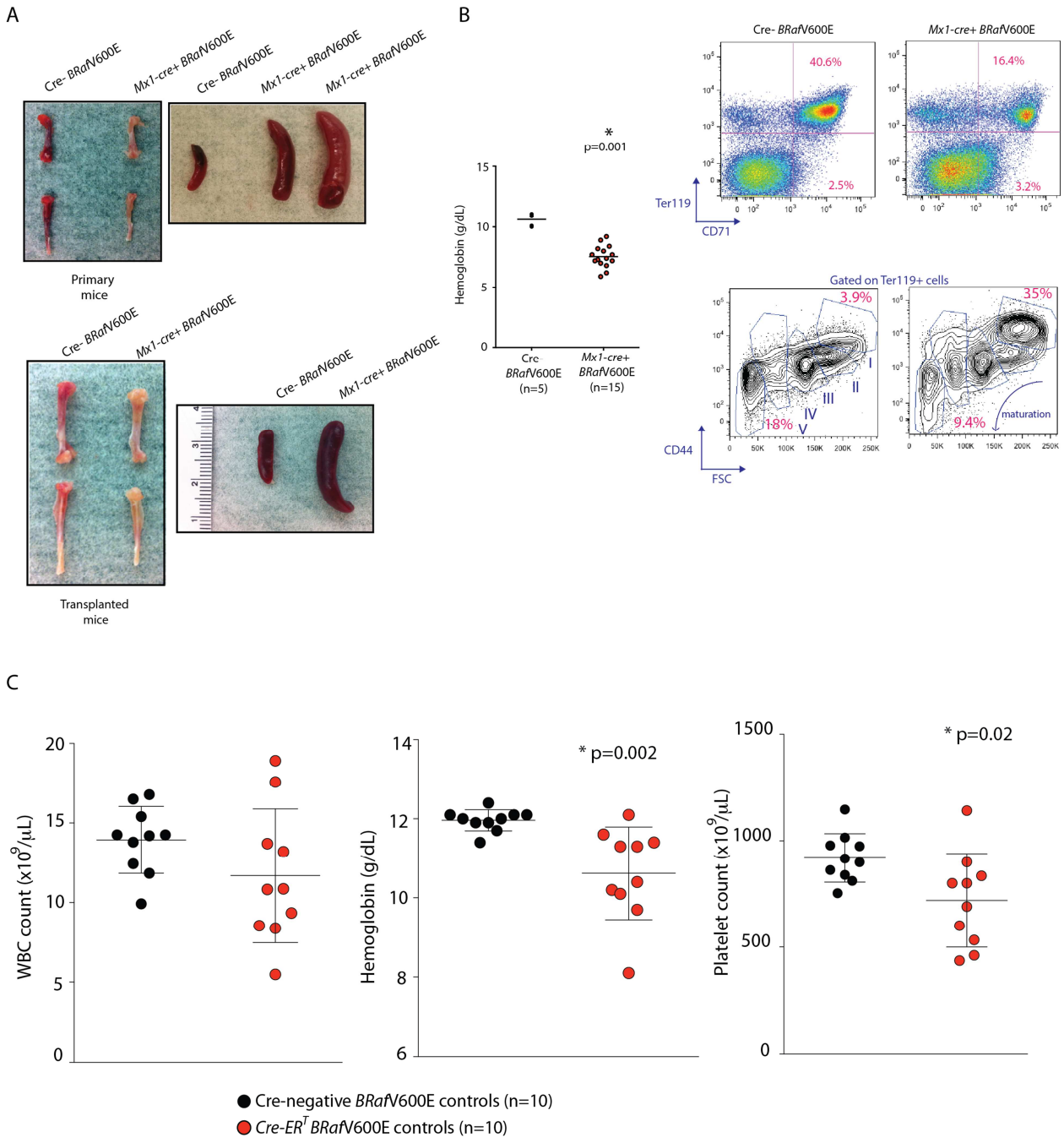

Fig. S2. Effect of BRafV600E expression at different time points and stages of hematopoiesis. (**A**) Femurs, tibiae, and spleens of primary *Mx1-cre BRaf*V600E mice and cre-negative controls (top) and wild-type recipient mice transplanted with cre-negative *BRaf*V600E or *Mx1-cre BRaf*V600E BM cells (bottom). (**B**) Quantification of hemoglobin and flow cytometric analysis of erythroid differentiation in BM of cre-negative *BRaf*V600E or *Mx1-cre BRaf*V600E mice with CD71/Ter119 analysis and

erythroblast sub-fractionation based on CD44 versus forward scatter (FSC) (*32*). (**C**) Blood counts of mice transplanted with BM from *CreER*^{T} *BRaf*V600E (n=10) or Cre-negative (n=10) control mice at 2 weeks after tamoxifen administration. Tamoxifen was administered to recipient mice at 4 weeks post-transplantation.

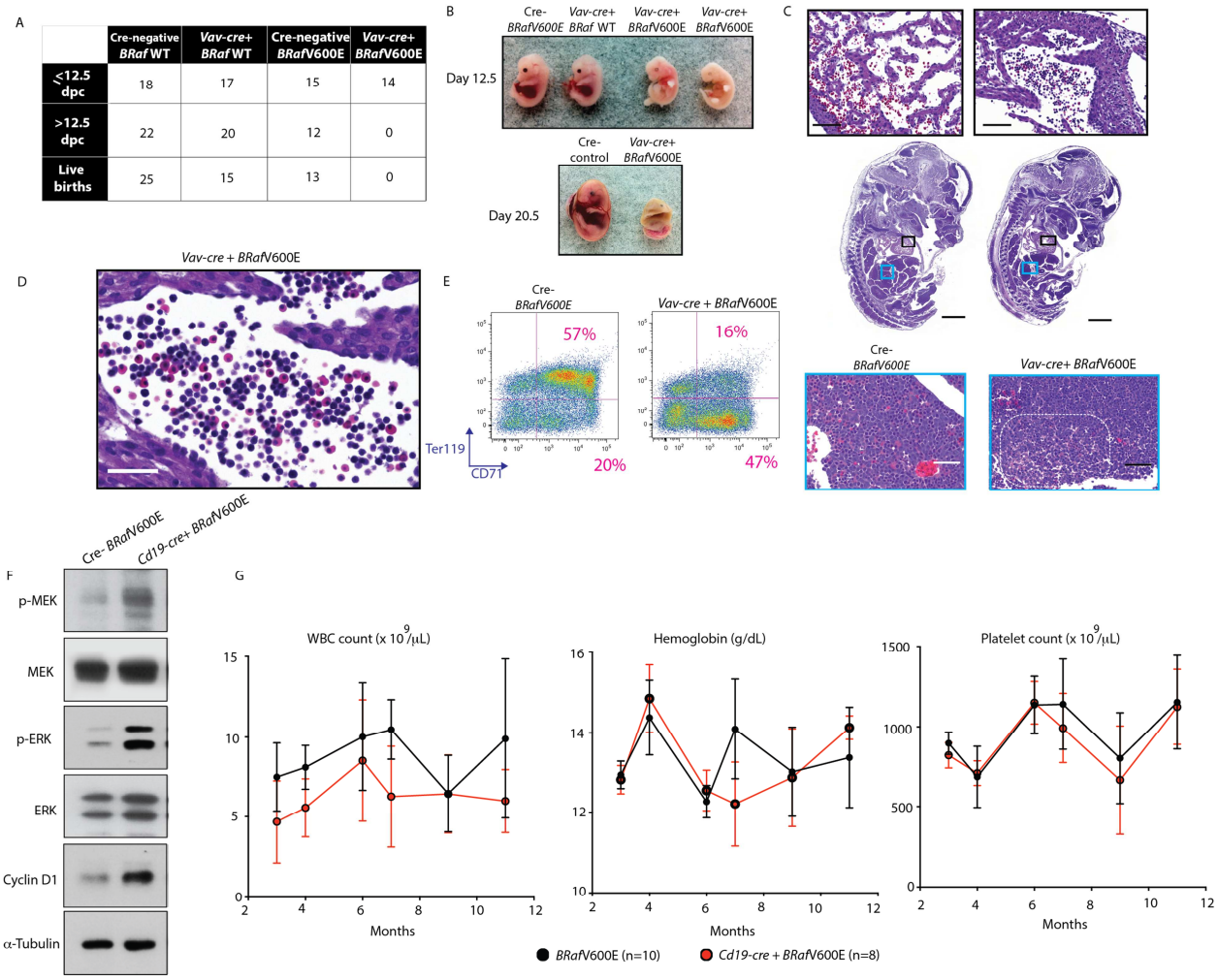

Fig. S3. Effect of *BRaf*V600E expression on fetal hematopoiesis and in mice with B cell–restricted expression of the mutant allele. (**A**) *Vav-cre BRaf*V600E mice are associated with 100% lethality beyond embryonic day 12.5. (**B**) *Vav-cre BRaf*V600E mice observed at <12.5 day post-conception (dpc) are marked by pallor and are resorbed before birth. (**C**) Analysis of *Vav-cre BRaf*V600E mice before 12.5 dpc reveals leukocytosis (top; scale bar: 50 μm) and splenic infarcts (bottom; white encircled area (scale bar: 50 μm)). (**D**) Magnified view of leukocytes within 11.5 dpc *Vav-cre BRaf*V600E (scale bar: 50 μm). (**E**) CD71/Ter119 FACS analysis of fetal liver 11.5 dpc. (**F**) Immunoblot of B220+ cells after culturing lineage-negative Sca1+ c-Kit+ (LSK) cells sorted from *Cd19-cre BRaf*V600E or cre-negative control mice in IL-7 for 7 days. (**G**) Monthly blood counts of *Cd19-cre BRaf*V600E and littermate cre-negative control mice. Error bars represent mean ± SD.

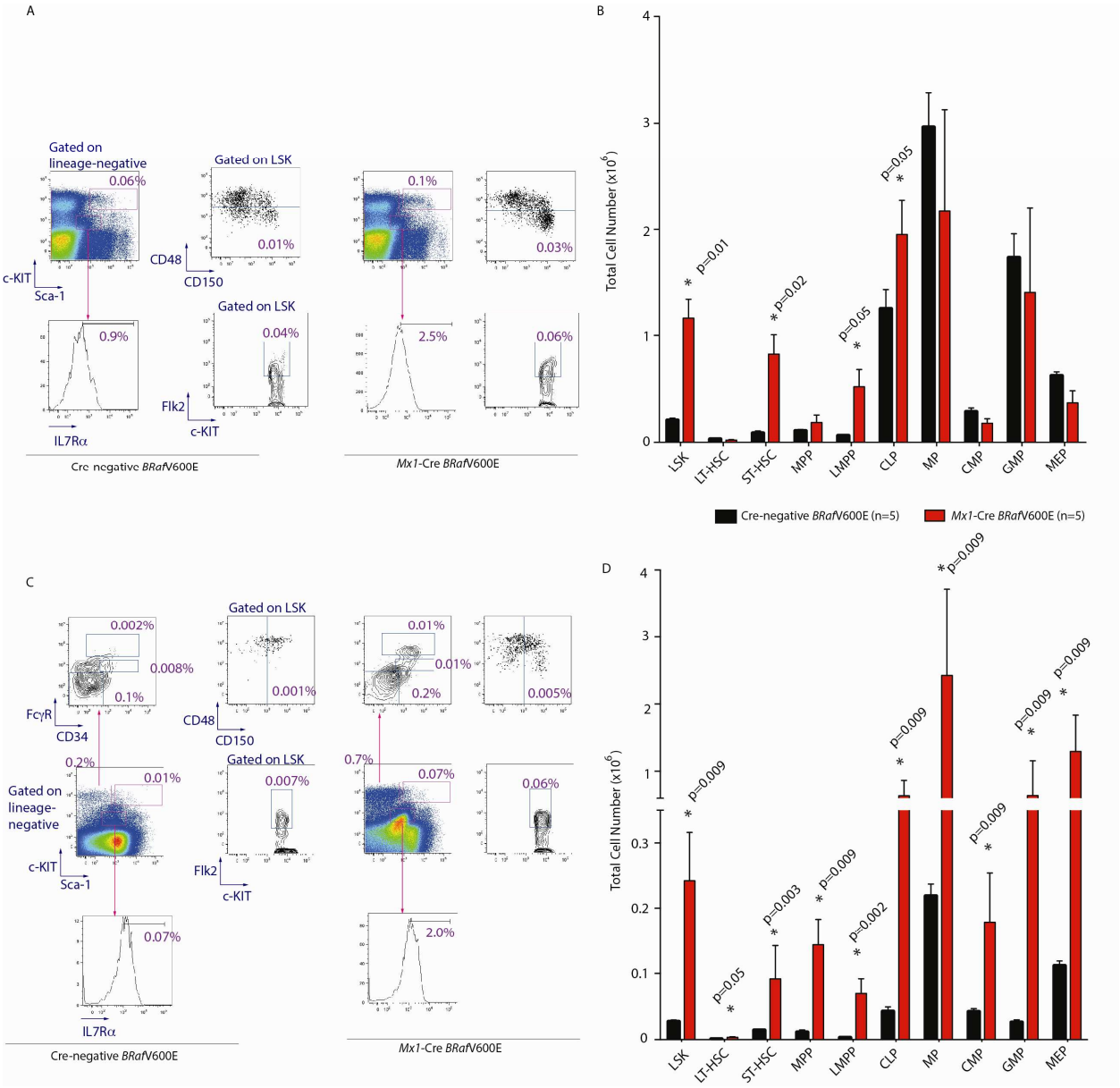

Fig. S4. Effect of *BRaf*V600E expression on HSPC numbers and frequencies. HSPCs from the BM and spleen of 5 secondarily transplanted *Mx1-Cre BRaf*V600E mice and 5 similarly treated Cre-negative control mice were analyzed by FACS. Representative frequencies of each HSPC population amongst live cells (A) as well as the absolute number of each population of cells (B) in BM is shown (LT-HSC: long-term hematopoietic stem cell; ST-HSC: short term hematopoietic stem cell; MPP: multipotent progenitor; CLP: common lymphoid progenitor; MP: myeloid progenitor; CMP: common myeloid progenitor; MEP: megakaryocyte/erythroid progenitor). Similarly, representative frequencies (C) as well as the absolute

number of each population of cells (D) in spleen are also shown. Error bars represent mean ± SD. Asterisk represents $p<0.05$ (Mann-Whitney U test).

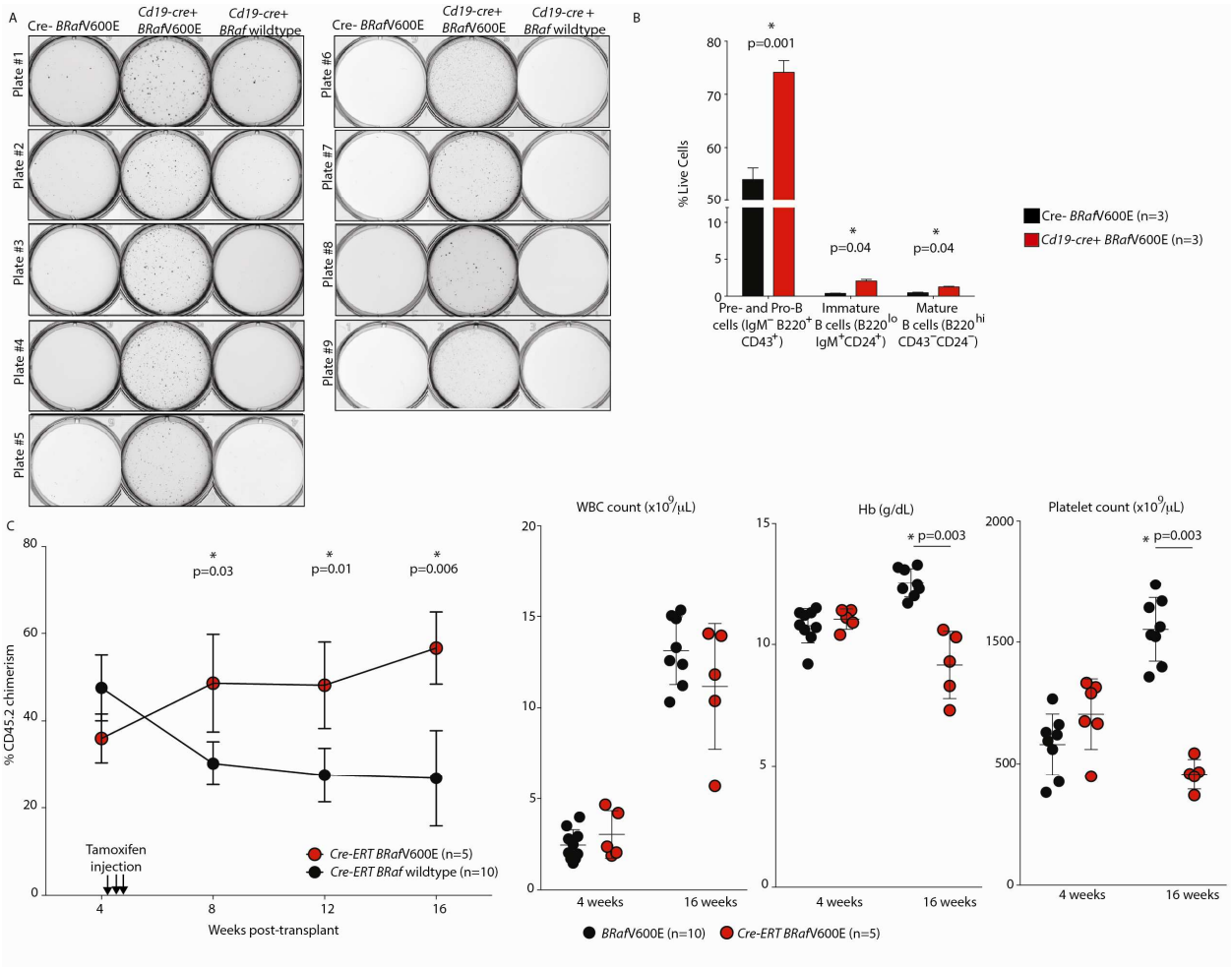

Fig. S5. Effect of *BRaf*V600E expression on B cell development and self-renewal. (**A**) Photograph of methylcellulose colony replating assays of whole BM from *Cd19-cre BRaf*V600E and control mice grown in rhIL-7. *BRaf* mutant B-cell progenitors could be replated for >9 platings in the presence of rhIL-7, whereas control BM samples could be replated for no more than 3 platings. (**B**) Flow cytometric assessment of B lineage cells from *BRaf*V600E BM cultured in methylcellulose containing rhIL-7. (**C**) Competitive transplantation of *Cre-ER*^{T} *BRaf*V600E or *Cre-ER*^{T} *BRaf* wildtype BM cells followed by activation of the BRafV600E mutation by tamoxifen injection 4 weeks after transplantation reveals the competitive advantage of *BRaf*V600E mutant hematopoietic cells (arrows indicate time of tamoxifen intraperitoneal injection into recipient mice). Recipient wild-type mice transplanted with *Cre-ER*^{T} *BRaf*V600E HSPCs in a competitive manner developed anemia and thrombocytopenia by 16 weeks. Error bars represent mean ± SD. Asterisk represents $p<0.05$ (Mann-Whitney U test).

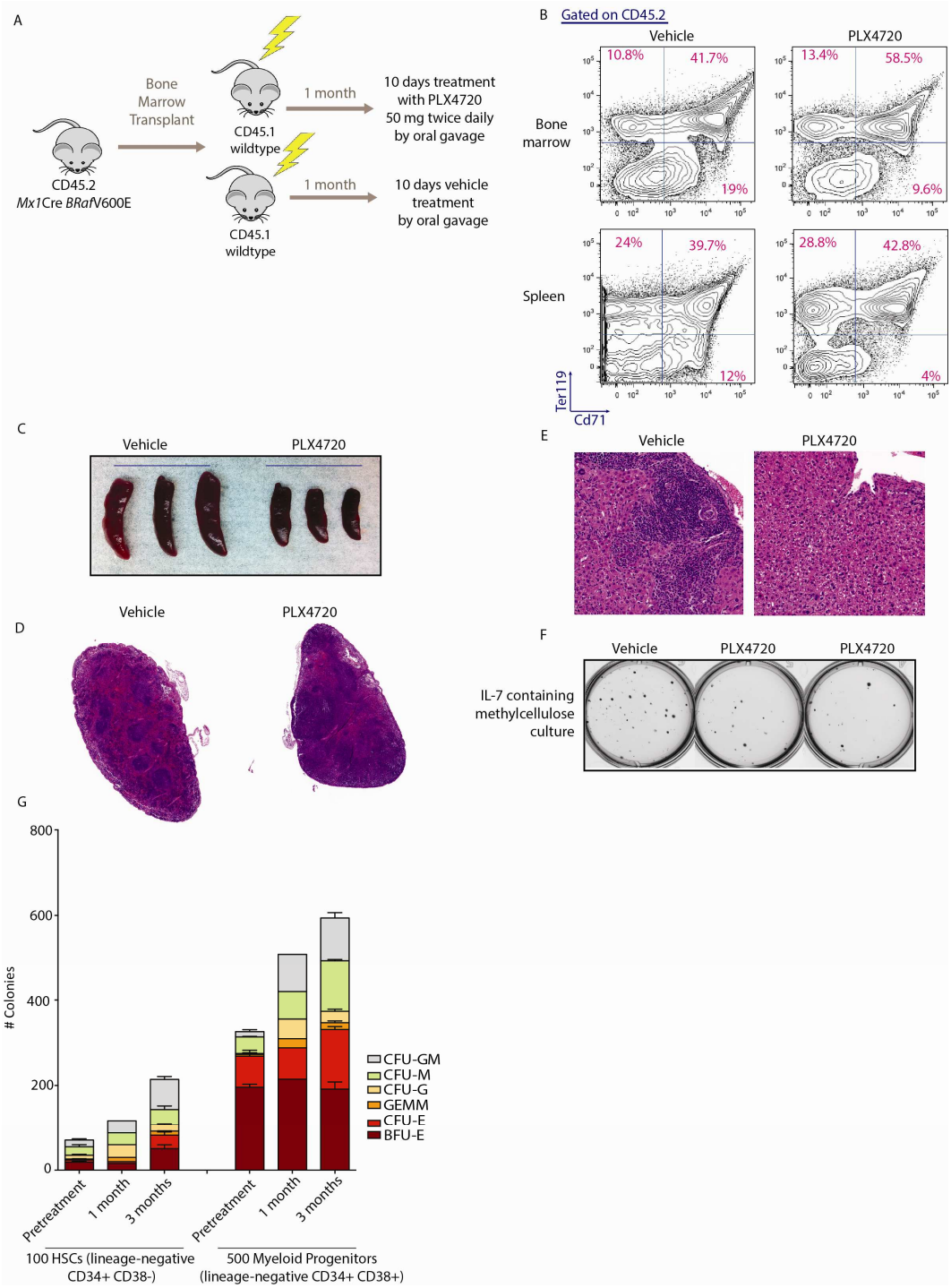

Fig. S6. Normalization of HSPC compartment and increased myeloid/erythroid output from *BRAF*V600E-mutant HSPCs after BRAF inhibition. (**A**) Schema describing in vivo PLX4720 treatment. Wild-type CD45.1 recipient mice were lethally irradiated and then transplanted with *Mx1-cre* *BRaf*V600E BM cells. Four weeks later, mice were randomized to receive treatment with PLX4720 at 50

mg/kg twice daily or vehicle (5% DMSO, 1% methylcellulose) for 10 days continuously. (**B**) Erythropoiesis in BM and spleen of *Mx1-cre BRaf*V600E mice treated with PLX4720. (**C**) Photographs of gross spleen and (**D**) spleen sections after treatment of *Mx1-cre BRaf*V600E mice with vehicle or PLX4720. Drug-treated mice have smaller spleens with decreased red pulp compared with vehicle-treated mice. (**E**) PLX4720 decreases extramedullary hematopoiesis in liver and (**F**) the ability of B-cell progenitors to proliferate in vitro in rhIL-7. (**G**) Plating of 100 HSCs and 500 myeloid progenitors sorted from BM aspirates of HCL patients before treatment and after 1 and 3 months of therapy (n=3 patients for each timepoint) into myeloid and erythroid cytokine-containing methylcellulose revealed increased clonogenic capacity of these cells after vemurafenib therapy. Error bars represent mean ± SD.

Table S1: Genotyping results of cell populations sorted from HCL patient BM aspirates.

Sample	*BRAF*V600E Allele Specific PCR Qualitative Result *BRAF*V600E Variant Allele Frequency from MiSeq						Comments
	HSC	Hematogone	MP	HCL	CD3+	CD14+	
1	Mutant	Mutant	Wildtype	Mutant	N/A	N/A	-
	N/A	N/A	N/A	N/A	N/A	N/A	
2	Mutant	Mutant	Wildtype	Mutant	N/A	N/A	-
	N/A	N/A	N/A	N/A	N/A	N/A	
3	Mutant	Mutant	N/A	Mutant	N/A	N/A	-
	N/A	N/A	N/A	N/A	N/A	N/A	
4	Wildtype	Mutant	Wildtype	Mutant	N/A	N/A	ARID1A p.V1427fs
	0.06%	91.5%	0.14%	95.3%	N/A	N/A	
5	Mutant	Mutant	Wildtype	Mutant	N/A	N/A	-
	0.69%	N/A	N/A	42.3%	N/A	N/A	
6	Mutant	Mutant	Wildtype	Mutant	N/A	N/A	MLL3 p.C394Y
	N/A	N/A	N/A	N/A	N/A	N/A	
7	Mutant	Wildtype	Wildtype	Mutant	N/A	N/A	-
	8%	0.17%	0.1%	40.7%	N/A	N/A	
8	N/A	N/A	N/A	N/A	N/A	N/A	-
	8.7%	0.16%	0.08%	40.7%	0.08%	0.08%	
9	N/A	N/A	N/A	N/A	N/A	N/A	-
	N/A	N/A	N/A	N/A	0.07%	0.04%	
10	N/A	N/A	N/A	N/A	N/A	N/A	-
	N/A	N/A	N/A	N/A	0.08%	0.05%	
11	Mutant	Wildtype	Wildtype	Mutant	N/A	N/A	-
	7.4%	0.05%	0.08%	89.8%	N/A	N/A	
12	N/A	N/A	N/A	N/A	N/A	N/A	-
	N/A	N/A	N/A	N/A	0.04%	0.08%	
13	N/A	N/A	N/A	N/A	N/A	N/A	-
	N/A	N/A	N/A	N/A	0.05%	0.09%	
14	Mutant	Mutant	Wildtype	Mutant	N/A	N/A	No mutations seen in extended targeted sequencing other than *BRAF*V600E
	N/A	N/A	N/A	N/A	N/A	N/A	

Table S2: Three hundred genes sequenced in three HCL leukemic cell and granulocyte genomic DNA samples.

Gene Symbol of Genes Sequenced					
ABL1	CEBPA	FBXO11	KCNJ5	NOTCH2	RICTOR
ABL2	CHEK1	FBXW7	KDM5C	NOTCH3	RNF43
AKT1	CHEK2	FGFR1	KDM6A	NOTCH4	ROR2
AKT2	CIC	FGFR2	KDR	NPM1	ROS1
AKT3	CREBBP	FGFR3	KEAP1	NRAS	RPS6KB1
ALK	CRKL	FGFR4	KIT	NTRK1	RPTOR
ALOX12B	CRLF2	FH	KLF6	NTRK2	RUNX1
APC	CSF1R	FKBP1A	KRAS	NTRK3	SDHB
AR	CTCF	FLCN	LDHA	PAK7	SETD2
ARAF	CTNNB1	FLT1	LGR6	PALB2	SF3B1
ARHGAP26	CYLD	FLT3	LMO1	PARK2	SHQ1
ARID1A	DAXX	FLT4	MAGI2	PARP1	SMAD2
ARID1B	DDR2	FOXL2	MAP2K1	PAX5	SMAD3
ARID2	DICER1	FUBP1	MAP2K2	PBRM1	SMAD4
ASXL1	DIS3	GATA1	MAP2K4	PDGFRA	SMARCA4
ATM	DNMT1	GATA2	MAP3K1	PDGFRB	SMARCB1
ATR	DNMT3A	GATA3	MAP3K8	PHOX2B	SMO
ATRX	DNMT3B	GLI1	MAPK1	PIK3CG	SOCS1
AURKA	E2F3	GLI3	MCL1	PIK3CA	SOX2
BAP1	EGFR	GNA11	MDM2	PIK3CB	SPOP
BCL2L1	EIF4EBP1	GNAQ	MDM4	PIK3CD	SRC
BCL2L11	EP300	GNAS	MED12	PIK3CG	SRSF2
BCL6	EPHA10	GOLPH3	MEF2B	PIK3R1	STAG2
BCOR	EPHA2	GRIN2A	MEN1	PIK3R2	STK11
BIRC2	EPHA3	GRM3	MET	PIK3R3	SUFU
BRAF	EPHA4	GSK3B	MITF	PKM2	SYK
BRCA1	EPHA5	HDAC2	MLH1	PLK2	TBK1
BRCA2	EPHA6	HIF1A	MLL	PMS2	TEK
BUB1B	EPHA7	HLA-A	MLL2	PNRC1	TERT
CARD11	EPHA8	HMGA2	MLL3	POLE	TET1
CBL	EPHB1	HNF1A	MLST8	PPP2R1A	TET2
CBLB	EPHB2	HRAS	MPL	PPP6C	TGFBR2
CBLC	EPHB3	HSP90AA1	MRE11A	PRDM1	TMPRSS2
CCND1	EPHB4	IDH1	MSH2	PREX2	TNFAIP3
CCND2	EPHB6	IDH2	MSH6	PRKAA2	TNFRSF14
CCND3	ERBB2	IGF1R	MTOR	PRKAR1A	TOP1
CCNE1	ERBB3	IGFBP7	MYB	PRKCI	TP53
CD79B	ERBB4	IKBKE	MYC	PTCH1	TP63
CDC42EP2	ERCC2	IKZF1	MYCL1	PTEN	TSC1
CDC73	ERCC5	IL7R	MYCN	PTPN11	TSC2
CDH1	ERG	INPP4A	MYD88	PTPRD	TSHR
CDH11	ESR1	INPP4B	NBN	PTPRS	U2AF1
CDK12	ETV1	INSR	NCOA2	PTPRT	VHL
CDK4	ETV6	IRF4	NF1	RAC1	WAS
CDK6	EZH2	IRS1	NF2	RAD50	WNK1
CDK8	FAM123B	IRS2	NFE2L2	RAF1	WT1
CDKN1A	FAM46C	JAK1	NFKB1	RARA	XPO1
CDKN2A	FAS	JAK2	NFKB2	RB1	YAP1
CDNKN2B	FAT1	JAK3	NKX2-1	REL	YES1
CDKN2C	FAT4	JUN	NOTCH1	RET	ZSRSR2