

SUPPLEMENTARY INFORMATION

Effective and selective targeting of Ph⁺ leukemia cells using a TORC1/2 kinase inhibitor

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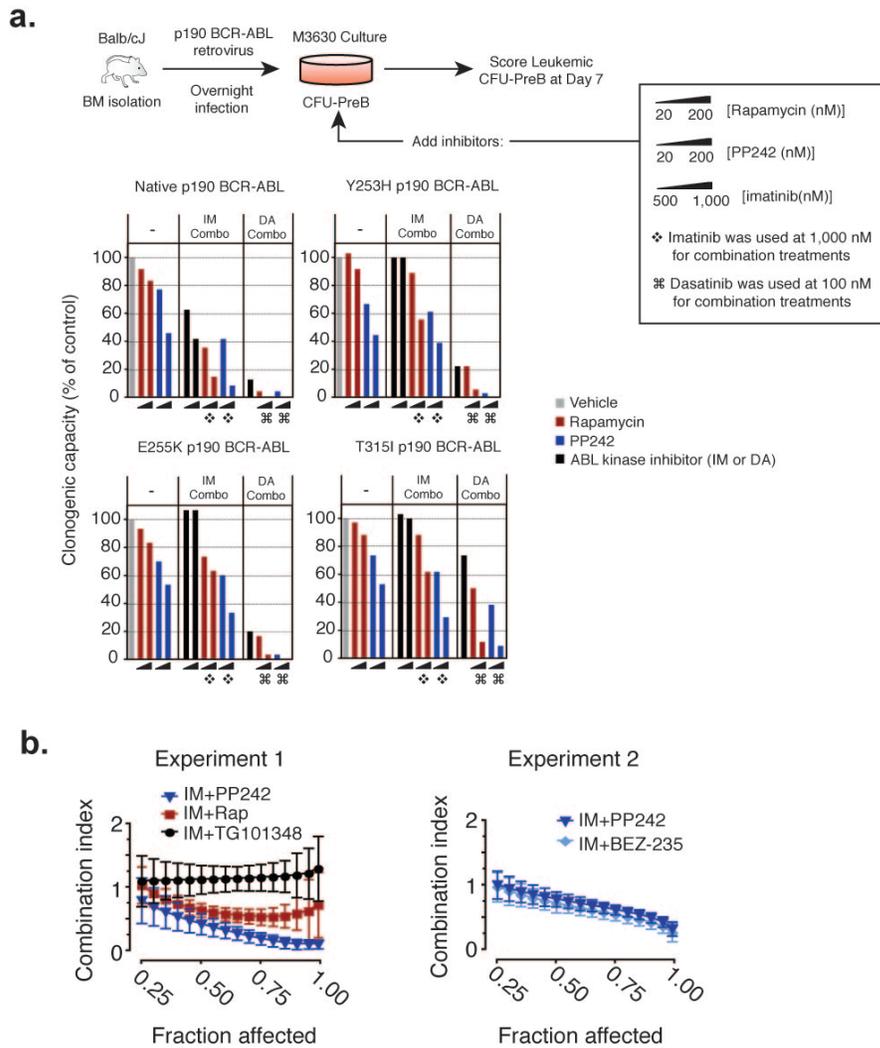
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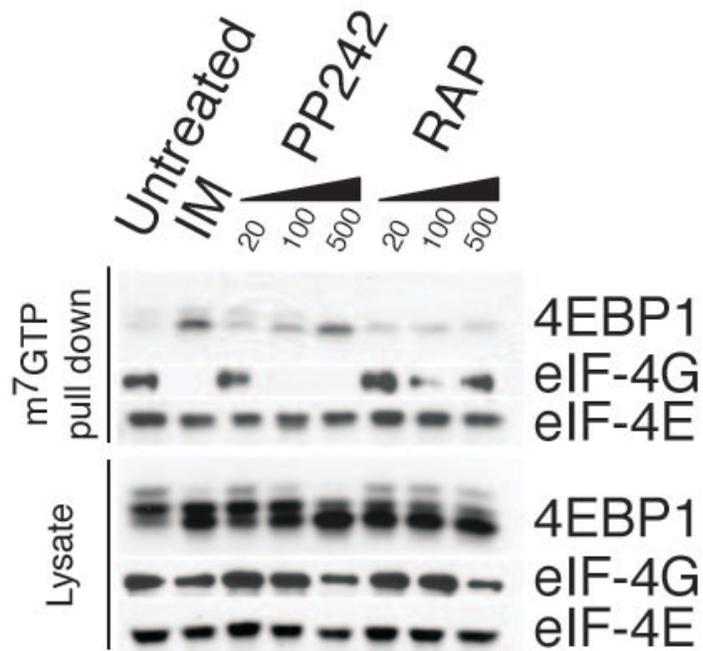
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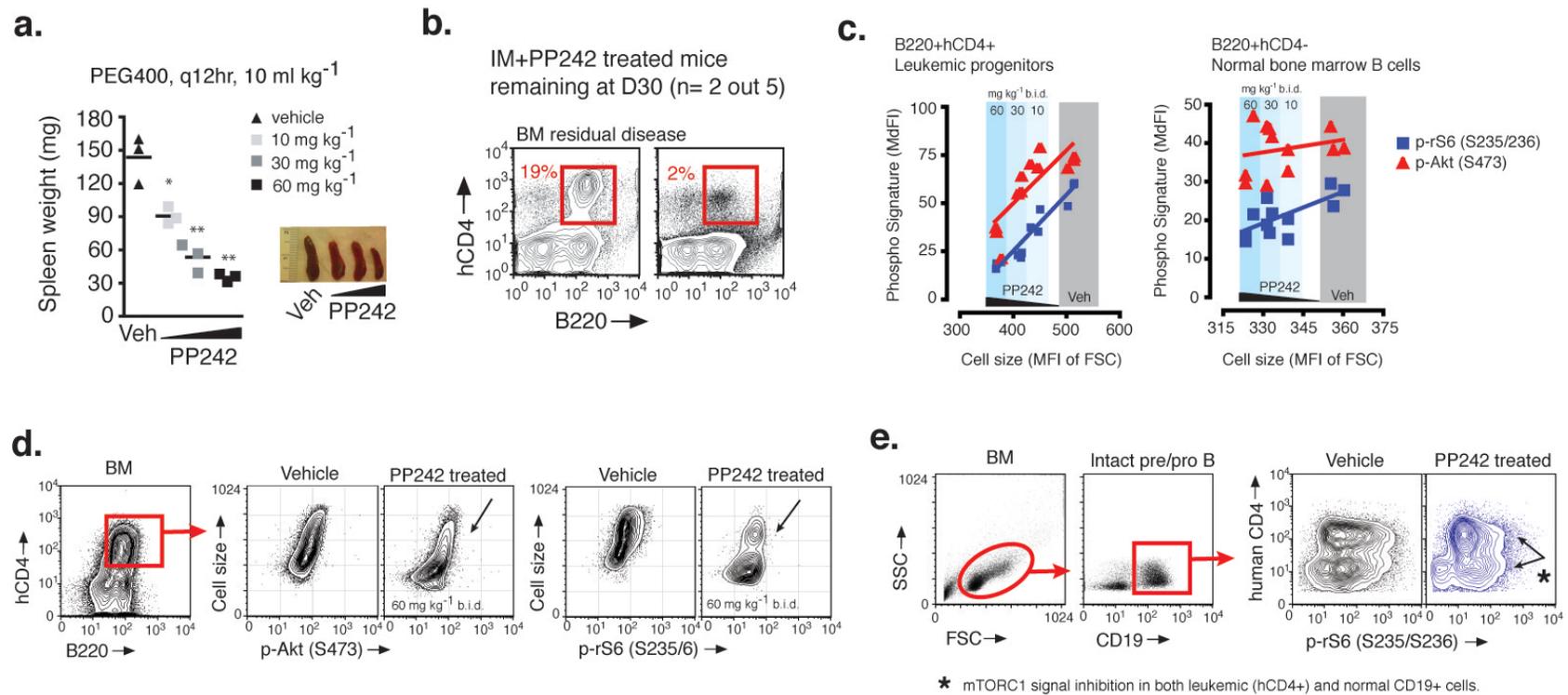


Supplementary Figure 1. TKI combination studies with mouse BM hematopoietic cultures transformed with native and mutant p190 BCR-ABL. **(a)** Bone marrow (BM) was collected and infected with native p190-BCR-ABL, imatinib-resistant (Y253H and E255K) p190-BCR-ABL, or imatinib/dasatinib-resistant (T315I) p190 BCR-ABL overnight. M3630 cultures of infected bone marrow was scored for CFU-pre B formation 7 days following treatment with indicated inhibitors. Clonogenic capacity signifies the amount of CFU-preB normalized to the vehicle control condition. Cultures were performed in duplicate 35mm dishes and averaged. **(b)** p190 cells were cultured for 48 hr with the indicated combinations of compounds and assessed for growth inhibition

using the median effect method. TG101348 is a JAK2 inhibitor. Drug combinations were assessed for synergy by calculating the combination index (CI). The CI was plotted as a function of the fraction affected by treatment. $CI < 1$, $=1$, or > 1 indicate synergism, additive effect, or antagonism, respectively. Growth was quantified using the MTS assay in 96 well format.

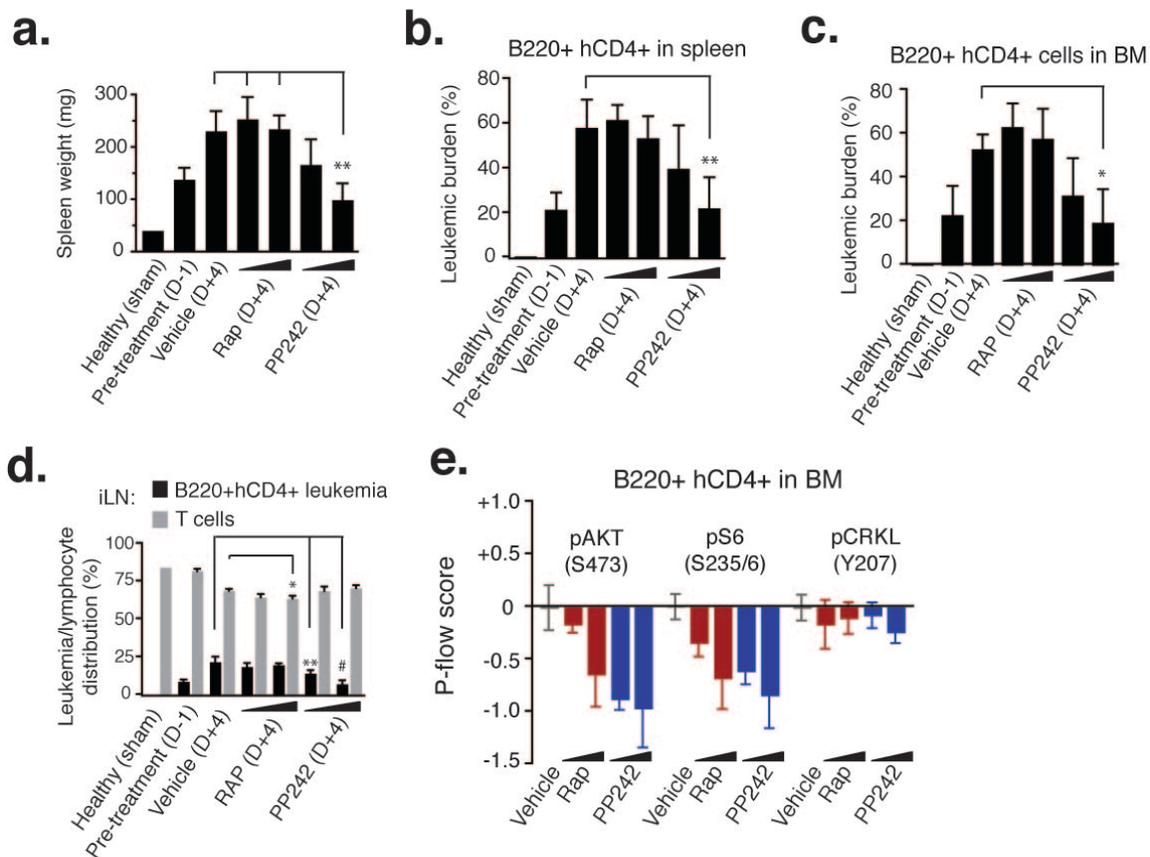


Supplementary Fig 2. Distinct effects of PP242 and rapamycin in cap-binding assay. p190 cells were cultured with PP242, RAP, and high concentrations of IM (5 μM) for 4 hr. Cap-binding proteins in lysates were purified by 7-methyl GTP (m⁷GTP) affinity and analyzed by western blotting. Similar results were obtained in a separate experiment.



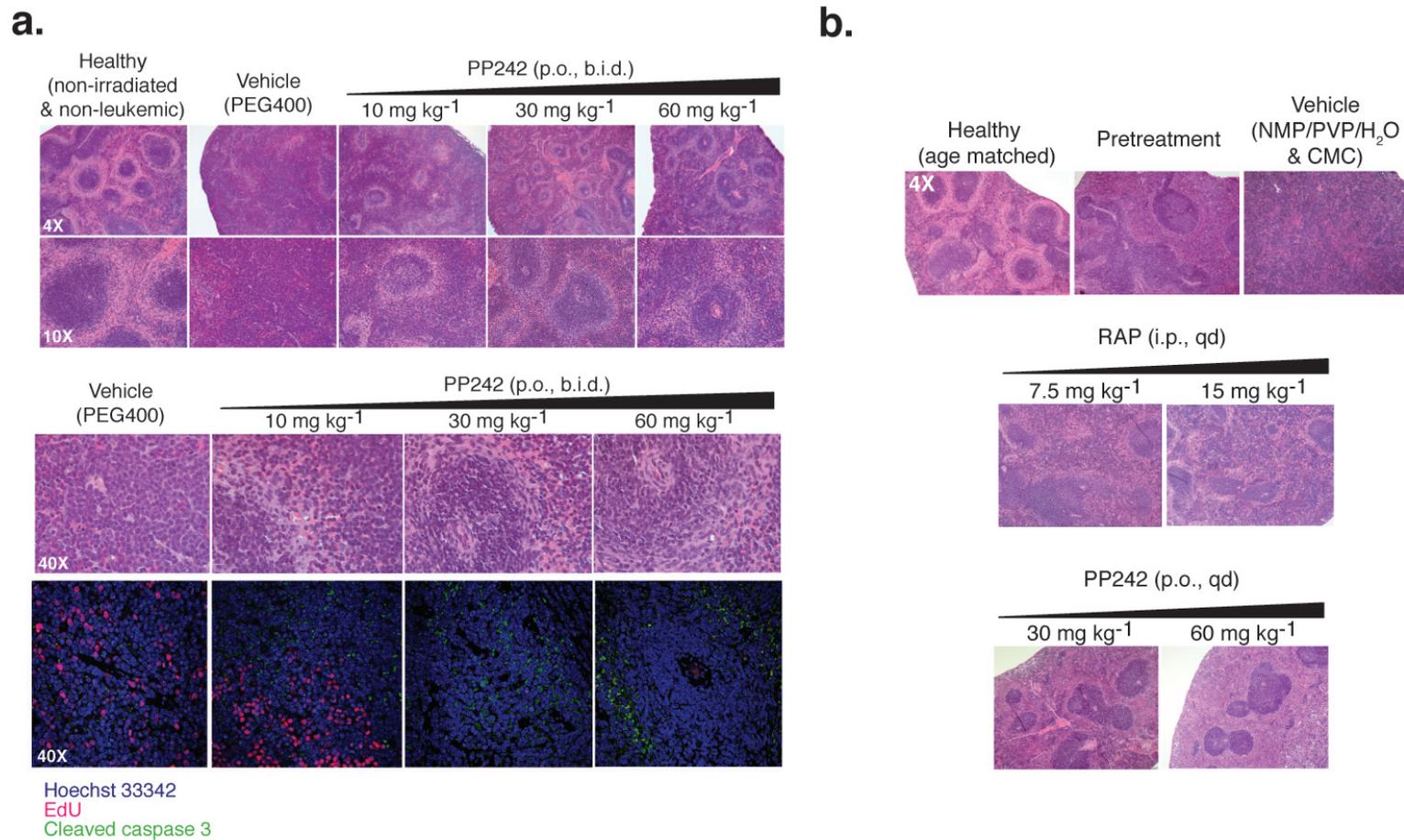
Supplementary Fig 3. PP242 induces leukemia regression *in vivo* by inhibiting mTORC2 and mTORC1 activation that correlates with loss in cell size. **(a)** Spleen weights of mice treated with PP242 following short-term administration as in **Fig. 4a–d**. (* $P < 0.05$, ** $P < 0.01$; ANOVA). **(b)** Residual disease (B220⁺hCD4⁺) in the BM of mice treated with IM + PP242 (60 mg kg⁻¹) as in **Fig. 4f**. Note that were euthanized all had > 70% leukemic burden (data not shown). **(c)** Mouse p190 cells compared to endogenous normal precursor/progenitor B cells were analyzed by flow cytometry. Mice were sacrificed 2 hr following the last dose. BM was quickly collected in waves, processed to single cell suspensions, fixed, and assessed for phospho-signatures and cell size. Median fluorescent intensity (MdFI) values representing the phospho-signatures from each population of treated mice were determined (see Supplementary methods for more details). In the leukemic cells, inhibition of both p-rS6 ($P < 0.0001$; +0.96 Pearson r) and p-Akt ($P = 0.0016$; +0.69

Pearson r) significantly correlate with a loss of cell size. The low basal phospho-signatures of normal bone marrow B cells are marginally inhibited where the loss of p-rS6 significantly correlated with a loss in cell size ($P = 0.0104$; -0.71 Pearson r) but p-Akt lacked any significant trend ($P = 0.4781$; $+0.2271$ Pearson r). **(d)** Representative example of leukemic-specific mTOR substrate inhibition correlating with cell size loss (FSC) following *in vivo* administration of PP242. **(e)** Short-term dosing (4 days; qd, p.o. 60 mg kg^{-1}) with PP242 using 5% NMP, 15% PVP, 80% water vehicle exhibits similar pharmacodynamics as the PEG400 vehicle (and data not shown).



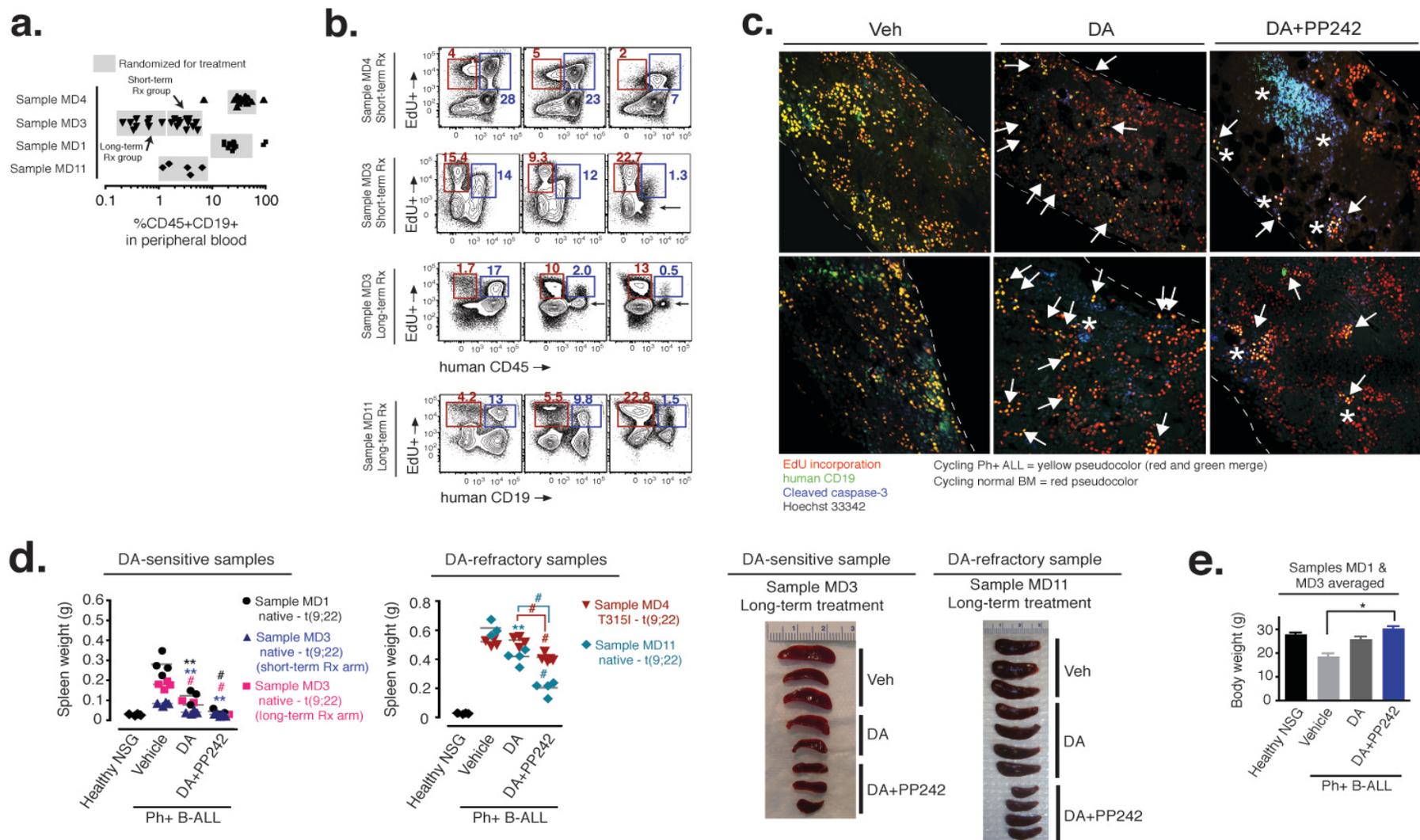
Supplementary Fig 4. Comparison of PP242 and rapamycin *in vivo*, in short-term p190 leukemia model with non-irradiated immunocompetent recipients. The rationale for using non-conditioned mice was to exclude confounding effects of radiation on normal splenic architecture (**Supplementary figure 5b**). **(a)** Mice were treated with RAP (7.5 and 15 mg kg⁻¹; i.p.) or PP242 (30 and 60 mg kg⁻¹; p.o.) every 24 hr. NMP/PVP/water vehicle was used for PP242 oral delivery. All treated mice received both i.p. and p.o. injections for fidelity. PP242 significantly reduced the size of the spleen compared to vehicle and RAP treatments (***P* < 0.01, ANOVA). **(b-c)** The leukemic burden in the spleen (***P* < 0.01, ANOVA) and BM (**P* < 0.05, ANOVA) was significantly reduced with PP242 (60 mg kg⁻¹) compared to vehicle treated mice. **(d)** The leukemic burden in the inguinal lymph nodes (iLN) were compared to the amount of normal lymphocytes in RAP or PP242 treated mice. PP242 significantly reduced the leukemic burden while preserving normal lymphocyte abundance. RAP significantly reduced the amount of T

cells (* $P < 0.05$, ** $P < 0.05$, # $P < 0.001$, two-way ANOVA). (e) The effects on mTORC1 (p-rS6), mTORC2 (p-Akt), and BCR-ABL (p-CRKL) substrates on a per cell basis was assessed by intracellular staining and displayed as a P-flow score (see **Supplementary Methods** for further details). RAP and PP242 showed dose dependent inhibition of mTOR signaling without directly affecting BCR-ABL activation.



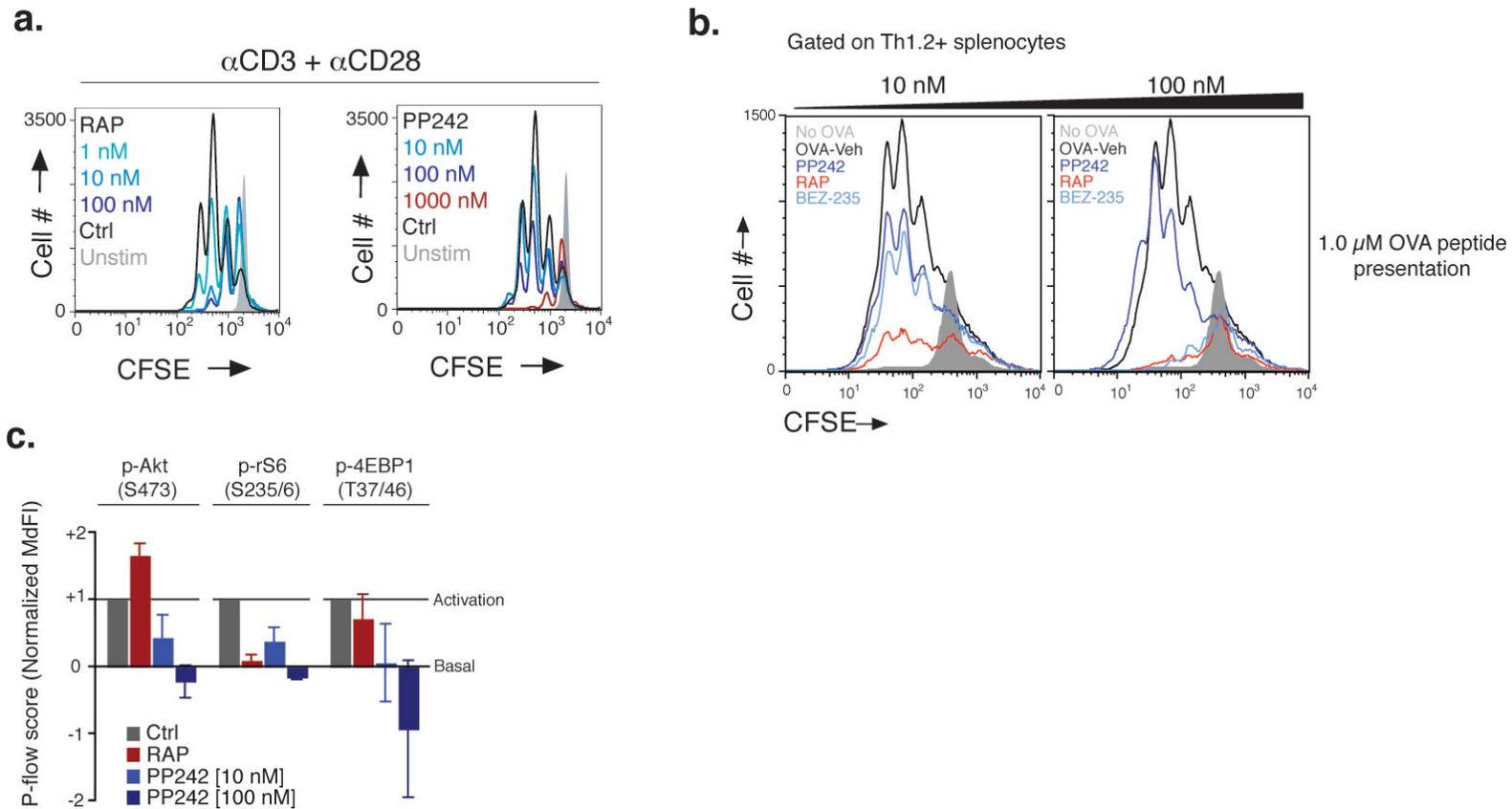
Supplementary Fig 5. Spleen pathology of p190 leukemia model comparing PP242 and rapamycin treatment. (a) H&E stained-formalin fixed paraffin-embedded (FFPE) spleen sections from healthy and leukemic mice. Note the complete effacement of splenic architecture in Veh treated mice and the preservation of splenic architecture in mice treated with PP242. Assessment by confocal

microscopy depicts a major inhibition of proliferation (EdU accumulation), preservation of splenic architecture, and induction of apoptosis in mice treated with PP242 in a dose-dependent manner. Note, healthy age-matched control mice lacked significant EdU accumulation (data not shown). **(b)** In this experiment, we used non-irradiated mice to prevent confounding pathology due to the response to sublethal irradiation. Spleens from p190-transplanted mice were harvested before and after treatment with the indicated compounds and doses, and compared to spleens from healthy controls.



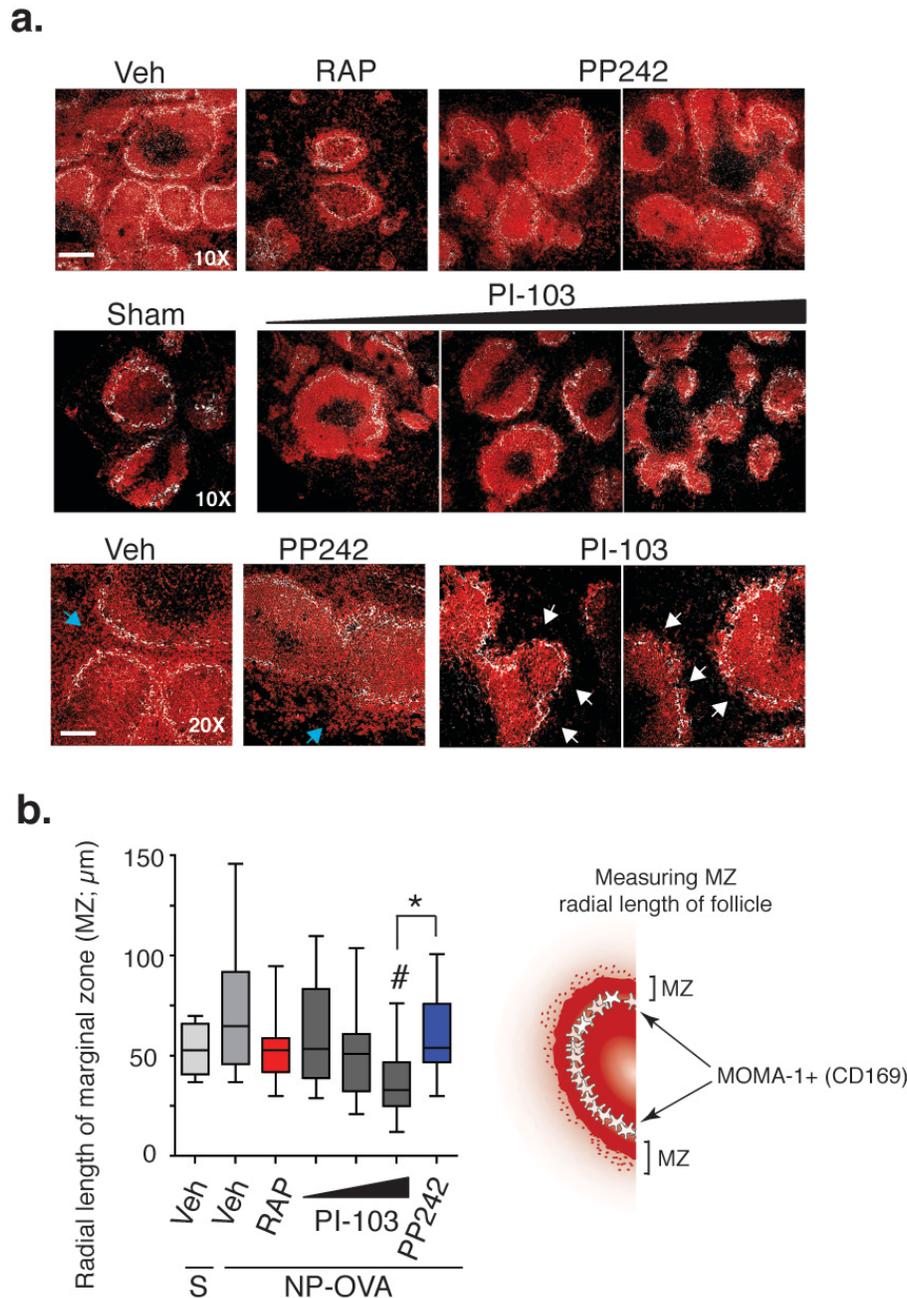
Supplementary Figure 6. TKI combination studies with PP242 in primary Ph⁺ B-ALL xenograft model. (a) CD3-depleted bone marrow samples were injected (i.v.) into non-conditioned NSG mice and were monitored for engraftment by flow cytometry and/or

peripheral blood smears and randomized into treatment groups where indicated. **(b)** Flow cytometry analysis of bone marrow (as in **Fig. 5c** and representative from **Fig. 5d–h**) from NSG mice engrafted with groups of 4 independent Ph⁺ ALL samples. **(c)** Representative confocal fluorescent analysis of the tibia in recipients engrafted with sample MD3. Long-term treatment (3 weeks) caused a reduction in cycling leukemia cells (yellow) accompanied by apoptosis (light blue). Residual disease (arrows) and apoptosis (asterisks) are noted frequently in DA and DA + PP242 treated mice respectively. Images are representative of BM from 3 mice of each treatment arm. Flow cytometry accompanies the marrow in **Fig. 5g** and panel **b** above. **(d)** Spleen weights of mice engrafted with indicated samples following both short- or long-term treatment. Note, DA response was grouped according to achieving leukemic clearance ($P < 0.05$) in the bone marrow. Pictures show representative gross spleen morphology of all treatments. **(e)** There was no overt toxicity from treatments as determined by changes in body weight (* $P < 0.05$).



Supplementary Fig 7. Lymphocyte function in the presence of pharmacologic mTOR kinase inhibition. **(a)** CFSE-labeled purified T cells were stimulated with anti-CD3 and anti-CD28 antibodies or left unstimulated to model polyclonal T cell activation. 15 min prior to activation cells were cultured with indicated inhibitors. After 3 days of culture with inhibitors cells were harvested, stained, and analyzed by flow cytometry to assess proliferation (CFSE-dilution). **(b)** CFSE-labeled DO11.10 transgenic CD4⁺ T cells were presented with OVA peptide by splenic-derived antigen presenting cells (APCs). Cells were either unstimulated or presented with indicated concentrations of OVA peptide and cultured for 72 hr in the presence or absence of RAP, BEZ-235, or PP242 as above.

Thy1.2⁺ T cells were subsequently analyzed by flow cytometry to assess proliferative ability by CFSE-dilution. (c) B cells were magnetically sorted from spleens and allowed to rest for 2 hr. Following the resting period, cells were pretreated for 15 min with indicated inhibitors followed by BCR activation with anti-IgM antibodies. Cells were stimulated for 15 min and immediately fixed with PFA, permeabilized, and stained for surface and intracellular antigens. The MdfI of control cells was normalized to 1 and unstimulated to 0.



Supplementary Fig 8. Comparison of splenic architecture in NP-OVA immunized mice treated with PP242, rapamycin, or PI-103. **(a)** IFA of frozen-spleen tissue from NP-OVA immunized mice from experiment 2 (**Fig. 6e–g**). B cells were stained with anti-B220 (red), and metallophilic macrophages with anti-CD169 (MOMA-1, white). Scale bar for 10x images (250 μm) and 20x images (120 μm). **(b)** The schematic highlights B cell areas of the marginal zone (MZ) surrounding the MOMA-1⁺ macrophages. Areas of

prominent MZ (blue arrows) and areas of MZ disruption (white arrows) are noted. At least three random areas on at least > 5 random follicles were enumerated for the radial length of the MZ extending out from the MOMA-1⁺ macrophages using Volocity imaging software. * $P < 0.05$, # $P < 0.001$ (1-way ANOVA).

Supplementary Table 1. PP242 pharmacologic properties.

a.

ADMET properties of PP242

Caco-2 apparent permeability coefficients (P _{app}):	Apical–basal 75.9 x 10 ⁻⁶ cm sec ⁻¹	Basal–apical 34.0 x 10 ⁻⁶ cm sec ⁻¹
Aqueous solubility:	at pH 7.4: 33 μM Sol	at pH 2–3: > 200 μM Sol
Liver microsome stability, t _{1/2} :	Mouse: 30 min	Human: 37 min
Cytochrome P450 inhibition (μM):	1A (> 10), 2C9 (> 10), 2D6 (> 10), 3A4/5 (> 10)	
Ames test (+/- S9):	Negative	
hERG [IC ₅₀]:	> 10 μM	
Plasma protein binding (h):	80%	

b.

Pharmacokinetic properties of PP242

Treatment:	AUC _(0-t) (μg L ⁻¹ hr)	AUC _(0-∞) (μg L ⁻¹ hr)	t _{1/2z} (hr)	T _{max} (hr)	V _z (L kg ⁻¹)	CL _z (L hr ⁻¹ kg ⁻¹)	C _{max} (μg L ⁻¹)	F (%)
2 mg kg ⁻¹ (i.v.)	117	119	0.9	-	20.7	16.9	439	-
20 mg kg ⁻¹ (p.o.)	704	970	5	0.25	-	-	268	60

Supplementary Table 2. Drug GI₅₀ values and synergy studies in p190 cells.**a.**

Compound	GI ₅₀ [nM] (95% confidence intervals)		
	p190-transformed murine BM	SUP-B15	K562
Imatinib	185 (125–275)	575 (345–950)	230 (170–300)
Dasatinib	2 (1–3)	42 (32–58)	1 (0.4–2.5)
Rapamycin	6.5 (0.4–90)	8 (2.5–25)	20 (0.5–60)
PP242	12 (8–15)	90 (60–130)	85 (50–155)
Ku-0063794	36 (21–60)	110 (90–130)	650 (289–1,892)
BEZ-235	4 (3–5)	63 (31–130)	55 (35–85)
PI-103	86 (52–143)	195 (120–220)	300 (170–520)
TG101348	> 20,000	> 20,000	N/A
IC87114	> 10,000	> 20,000	N/A

b.

Compound	Combination index (CI)		
	CI (at GI ₅₀)	CI (at GI ₇₅)	CI (at GI ₉₀)
Imatinib alone	NA	NA	NA
Imatinib + Rapamycin	0.68	0.53	0.54
Imatinib + PP242	0.61	0.32	0.18
Imatinib + BEZ-235	0.60	0.29	0.17

Supplementary Table 3. Drug GI₅₀ values in solid tumor cell lines.

Drug	GI ₅₀ (μM)			
	Cell line and <i>mutation</i>			
	SKOV3	PC3	786-O	U87
	<i>PIK3CA</i>	<i>PTEN</i>	<i>PTEN</i>	<i>PTEN</i>
Rapamycin	24.4	None	23.9	16.7
PP242	0.49	0.19	2.13	1.57
PI-103	0.97	0.35	3.7	2.8

Supplementary Table 4. Clinical history and characteristics of human samples.

(* used for Leukemic-CFC cultures, † used for xenograft studies)

Ph⁺ B-ALL samples:

*†**MD-1:** Bone marrow sample, t(9;22)(q34;q11.2). Age 59. Patient with pre-B ALL tested positive for the e1a2 BCR-ABL fusion transcript coding for the p190 kDa BCR-ABL protein. Sample was collected before any treatment regimens. Patient had a complete response (CR) to hCVAD+dasatinib.

†**MD-3:** Bone marrow sample, t(9;22)(q34;q11.2). Age 66. Patient with pre-B ALL tested positive for the e1a2 BCR-ABL fusion transcript. Sample was collected before any treatment regimens. Patient had a temporary CR to hCVAD+dasatinib. (#) Sample did not grow in culture.

*†**MD-4:** Bone marrow sample, t(9;22)(q34;q11.2). Age 9. Patient with pre-B ALL tested positive for the e1a2 BCR-ABL fusion transcript. Patient was treated with multiple chemo protocols. Patient failed double dose-imatinib and developed a BCR-ABL kinase mutation T315I. Sample was collected after 2nd relapse.

***MD-6:** Bone marrow sample, t(9;22)(q34;q11.2). Age 28. Patient with pre-B ALL tested positive for the e1a2 BCR-ABL fusion transcript. Sample was collected before any treatment regimens. Patient achieved CR with hCVAD+dasatinib. Patient underwent allogeneic stem cell transplant and is currently in CR.

***MD-7:** Bone marrow sample, t(9;22)(q34;q11.2). Age 49. Patient with pre-B ALL tested positive for the e1a2 BCR-ABL fusion transcript. Sample was collected before any treatment regimens. Patient achieved CR with hCVAD+dasatinib. Patient is currently on dasatinib maintenance therapy and is negative for BCR-ABL transcripts by PCR.

***MD-8:** Bone marrow sample, t(9;22)(q34;q11.2), -9, +7. Age 72. Patient was diagnosed with ALL. Patient achieved CR with hCVAD chemo alone. Two years later patient relapsed and tested positive for the e1a2 BCR-ABL fusion transcript. The sample was taken before the patient was treated with hCVAD+imatinib. No further treatment response information is available.

***MD-9:** Bone marrow sample, t(9;22)(q34;q11.2). Age 54. Patient was diagnosed with ALL. Patient achieved CR with hCVAD chemo alone. One half-year later patient relapsed and tested positive for the e1a2 BCR-ABL fusion transcript. The sample was taken before treatment with hCVAD+imatinib. The patient achieved CR. One half-year later patient relapsed and was switched to dasatinib. Patient failed therapy and died 3 months later.

***MD-10:** Bone marrow sample, t(9;22)(q34;q11.2). Age 28. Patient was diagnosed with pre-B ALL tested positive for the e1a3 BCR-ABL fusion transcript. Sample was

collected before any treatment regimens. Patient achieved CR with hCVAD+imatinib. One half-year later patient relapsed from therapy. A second sample was collected at this time, designated **MD-10R**. No further treatment response information is available.

†**MD-11**: Bone marrow sample, t(9;22)(q34;q11.2). Age 53. Patient with pre-B ALL tested positive for the b2a2 BCR-ABL fusion transcript coding for the 210 kDa BCR-ABL protein. Sample was collected before any treatment regimens. Patient achieved CR with hCVAD+imatinib. Patient subsequently underwent allogeneic stem cell transplant. One half-year later, patient presented with relapsed disease. Patient was switched to salvage chemotherapy with hCVAD+imatinib (off protocol). Patient failed to respond and died 3 months later.

***MD-12**: Bone marrow sample, t(9;22)(q34;q11.2), +X, +2, +6, +14, +18, +21, der(22). Age 53. Patient with pre-B ALL tested positive for the e1a2 BCR-ABL fusion transcript. Sample was collected before any treatment regimens. Patient achieved CR with hCVAD+dasatinib. Patient is currently on dasatinib maintenance and remains in CR.

***MD-13**: Bone marrow sample t(9;22)(q34;q11.2), -7. Age 51. Patient was diagnosed with preB-ALL tested positive for the e1a3 BCR-ABL fusion transcript. Sample was collected before any treatment regimens. Patient achieved CR with hCVAD+dasatinib. Two years later patient relapsed and tested positive for V299L mutation. Patient was switched to hCVAD+imatinib regimen but failed to respond. Patient achieved 2nd CR to nilotinib. Patient currently remains in 2nd CR.

***UCI-1**: Peripheral blood sample, t(9;22)(q34;q11.2), t(3;10), trisomy 8. Age 15. The patient failed one combination chemotherapy regimen with standard dose imatinib. The blood sample was collected following a 2nd relapse and immediately before starting dasatinib, which induced CR.

***UCI-2**: Peripheral blood sample, t(9;22)(q34;q11.2), der(7;12). Age 63. The patient failed two combination chemotherapy regimens, one with standard dose imatinib. The blood sample was collected following 2nd relapse and immediately before starting a dasatinib protocol, which induced a temporary CR.

***UCI-4**: Peripheral blood sample, t(9;22)(q34;q11.2), der(16), t(1;16)(q21;q13). Age 36. Sample was collected before any treatment regimens. Patient achieved CR with hCVAD+dasatinib.

***UCI-5**: Peripheral blood sample, t(9;22)(q34;q11.2). Age 42. Blood was collected before any treatment regimens. Patient achieved CR with hCVAD+imatinib.

Ph⁺ CML samples:

***UCI-6**: Peripheral blood sample, t(9;22)(q34;q11.2). Age 30. Patient diagnosed with CML in chronic phase. Patient progressed to LyBC-CML before blood sample was collected. Sample was obtained immediately before starting dasatinib treatment. The patient failed one combination chemotherapy regimen with standard dose imatinib.

***UCI-7:** Peripheral blood sample, t(9;22)(q34;q11.2) with multiple additional karyotype anomalies and three BCR-ABL point mutations. Age 31. Patient diagnosed with CML in chronic phase. Patient achieved CHR on imatinib, but was non-compliant. Patient progressed to LyBC-CML 2 years later. Patient achieved CHR with dasatinib. Sample was collected after initial period of dasatinib. Patient was non-compliant with dasatinib and subsequently relapsed. No further treatment response information is available.

***UCI-8:** Peripheral blood sample, t(9;22)(q34;q11.2). Age 45. Patient diagnosed with CML in chronic phase. Patient progressed to LyBC-CML several years after initial diagnosis. Initial treatment was interferon and hydroxyurea. Patient was treated at UCLA on the "first-in-human" imatinib trial. Sample was obtained at initial relapse on imatinib.

***UCI-9:** Peripheral blood sample, t(9;22)(q34;q11.2). Age 63. Patient diagnosed with CML in accelerated phase. Patient failed imatinib, then progressed to LyBC-CML one half-year later. Sample was collected at this point. Patient subsequently failed two chemotherapy regimens.

***UCI-10:** Peripheral blood sample, t(9;22)(q34;q11.2). Age 54. Patient diagnosed with CML in chronic phase. Patient progressed to MyBC-CML before blood sample was collected. Patient was refractory to dasatinib, nilotinib, double dose imatinib, and multiple chemo protocols.

Abbreviations:

hCVAD = Hyper-CVAD chemo regimen comprised of (1) dose-intense systemic chemotherapy and intensified consolidation; (2) prolonged maintenance therapy; (3) CNS prophylaxis; and (4) supportive care (hematopoietic growth factors and prophylactic antibiotics).

CR = Complete response or complete remission

CHR = Complete hematologic response

LyBC-CML = Lymphoid blast crises phase of chronic myelogenous leukemia

MyBC-CML = Myeloid blast crises phase of chronic myelogenous leukemia

Supplementary methods:

Antibodies. For immunoblotting and microscopy staining we used: a purified mouse IgM mAb specific for PIP3 (catalog #Z-P345, Echelon Biosciences), rabbit and mouse antibodies specific for total and phosphorylated forms from Cell Signaling Technologies: AKT (#2965, #9267, #4058, #4685), FOXO (#9464, #9461, #2880 or clone C29H4 for confocal microscopy analysis), NDRG1 (#3217), ERK1/2 (#4370), PRAS40 (#2640), GSK-3 α/β (#9327) mTOR (#2974, #2983), rS6 (#4838, #2215, #2211), 4EBP1 (#2855, #9455, #9451), eIF-4G (#2617), eIF-4E (#2067), cleaved caspase-3 (#9664 or clone 5A1E). An antibody recognizing tubulin (clone B-5-1-2) was obtained from Sigma-Aldrich. An antibody recognizing phospho-tyrosine (clone 4G10) was obtained from Millipore. An antibody recognizing LC3 (clone 4E12) was obtained from MBL international. For flow cytometry, anti-mouse antibodies were: IgM-FITC (II/41), -PC7 (R6-60.2), CD8-PerCP or PerCP/Cy5.5 (5H10-1), IgD-FITC (11-26c.2a), CD19-PE (1D3), B220-PE, -APC, -Ax647, -APC/efluor780, and -APC/Ax750 (RA3-6B2), Fas-PE (15A7), CD4-Ax488, -PE (GK1.5), CD25-PC7 (PC61), CD21-PerCP/Cy5.5 (4E3), CD23-PE/Cy7 (B3B4), CD38-PC7 (90), CD11b-APC (M1/70). Anti-human antibodies were: CD4-Ax488, -Pacific blue, -Ax647 (OKT4), CD3-APC (OKT3), CD8-APC (RPA-T8), CD45-PerCP/Cy5.5 (HI30), CD19-Ax488 (HIB19), CD34-PE (4H11). All flow cytometry antibody reagents were purchased from Invitrogen, eBioscience, and Biolegend. Succinimidyl ester 5-(and -6) carboxyfluorescein diacetate, (CFDA or CFSE), DAPI, hoechst 33342, propidium iodide and 7-amino-actinomycin D (7AAD, both used to detect DNA content), Live/dead fixable blue (UV-excitable viable dye), EdU

Click-iT microscopy kit, and EdU Click-iT flow cytometry kit were all obtained from Invitrogen.

Retroviral vectors. Bicistronic retroviral vectors were used to express exogenous genes and a marker gene from the murine stem cell virus (MSCV) long terminal repeats. These vectors have been previously described elsewhere ¹.

Bone marrow progenitor cell transduction and establishment of p190 BCR-ABL transformed cells. BM cells were flushed from the long bones (tibias and femurs) of 3- to 4-week-old mice. Cells were spinoculated with retroviral supernatants (p190 BCR-ABL-IRES-hCD4 or mutated p190 where indicated) in the presence of 5 $\mu\text{g ml}^{-1}$ polybrene for 45 min at 450 g and 37°C with RPMI+20% FCS culture medium supplemented with recombinant mouse IL-7 (10 ng ml⁻¹; Invitrogen) to promote cell cycle entry. Following spinoculation, cells were incubated overnight at 37°C, 5% CO₂, expanded, until a pure 100% human CD4⁺ cytokine- and stromal- independent culture was established as previously described ¹. For colony formation of mouse p190 transformed cells, 5 x 10⁴ cells were plated in M3630 methylcellulose cultures (StemCell Technologies) that contained indicated amounts of inhibitor. M3630 cultures were scored 7 days later. Mutant p190 BCR-ABL constructs were generated with the Quicksite mutagenesis kit (Stratagene) using the manufacturer's recommended instructions.

***In vitro* proliferation and drug combination experiments.** Cells were seeded in triplicate wells of 96-well flat bottom culture plates for 48 hr in the presence of increasing concentrations of indicated inhibitors. Cell viability and median-effect dose affecting growth (GIC₅₀) was determined using the MTS assay (Cell Titer 96 Aqueous One solution cell proliferation assay kit; Promega). Absorbance values (490 nm) were normalized to controls and expressed as %MTS conversion. Wells lacking cells but with MTS added were used as the zero value when normalizing. For drug combination experiments, a range of fixed ratios of inhibitors was used to assess synergy using the combination index (CI) with CalcuSyn software (Biosoft) according to the median-effect method as previously described². For proliferation experiments with PC-3, SKOV3, 786-O, and U87 cells, the CellTiter-Glo Luminescent reagent (Promega) was used following the manufacturer's instructions. Quantitation was performed as mentioned above.

Western blotting. Western blots were performed using standard procedures where cells were harvested, washed with HBSS and lysed in buffer containing 40 mM HEPES (pH 7.4), 120 mM NaCl, 1% Triton X-100, 1mM Na₂EDTA, 10 mM Na pyrophosphate, 10 mM Na glycerophosphate, 50 mM NaF, 0.5 mM Na₃VO₄, supplemented with fresh phosphatase inhibitor cocktails I and II (Sigma-Aldrich), and protease inhibitor cocktail (Calbiochem) for 25 min on ice. Lysates were centrifuged at 16,000 rpm for 10 min and protein concentration in the supernatant was determined by the Bradford method (Pierce) or by cell number equivalent. Equal amounts of total protein were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Blots were probed overnight at

4°C with antibodies against the proteins of interest. After incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were detected using chemiluminescence (Pierce).

Cap binding assay. Cells treated with indicated conditions were washed with ice-cold HBSS and lysed in cap binding buffer (150 mM NaCl, 50 mM Tris pH 7.5, 50 mM NaF, 10 mM Na pyrophosphate, 1 mM EDTA, supplemented with fresh phosphatase inhibitor cocktails I and II (Sigma-Aldrich), and protease inhibitor cocktail (Calbiochem) by freeze/thawing 3 times using liquid nitrogen. 200 µg of lysate in 200 µl of cap binding buffer was added to 50 µl pre-washed 7-methyl GTP sepharose beads (Amersham Biosciences). Slurry was put on a shaker (high setting) for 1 hr at RT. Samples were subsequently washed with cap binding buffer, and quenched with sample buffer. Samples were boiled and equal amounts of eluate and 10 µg of lysate were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Blots were probed as mentioned above.

Confocal and light microscopy. PIP3 levels were determined in mouse p190 transformed cells as has been previously described¹. In brief, cells were treated in 12 well plates with the indicated inhibitors. Cells were subsequently seeded onto sterile glass chamber slides (BD biosciences) that were previously coated with poly-*L*-lysine (Sigma-Aldrich) for 15 min. Subsequently, cells were washed in HBSS, fixed with 4% PFA for 20 min, and permeabilized with 0.5% saponin in TBS for 20 min. Cells were

then blocked for 1 hr in 10% goat serum (Sigma-Aldrich) and incubated with primary antibody overnight at 4°C in PBS with 0.5% BSA and 1% goat serum. After washing, incubation with a secondary antibody (goat anti-IgM TRITC conjugated) was carried out for 1 hr at RT. Cells were washed and counterstained with DAPI-containing Vectashield mounting medium (Vector Laboratories) and examined using a confocal fluorescent microscope (Olympus Fluoview FV1000) using 405 nm (DAPI) and 559 nm (TRITC) laser lines for excitation and a 40x oil, 1.3NA objective. Images were rendered using Volocity 3D/4D image analysis software (Version 5.1.0). Measurement parameters to locate cellular objects was performed by excluding objects < 2 µm, and thresholding by s.d. intensity. This protocol was applied to the DIC channel and PIP3 fluorescent channel. Automatic thresholding was used to generate colocalization of DIC and PIP3 channels. All PIP3 intensity colocalized 100% with DIC channels for fidelity. This protocol was batched applied to all treatment conditions. The pixel intensities and total PIP3 area per cellular object was calculated. The data were calculated and exported into Excel and presented using GraphPad Prism software (version 4.0c).

For detection of EdU accumulation, and Foxo1 translocation studies, p190 cells were treated with inhibitors for the indicated amount of time in 12 well plates. 1 hr before harvesting cells, 2 µM of EdU (Invitrogen) was added to the cells, mixed, and allowed to incubate for 1 hr. Cells were subsequently seeded onto sterile glass chamber slides (BD biosciences) that were previously coated with poly-*L*-lysine (Sigma-Aldrich) for 15 min. Subsequently, cells were washed in HBSS, fixed with 3.7% PFA for 20 min. Cells were washed with HBSS, and half were processed for either 1). EdU accumulation or 2). Foxo1 nuclear translocation. Cells processed for 1) were permeabilized with

0.5% triton X-100 and were subsequently stained for EdU accumulation following the manufacturer's instructions for EdU detection. Click-iT reaction cocktail with the Ax594-conjugated azide was used. Cells processed for 2) were permeabilized with 90% methanol and were subsequently blocked for 1 hr in 10% goat serum (Sigma-Aldrich) and incubated with primary antibody (anti-FOXO1 clone C29H4, #2880, Cell signaling technologies) overnight at 4°C in PBS with 0.5% BSA and 1% goat serum as in 1). Cells were subsequently washed and incubated with goat anti-rabbit IgG-Ax594 (Invitrogen) for 1 hour at RT. Cells were counterstained with mounting medium Vectashield (Vector Laboratories) supplemented with Hoechst 33342 and examined using a confocal fluorescent microscope (Zeiss LSM710 Meta NLO) using 405 nm (Hoechst 33342), and 543 nm (Ax594) laser lines with a 40x water objective. Images were rendered using Volocity 3D/4D image analysis software (Version 5.1.0).

For detection of EdU, and cleaved caspase-3 in formalin-fixed paraffin embedded (FFPE) tissues, spleens of treated mice as indicated in the figure legends were fixed in buffered formalin and processed using standard methods. Slides were subsequently dewaxed as has been previously described³. Antigen retrieval (Dako target retrieval solution, High pH) was performed at 95°C for 30 min and cooled for 20 min at RT. Slides were subsequently washed and the detection of EdU accumulation was assessed using the Click-iT reaction buffer (as mentioned above). Slides were subsequently washed and stained with primary antibody (anti-cleaved caspase-3), followed by subsequent washes and secondary stain with goat anti-rabbit IgG-Ax488 (Invitrogen). All antibodies were diluted in Dako Antibody Diluent containing background reducing components. Slides were mounted using the same procedures as above. Images were

captured on a confocal fluorescent microscope (Zeiss LSM510 META NLO) using 488 nm (cleaved caspase-3), 561 nm (EdU), and 710-930 nm 2-photon coherent chameleon (DAPI) laser lines with both 40x water, and 63x oil objectives. Images were rendered using Volocity 3D/4D image analysis software (Version 5.1.0).

For detection of EdU, human CD19, and cleaved caspase 3 in FFPE bone marrow tissues, the long bones of the femur and tibia were dissected off the mouse immediately following sacrifice. The skin and muscle from the hip down was removed, and the bones were fixed in Cal-Ex II (Fisher) for 3 days. Samples were subsequently transferred to 70% ethanol and processed using standard methods. Dewaxing and antigen retrieval was performed as above. Slides were subsequently stained with Click-iT detection reagents as mentioned above. Slides were subsequently washed, blocked with 10% goat serum and Fc block (rat anti-mouse CD16/CD32; Biolegend) for 1hr, and stained with primary antibodies (mouse IgG1 anti-human CD19 clone LE-CD19, Dako) and rabbit anti-cleaved caspase-3 (cell signaling technologies) followed by subsequent washes and secondary stain with goat anti-rabbit Ig-Ax488 (Invitrogen) and goat anti-mouse Ig-Ax647 (Invitrogen). All antibodies were diluted in Dako Antibody diluent (with background reducing components). Cells were counterstained with mounting medium Vectashield (Vector Laboratories) supplemented with Hoechst 33342 and examined using a confocal fluorescent microscope (Zeiss LSM710 Meta NLO) using 405 nm (Hoechst 33342), 488 nm (Ax488), 543 nm (Ax594), and 633 nm (Ax647) laser lines with a 20x air or 40x water objective. Images were rendered using Volocity 3D/4D image analysis software (Version 5.1.0).

For detection of marginal zone (MZ) B cells, spleens were quickly frozen in an OCT compound (VWR) using a slurry of 2-butanol in a container cooled by liquid nitrogen. 8 micron frozen sections were desiccated overnight at RT and fixed in acetone at -20C for 20 min. Slides were washed and blocked in 10% goat serum for 30 min at RT. Slides were subsequently stained with rat anti-mouse B220 (BD biosciences, clone RA3-6B2, 1:50 in 10% goat serum) for 2 hr at RT. Slides were washed 3x for 5 min with PBS+0.5% Tween-20. Slides were next stained with goat anti-rat-Ax594 (Invitrogen, 1:1000 in 10% goat serum) for 1 hr at RT. Slides were washed 3x for 5 min with PBS+0.5% Tween-20. Slides were next stained with rat anti-mouse CD169-FITC (Serotec, clone MOMA-1, 1:100 in 10% goat serum) for 1 hr at RT. Slides were washed 3x for 5 min with PBS+0.5% Tween-20. Slides were mounted with Vectashield (Vector Laboratories) and examined using a confocal fluorescent microscope (Olympus Fluoview FV1000) using 488 nm (FITC) and 559 nm (Ax594) laser lines for excitation and a 10x/0.4NA and 20x/0.75NA objectives. Images were rendered using Volocity 3D/4D image analysis software (Version 5.1.0).

For histology slides, spleens were stained with hematoxylin and eosin (H&E) using standard methods. Light microscopy pictures were captured on an Olympus BX60 using 4x, 10x, 20x, and 40x objectives where indicated. Images were processed in Photoshop CS2.

Flow cytometry. For cell cycle analysis, cultures were washed cell cycle buffer (PBS containing 5 mM EDTA) and subsequently fixed with ice-cold 70% EtOH

dropwise while being vortexed (medium setting). Cells were fixed overnight at -20°C . Samples were subsequently washed with cell cycle buffer and incubated with 2 mg ml^{-1} RNase A solution for 3 hr at RT. Cells were subsequently stained with prodium iodide (0.5 mg ml^{-1}) for 1 hr at RT. To prepare RNase A solution, DNase-free RNase from bovine pancreatic RNase, type IIA (Sigma) was dissolved in 10 mM sodium acetate (pH 5.2) and heated to 100°C for 15 min. Solution was cooled and adjusted to pH 7.4 by adding 90% volume 1M Tris-HCl (pH 7.4). Solution was stored at -20°C for fresh aliquots. The DNA fluorescence parameter (585/42 nm bandpass) using linear amplification was collected on a FACS Caliber equipped with a 488 nm laser line. Doublets and aggregates were excluded by pulse width versus area. For all cell surface staining, cells were stained with pre-titrated concentrations of indicated antibodies at RT for 15 min in FACS buffer (HBSS containing 0.5% BSA and 1% FCS). Cells were washed, transferred to tubes and analyzed on an LSR II (BD biosciences) equipped with 350 nm, 405 nm, 488 nm, and 633 nm laser lines or a FACS Caliber equipped with 488 nm and 635 nm laser lines.

Intracellular phospho-staining by flow cytometry. Intracellular phospho-staining was performed as previously described with a few modifications⁴. In brief, single cell suspensions were obtained from either 1) bone marrow (BM), 2) peripheral blood (PB), or 3) cell culture suspension cells. 1). BM cells were flushed from the long bones (tibiae and femurs) of mice into ice-cold RPMI+20% FCS. 1 ml of single cell suspensions were immediately fixed with 16% paraformaldehyde (PFA) stock solutions (EM grade, Electron Microscopy Sciences) for a final 2% concentration for 10 min at RT. Cells were

subsequently washed, then permeabilized with ice-cold 95% methanol (20 minutes) and stored at -20°C before staining for flow cytometry. 2). Peripheral blood was collected from retro-orbital eye bleeds from mice. 100 μl of blood was diluted (1:4) in PBS+5 mM EDTA to prevent coagulation. Suspensions were washed once at RT with FACS buffer and immediately fixed as above. 3). Single cell suspensions in culture were likewise fixed immediately in culture media by adding a 1:10 volume of 16% PFA and processed as above.

Before staining, cells were washed twice with FACS buffer (HBSS containing 0.5% BSA and 1% FCS) and incubated for 10 min with Fc Block (1:100, Biolegend) at room temperature. Unconjugated primary antibodies were added (p-AKT (S473) 1:50, p-rS6 (S235/236) 1:300, p-CrkL (Y207) 1:75, p-4EBP1 (T37/46) 1:50) in 1.5 ml eppendorf tubes and incubated at RT for 1 hour. Samples were washed by adding 1 ml of FACS buffer, allowed to sit for 5 min followed by centrifugation at 6,000 rpm for 5 min). In between all washes, supernatant was carefully aspirated preserving the intact pellet. Secondary antibody (goat anti-rabbit IgG-biotin) was used to amplify primary signal by staining in a new eppendorf tube (1:300, Jackson Laboratories). Cells were stained at room temperature in the dark for 30 min. Antibodies to lineage antigens were included in the sample master mix at optimal concentrations that were pre-determined. After incubation, samples were washed, transferred to new tubes and incubated in streptavidin-conjugated with PE or APC (1:500–1:1000, ebioscience) for 20 min at RT. Cells were washed, transferred to tubes and analyzed on an LSR II (BD biosciences) equipped with 350 nm, 405 nm, 488 nm, and 633 nm laser lines or a FACS Caliber equipped with 488 nm and 635 nm laser lines.

Data collection and analysis for flow cytometry. Data were collected using DIVA software or Cell Quest Pro and analyzed using Flowjo software. To determine the P-flow signature where indicated in the figure legends, dead cells were gated out by use of either size exclusion by forward/side scatter parameters or with use of LIVE/DEAD fixable blue dye (Invitrogen). Surface lineage antigens were then profiled for population of interest. The median fluorescent intensity (MdFI) value for the particular intracellular antigen in each cell population of interest was then determined. The fold change (FC) was then calculated: $FC = \frac{\text{MdFI of treated mouse of interest (or vehicle control)}}{\text{the average MdFI of all vehicle treated mice}}$. This essentially gives you the fold change of treated/control. The FC value is then \log_2 transformed. Values are then tabulated in Prism Graphpad software. The $\text{Log}_2(FC) = \text{P-Flow score}$. Negative values = inhibition, positive values = activation, zero values = basal.

To determine the fractional phospho-signature in lymphocytes (**Supplemental Figure 7c**), B220⁺ events were analyzed for their MdFI values for each particular intracellular antigen. The unstimulated MdFI values were normalized to 0 and the corresponding stimulated MdFI values were normalized to 1.

Human Ph⁺ L-CFU assay. Mononuclear cells isolated from fresh bone marrow or peripheral blood were thawed, washed with IMDM containing 5% FCS. Peripheral blood samples were ficoll-paque plus (GE Healthcare) separated followed by isolation of the CD34⁺CD19⁺ (ALL) or CD34⁺ (CML) fraction by using the CD19 MultiSort kit and/or

CD34 microbeads (Miltenyi Biotec). For bone marrow samples, cell purity was determined by flow cytometry to assess lineage surface expression (CD45, CD34, CD38, CD19, CD20, CD3, CD4, CD8, CD15, CD16, CD11b), and viability using an LSRII (BD biosciences) equipped with 350 nm, 405 nm, 488 nm, and 633 nm laser lines. Samples greater than 90% pure leukemic blasts were used for colony assays without magnetic separation. Bone marrow cells that were less than 90% pure for leukemic blasts were magnetically enriched as stated above. Cells were kept on ice throughout the duration where possible.

Pure samples or enriched cells were subsequently plated in either IMDM supplemented with 30% FCS, 2 mM glutamine, 5 mM HEPES, antibiotics or StemPro-34 SFM media (Invitrogen). Both suspensions were additionally supplemented with the following human recombinant cytokines (Invitrogen): IL-3 (100 ng ml⁻¹), IL-7 (100 ng ml⁻¹), Flt-3L (100 ng ml⁻¹), SCF (100 ng ml⁻¹) at 3 x 10⁶ cells ml⁻¹. Following a 1 to 2-hour resting period, leukemic progenitors (3.3 x 10⁴ to 10 x 10⁴ cells) were added to complete MethoCult GF+H4435 (Stem Cell Technologies) at a 1:10 dilution (50 µl in 450 µl MethoCult) in 24 well plates. GF+H4435 MethoCult was additionally supplemented with recombinant human cytokines for a final concentration: IL-3 (100 ng ml⁻¹), IL-6 (20 ng ml⁻¹), GM-CSF (20 ng ml⁻¹), G-CSF (20 ng ml⁻¹), IL-7 (100 ng ml⁻¹), Flt-3L (100 ng ml⁻¹), SCF (100 ng ml⁻¹), EPO (3 U ml⁻¹), and antibiotics. Inhibitors were added at the indicated final concentrations at this point. GF+H4435 MethoCult cultures were then vigorously vortexed for 1 min, allowed to settle for 5 min, and 400 µl was dispensed into the inner 8 wells of a 24 well plate. The outer wells surrounding the

inner 8 wells were lined with 0.5 ml water to prevent dehydration. Colonies were scored after 8-10 days (CML cultures) or 12-16 days (ALL cultures).

Primary human bone marrow cultures. Normal donor bone marrow light density cells (Poietics Lot 07B21195) were thawed, washed with IMDM+2% FBS and evaluated for cell viability. Test compounds were added at indicated concentrations to MethoCult 84434 (Stem cell Technologies). Human marrow cultures were plated in triplicate with 1.0×10^4 cells per culture in 35 mm dishes. Following 14 days in culture, hematopoietic colonies were scored by Stem Cell Technologies using the following criteria: CFU-E (colony-forming unit-erythroid; mature erythroid colony containing 8–200 erythroblasts), BFU-E (burst-forming unit-erythroid; primitive erythroid colony containing > 200 erythroblasts), CFU-GM (colony-forming unit-granulocyte, macrophage; containing > 40 granulocytes-monocyte and/or macrophage cells), CFU-GEMM (colony forming unit-granulocyte, -erythroid, -macrophage, -megakaryocyte; primitive multipotent colony forming unit containing more than one lineage of > 20 or more cells).

***In vivo* transplant experiments with mouse p190 leukemia.** Mouse p190-transformed BM cells were used to initiate leukemia in either irradiated (conditioned with 450 rads) or non-irradiated (immunocompetent) syngeneic (Balbc/J) recipients. In all *in vivo* experiments p190 transformed BM was prepared fresh (< 4 week old cultures) to initiate leukemia. Leukemic engraftment was determined in anesthetized animals by retro-orbital

bleeds and analyzed by flow cytometry where indicated. Mice were euthanized as judged by signs of extreme hunched posture with piloerection, labored breathing, or weight loss.

SUP-B15 xenograft bioluminescent imaging. The human Ph⁺ ALL cell line SUP-B15 (ATCC# CRL-1929) infected with an EGFP-ffLuc lentiviral (pHIV7 backbone) plasmid (a kind gift from Michael Jensen, City of Hope, Duarte, CA.) was used for bioluminescent imaging. 8–10 wk-old female NSG mice were injected (i.v.) with 2.5×10^6 SUP-B15^{ffLuc} cells and 16 days later total body leukemia burden was assessed by bioluminescent imaging (BLI). Mice with established disease were randomly assigned into treatment groups and treated as reported in the figure legend. Imaging was performed again on days 23 and 30. Imaging was performed by injection of D-Luciferin firefly, potassium salt (Gold Biotechnology) at 150 mg kg^{-1} (i.p.) in awake animals. Following 7 min of distribution, mice were anesthetized (ketamine/xylazine) and placed on the imaging platform (IVIS Lumina, Caliper Life Sciences). All mice in groups of 3 were imaged between 10–12 minutes following the D-Luciferin injection/anesthesia procedure. Three mice were imaged at once following a 15 sec exposure for BLI in both supine (ventral, V) and prone (dorsal, D) positions. Regions of bioluminescence were drawn around each corresponding animal and the total photon flux sec^{-1} was determined using Living Image 3.2 software (Caliper Life Sciences).

***In vivo* xenograft experiments with human Ph⁺ leukemia.** NSG mice were used as recipients for human samples. For short term dosing experiments, non-irradiated NSG

mice were injected (i.v.) with leukemic samples (an equivalent amount of $0.3\text{--}10 \times 10^5$ cells per recipient). Leukemic engraftment was determined in anesthetized animals by retro-orbital bleeds or awake animals by puncture of the sub-mandibular facial vein and analyzed by flow cytometry. NSG mice were dosed with sterile disposable 20 G-1.5'' feeding needles (Popper). Mice were euthanized as judged by signs of extreme hunched posture with piloerection, labored breathing, or weight loss. All human samples were initially depleted with CD3-microbeads (Miltenyi Biotec) and purity verified by flow cytometry. At the time prior to being euthanized, recipient mice receiving EdU were injected 1 hr following last dose with indicated inhibitor. EdU (Invitrogen) was resuspended in sterile water at 5 mg ml^{-1} . Mice were injected i.p. with $100 \mu\text{l}$ into the left and right areas of the abdomen ($200 \mu\text{l}$ total). EdU was allowed 1 hr to distribute before mice were sacrificed. The femur and tibias were removed for single cell processing. Cells were cleared of RBCs using ACK lysis buffer. Cells were subsequently stained with live/dead fixable blue (Invitrogen) to remove dead cells during flow cytometric analysis. Following this, cells were stained for EdU accumulation following the manufacturer's instructions (using the saponin-based permeabilization step). Following EdU staining with the Click-iT staining reagent (Ax647 azide), cells were washed off twice with permeabilization buffer and resuspended in FACS buffer. Cells were subsequently stained for surface lineage antigens using standard flow cytometry staining methods and analyzed on a LSRII (BD biosciences) equipped with 350 nm, 405 nm, 488 nm, and 633 nm laser lines.

***In vivo* drug preparations.** PP242 was completely dissolved in PEG400 (Carbowax polyethylene glycol; Fisher-Scientific). PP242-PEG400 formulation was prepared fresh and used for dosages indicated in the figure legends by oral gavage (p.o.). An additional formulation was used for PP242 where indicated in the figure legends. PP242 was dissolved in NMP (1-methyl-2-pyrrolidinone; Sigma-Aldrich) and diluted to 5% in PVP (polyvinylpyrrolidone K 30; Fluka) diluted in water at a 15.8:84.2 wt vol⁻¹ ratio for a final 5% NMP, 15% PVP, 80% water vehicle. The PP242-NMP/PVP/water formulation was stored at room temperature and used within 72 hr at the dosages indicated in the figure legends by oral gavage. Rapamycin (LC Laboratories) was completely dissolved in 0.5% CMC (medium viscosity, carboxymethylcellulose sodium salt; Sigma-Aldrich) diluted in PBS. Rapamycin formulations were used fresh and administered by intraperitoneal (i.p.) injection. Imatinib was dissolved in water and prepared fresh for i.p. injection. Dasatinib was dissolved in a mixture of polypropylene glycol (Sigma-Aldrich) diluted in water (50:50) and administered by oral gavage. Dasatinib/PP242 combination was prepared as a 50:50 mixture of completely dissolved dasatinib (polypropylene glycol:water) combined with completely dissolved PP242 (NMP/PVP/water vehicle). The dasatinib/PP242 mixture had no overt effects on compound solubility. PI-103 was dissolved in 20% 4-hydroxypropyl β -cyclodextrin (MP biomedical) in saline and administered by i.p. injection. The imatinib/PI-103 mixture had no overt effects on compound solubility.

Serum ELISA. 96 well NUNC MaxiSorp plates (Nalgene) were coated with 50 μ l of NP(30)BSA (Biosearch Technologies) at a concentration of 50 μ g ml⁻¹ overnight at 4°C.

Serum dilutions were also plated out at 50 μ l per well. Serum Ig was detected with HRP-conjugated rabbit anti-mouse secondary antibodies against IgM and IgG1 (Invitrogen). Plates were developed with TMB peroxidase EIA substrate kit (BioRad) for colorimetric detection after which the reaction was stopped with 1N sulfuric acid and read on a plate reader at 450 nm.

Lymphocyte isolations. T cells were isolated from lymph nodes of 6–8 wk-old Balb/cJ mice using EasySep T cell enrichment kits (StemCell Technologies) according to the manufacturer's instructions. Where indicated, cells were labeled with CFSE in suspension with 2 μ M CFSE (Invitrogen) for 10 min in 37°C water bath. The labeling reaction was quenched by washing the cells twice with 10 ml of culture media followed by a final re-suspension in fresh culture media. B cells were purified from the spleens of 6–8 week old Balb/c mice by magnetic cell sorting using CD43 (Ly-48) microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Where indicated, cells were labeled in suspension with 2 μ M CFSE for 10 min in 37°C water bath. CFSE labeling was quenched by washing the cells twice with 10 ml culture media followed by re-suspension with fresh culture media.

***Ex vivo* lymphocyte stimulations.** T cells were stimulated with 10 μ g ml⁻¹ plate bound anti-CD3 ϵ (Southern Biotech, clone 145-2C11) and anti-CD28 (BD Pharmingen, clone 37.51) in 96 well NUNC MaxiSorp plates. Cells were pretreated with indicated concentrations of inhibitors for 15 min in 37°C water bath before adding cells to

antibody-coated wells. For OVA presentation studies, indicated amounts of OVA peptide were added to total splenocytes of DO11.10 T-cell receptor transgenic mice in 48 well plates. All cells were pretreated for 15 min with the indicated inhibitors prior to addition of OVA peptide. Splenocytes were subsequently cultured in the presence of inhibitors for 72 hours. T cells were stained with Thy1.2-PE (53-2.1, ebioscience). Cells were subsequently analyzed by flow cytometry. B cells were stimulated with $10 \mu\text{g ml}^{-1}$ of F(ab')₂ fragment anti-IgM, μ -chain specific antibody (Jackson ImmunoResearch, code 115-006-020). Cells were pretreated with the indicated concentrations of inhibitors for 15 min in 37°C water bath then plated into 48-well plates before adding an equal volume of warmed 2X anti-IgM stimulation solution.

Supplementary References

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