Supplemental Information Inventory

Supplemental Legends to Tables

Table S1. Related to Table 1. List of shRNAs targeting SLIM genes. Listed in alphabetical order based on HGNC symbol. FC; fold-change. Intensity values for a given probe set are provided: plus.1, plus.2 and plus.3 reflect detection of a given shRNA from the three imatinib treated cultures, and minus.1, minus.2 and minus.3 reflect detection of a given shRNA from untreated cultures.

Table S2. Related to Table 1. Biological functions overrepresented among SLIM genes. List was generated using Ingenuity Pathways Analysis (Ingenuity Systems).

Table S3. Related to Figures 2, 3, and 4. Combination index (CI values) for drug combinations tested in cell viability assays. CI values were calculated using CalcuSyn software (Biosoft). CI values less than 1 correspond to synergistic drug interactions, CI values equal to 1 correspond to additive interactions, and CI values more than 1 correspond to antagonistic interactions.

Figure S1

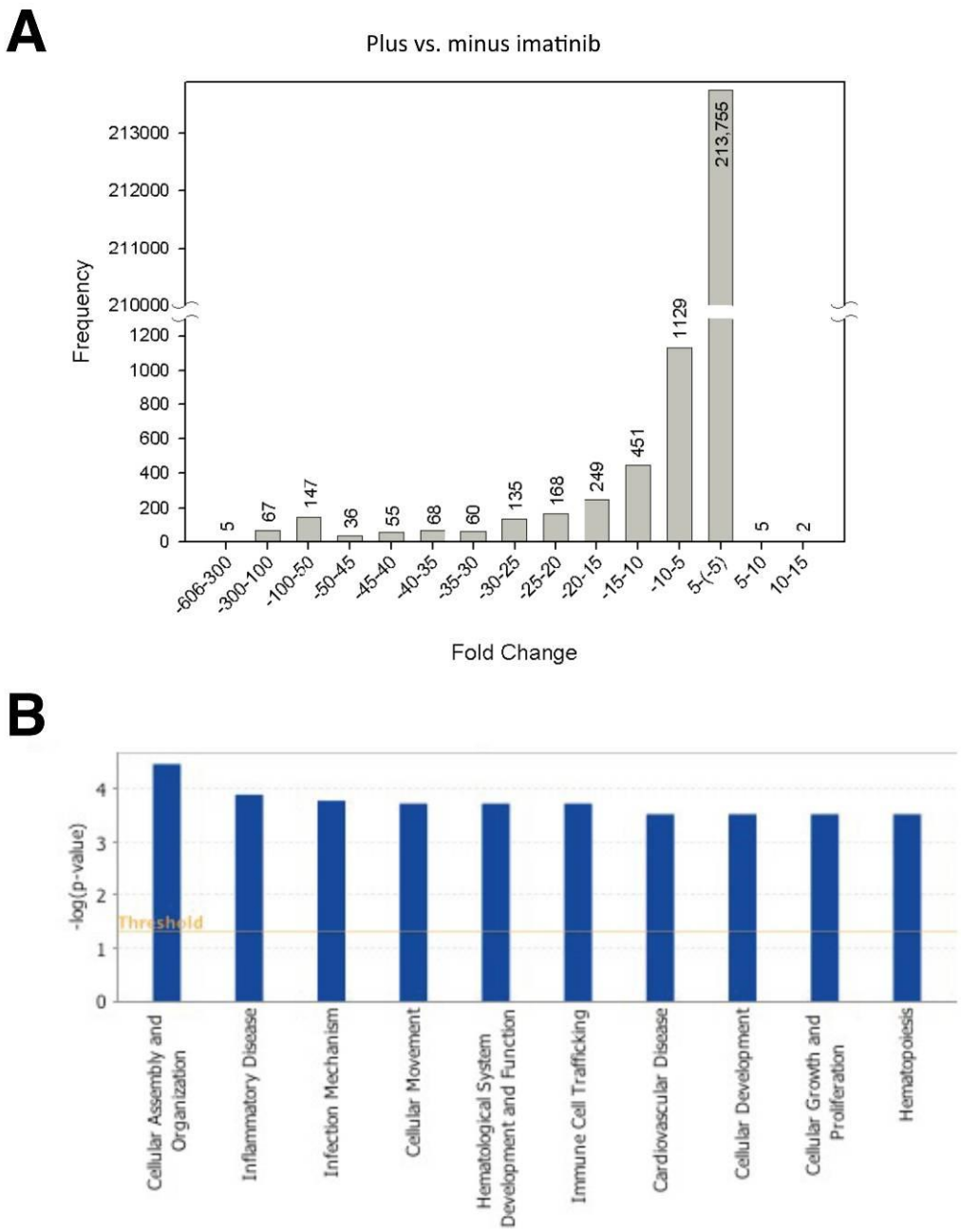

Figure S1. Related to Table 1. Fold-changes in the levels of individual shRNAs after imatinib treatment and biological functions of SLIM genes. A) Mean fold-changes for individual shRNA's were calculated as described in Supplemental Experimental Procedures and plotted versus frequency. The number above each bar shows the exact number of shRNAs that fall into each "bin" representing a range of fold-changes. The bin that includes shRNAs exhibiting no significant change (-5 to +5) would also include shRNAs from the library that were undetectable by the arrays. **B)** The SLIM gene list (Table S1) was analyzed using Ingenuity Pathways Analysis software (Ingenuity Systems). The top ten ranked functional categories identified by this analysis are shown. The log(p-value) refers to the significance of the overrepresentation of SLIM genes within a functional category.

Figure S2

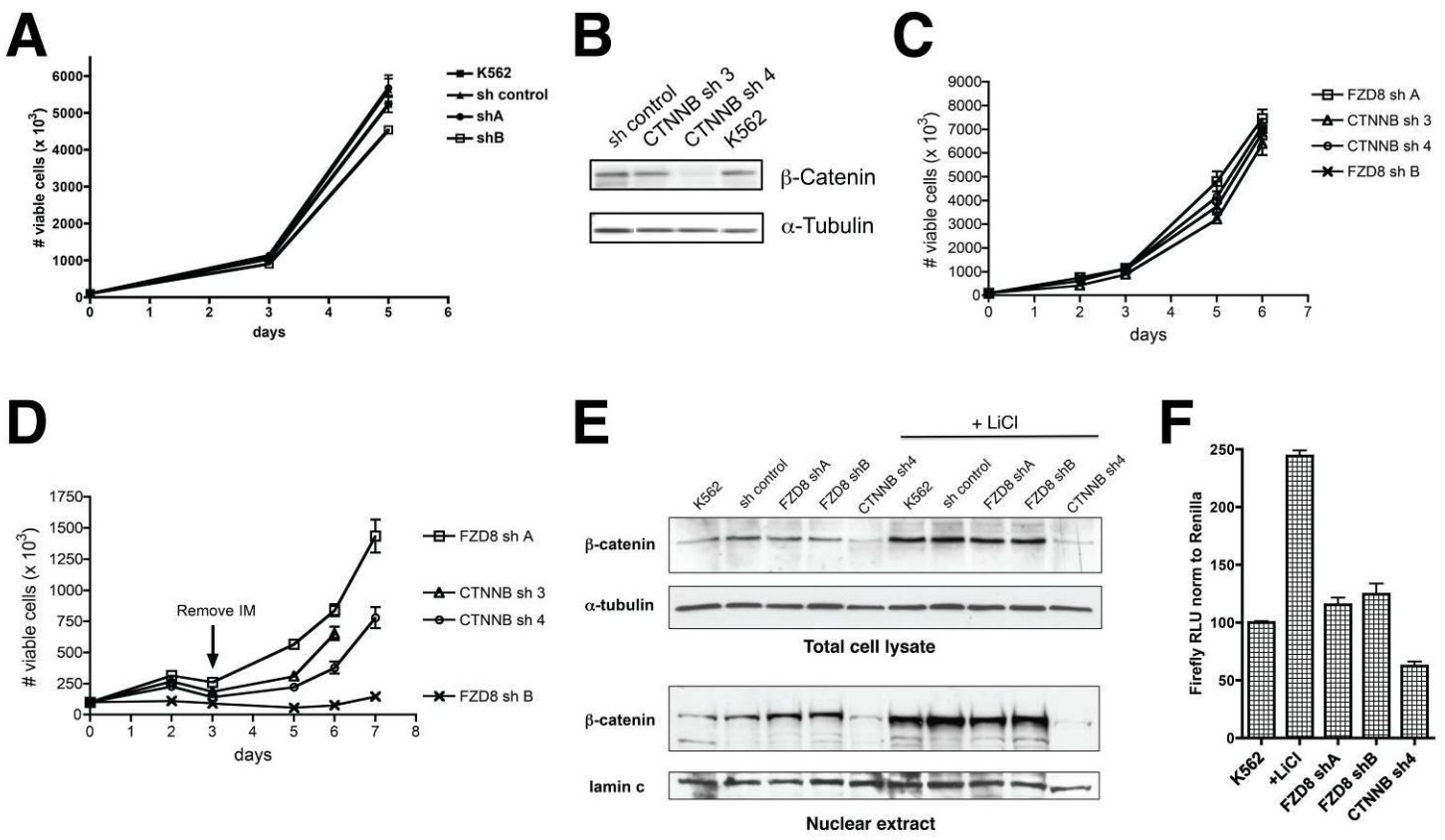

Figure S2. Related to Figure 1. FZD-8 does not signal through β-catenin. A) K562 cell lines expressing shRNAs targeting FZD-8 (shA and B) or a negative control shRNA were subjected to cell viability analysis: cells were seeded at 1 x 10^5/ml in triplicate in 24-well tissue culture plates and the number of viable cells (based on PI-exclusion) was counted by flow cytometry at the indicated time points and plotted. **B)** K562 cells were stably transduced with shRNAs targeting β-catenin (CTNNB) or negative control shRNA. Whole cell lysates were prepared and subjected to western blotting for β-catenin. The blot was stripped and reprobed for α-tubulin as a loading control. **C)** K562 cell lines expressing the indicated shRNA were left untreated or **D)** treated with imatinib mesylate at 1 μM for 72 hr, after which the cells were reseeded in the absence of drug and cultured for an additional 4 days. Viable (PI-excluded) cells were counted at the indicated time points using a flow cytometer. **E)** K562 shRNA-expressing cell lines were left untreated or treated with 10 mM LiCl for 24 hr (LiCl inhibits GSK-3, which leads to stabilization of β-catenin and its translocation to the nucleus). Whole cell lysates and nuclear extracts were prepared and subjected to Western blotting for β-catenin. The blots were stripped and reprobed for α-tubulin or lamin C as loading controls. **F)** K562 cell lines were transiently cotransfected with the TOP-FLASH luciferase reporter plasmid and pRL-TK (Promega) expressing renilla luciferase. After 24 hr, the cells were harvested and assayed for luciferase activity. TOP-FLASH promoter activity was normalized to renilla luciferase. Error bars +/- SD.

Figure S3

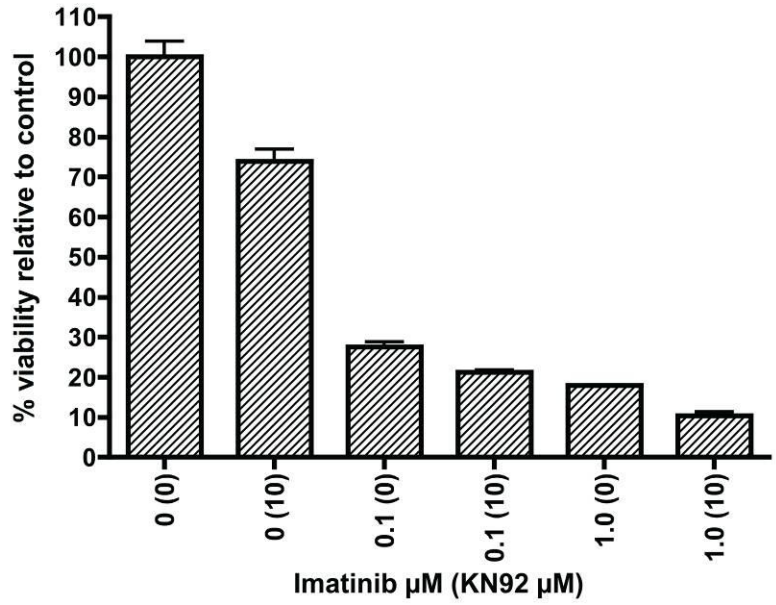

Figure S3. Related to Figure 2. The KN93 analog, KN92, does not effectively sensitize CML cells to imatinib. K562 cells were seeded at 1 x 10^5 cells/ml in 24-well tissue culture plates. Cells were treated with the indicated concentrations of imatinib and KN92 in triplicate. After 48 hr, the cells were stained with PI and viable (PI-excluded) cells were counted using a flow cytometer; error bars +/- SD.

Figure S4

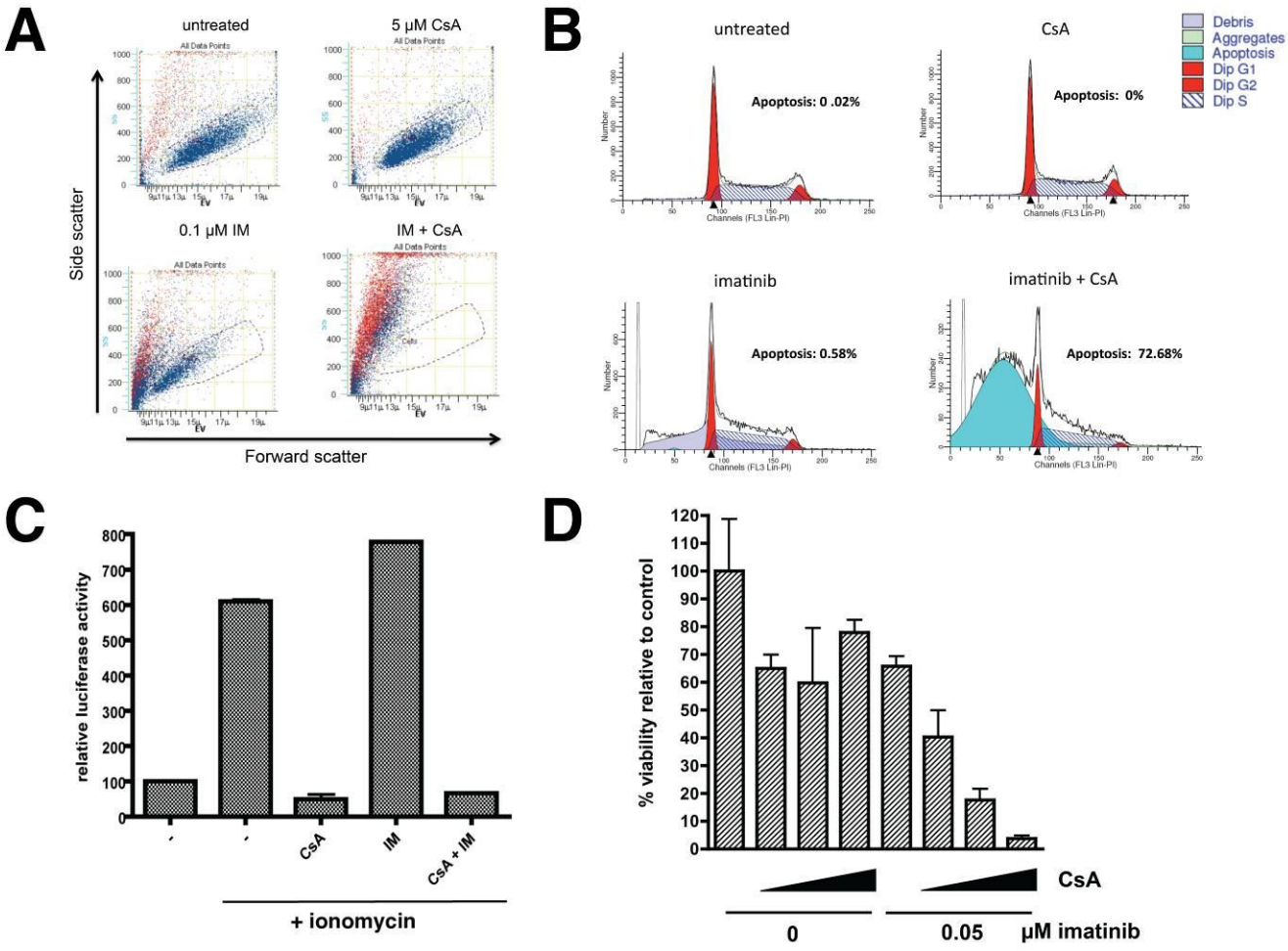

Figure S4. Related to Figure 3. CsA enhances imatinib-induced apoptosis of CML cells and imatinib does not influence NFAT activity. A) Representative flow profiles of K562 CML cells treated with imatinib and/or CsA. Cells were treated as described in the legend to Figure 3A. The "cell" gate was defined based on the scatter profile of untreated viable K562 cells (upper left panel). PI^+ cells are colored in red and PI^- in violet. **B)** K562 cells were seeded at 1 x 10^5 cells/ml and were left untreated or treated with CsA (5 µM) and/or imatinib (0.1 µM) as indicated for 72 hr. The cells were then resuspended in a saponin/propidium iodide solution (25 µg/ml propidium iodide, 0.3 % saponin, 0.1 mM EDTA, and 2 U/ml RNase), incubated overnight a 4° C, and analyzed for sub-G1 peak by flow cytometry. Modfit LT (Verity Software) was used for cell cycle and apoptotic peak modeling. **C)** K562 cells were infected with adenovirus containing the 9XNFAT-luc reporter. Thirty-two hours after infection, the cells were treated with ionomycin (1 µg/ml) and CsA (5 µM), imatinib (IM; 1 µM), or CsA /imatinib for 16 hr as indicated and lysates were subjected to standard firefly luciferase assays. **D)** KBM7 cells were seeded at 1 x 10^5 cells/ml in 24-well tissue culture plates. The cells were treated with the indicated concentrations of imatinib and 0, 1, 2.5 or 5 µM of CsA (increasing CsA concentrations indicated by blue triangles) in triplicate wells. After 72 hr, the cells were removed from the drugs, replated, and grown for an additional 72 hr. Viable (PI-excluded) cells were counted using a flow cytometer and the numbers plotted. Error bars +/- SD.

Figure S5

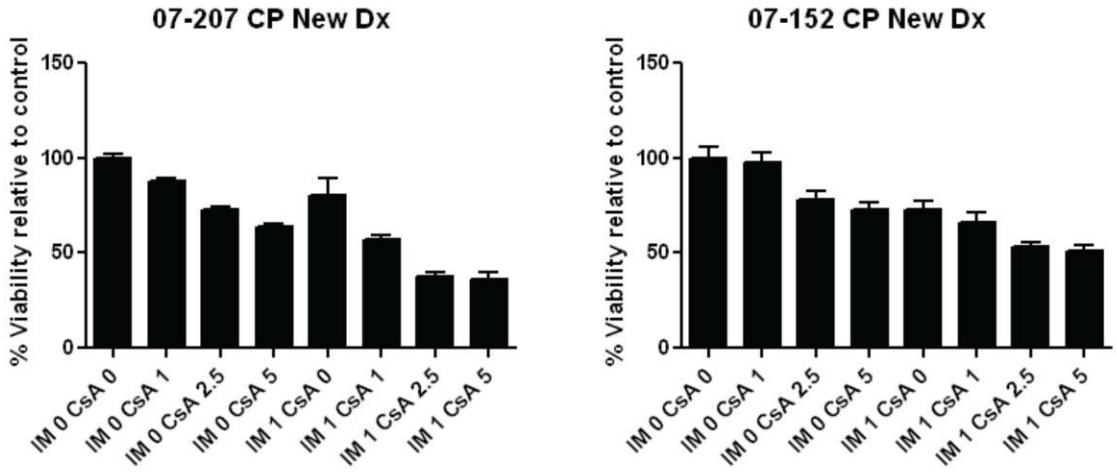

Figure S5. Related to Figure 4. CsA cooperates with imatinib in inhibiting proliferation of primary CML cells. Mononuclear cells taken from the bone marrow of 2 patients with newly diagnosed chronic phase CML (CP New Dx) were treated with imatinib and/or CsA for 72 hr at the indicated concentrations and were subjected to an MTS-based proliferation assay; error bars +/- SD.

Figure S6

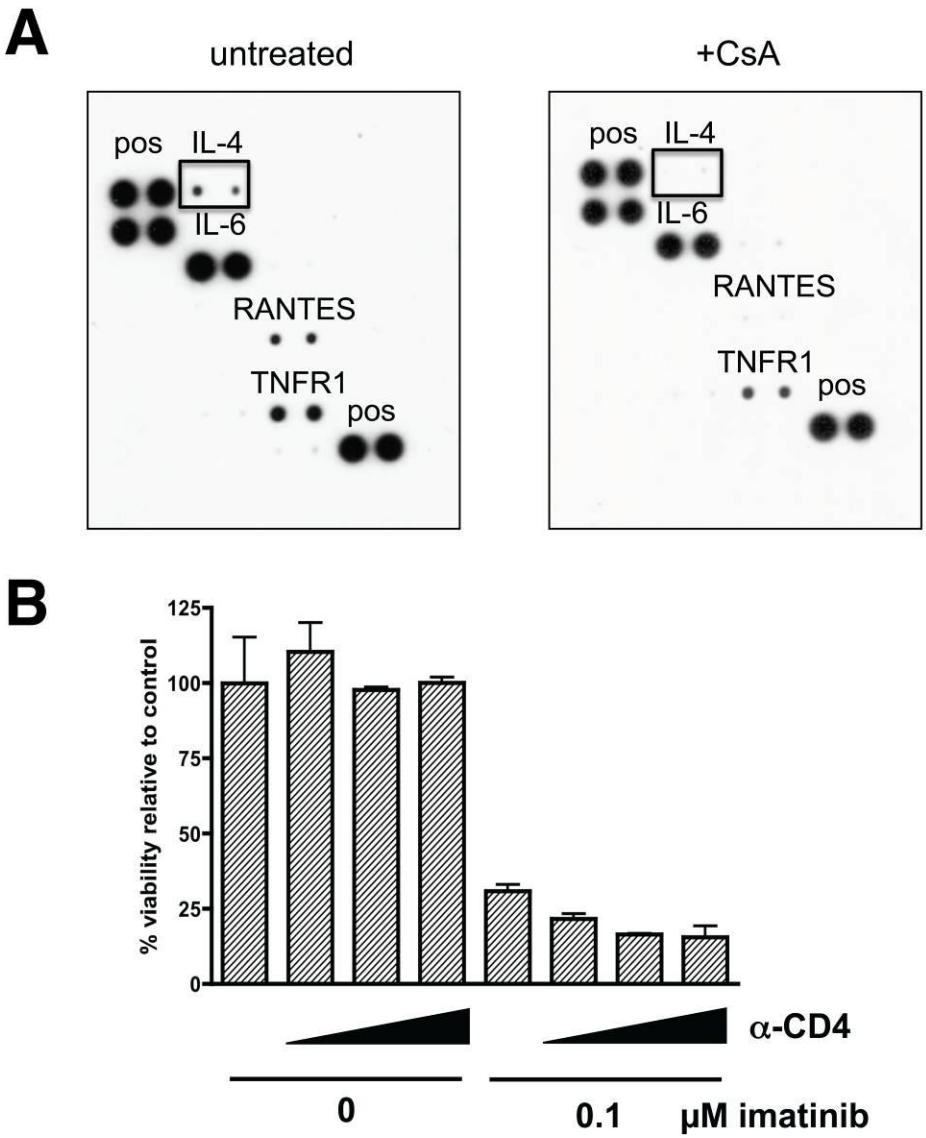

Figure S6. Related to Figure 6. CsA inhibits IL-4 production in Bcr-Abl$^+$ Ba/F3 and an antibody against CD4 does not effectively sensitize these cells to imatinib. A) Media was conditioned by incubating 2 x 10^6 Ba/F3 p210 Bcr-Abl cells in 2 ml of low serum media (0.2% FBS) in the presence or absence of 5 μM CsA for 24 hr. The conditioned media was incubated overnight at 4°C on antibody based cytokine arrays (RayBio Mouse Cytokine Antibody Array 1, Ray Biotech) followed by ECL-based detection according to the manufacturer's instructions. **B)** Bcr-Abl$^+$ Ba/F3 cells were treated with imatinib and neutralizing CD4 monoclonal antibody (α-CD4) alone or in combination as indicated (increasing α-CD4 concentrations of 0, 25, 50, and 100 μg/ml are indicated by the triangles) for 72 hr and viable cells were counted; error bars +/- SD.

Figure S7

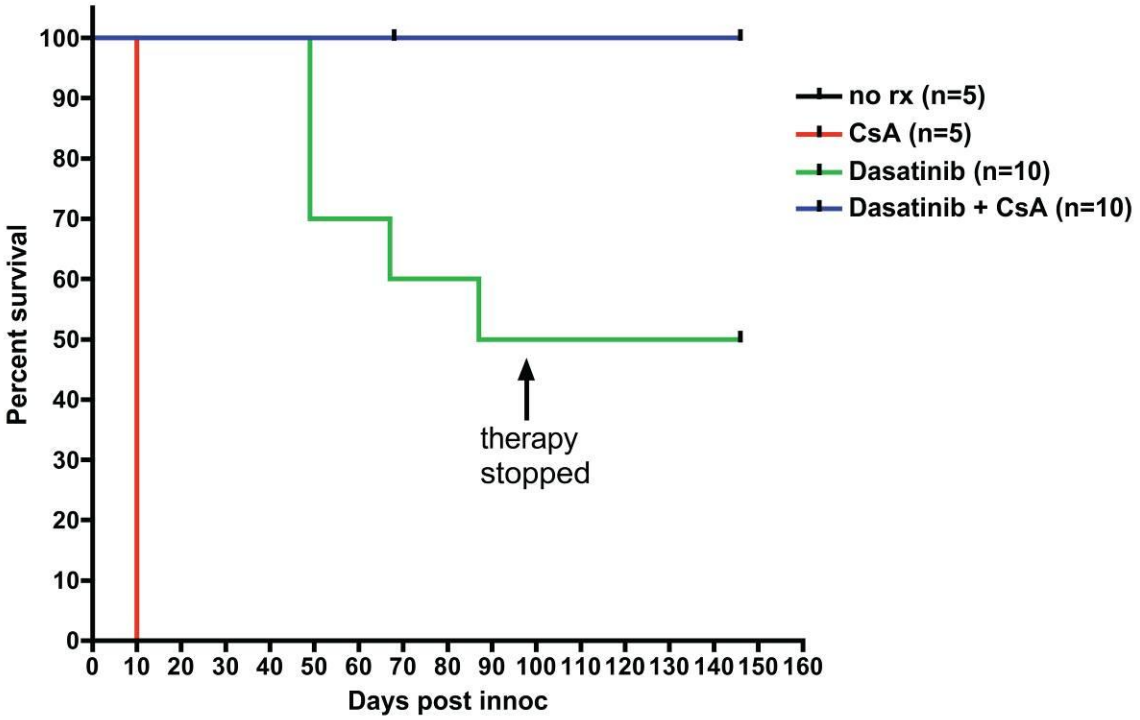

Figure S7. Related to Figure 7. CsA combined with dasatinib improves survival in a mouse model of Ph$^+$ALL. C57Bl/6 were inoculated intravenously with 5x10^5 Arf$^{-/-}$ p185 Bcr-Abl$^+$ B-ALL cells. After 3 days, groups of mice were treated once daily by oral gavage with vehicle, CsA (25mg/kg), dasatinib (20mg/kg) or both drugs for a period of 96 days. Kaplan-Meier curve shows overall survival. Surviving mice were sacrificed on day 146. Post-mortem analyses showed no leukemia (GFP$^+$) cells in the blood, bone marrow, or spleen.

Supplemental Experimental Procedures

Data analyses for shRNA screen

The intensity values from microarrays were first normalized by computing a mean value for each chip, and a global mean was then selected from the minimum of all the means. The normalization factors for each chip were then computed by dividing each individual mean by the global mean. The normalization process was performed by multiplying the intensity values for each chip by the respective normalization factor. 21,664 total probes were detectable (> 1.5-fold) above background. We then compared each "imatinib" chip to each "untreated" chip to compute the fold change for each probe. Given this configuration, a combination of 9 fold changes for each probe was produced. Linear models and empirical Bayes methods were used for assessing differential expression as described in Smyth, G. K. (2004). The probe-wise sample variances were shrinked towards a common value and the degrees of freedom for the individual variances were augmented. The (FDR) adjusted p-values were reported. Significant probes are defined by having at least 6 of the 9 fold changes greater than the percentile threshold.and a p-value of <0.05. Since the shRNA library manufacturer used duplicated probe IDs to represent probes selected from similar gene regions, these IDs were renamed by post-fixing a unique number. Using the NCBI "blastall" program, the proprietary probe IDs were then translated into meaningful annotation by aligning the corresponding short sequence against the NCBI RefSeq database. Finally, gene information was extracted using the Biomart Bioconductor package (Ensembl).

Reagents

KN92 and KN93 were obtained from Calbiochem. PMA (Phorbol 12-Myristate 13-Acetate) and ionomycin were obtained from Sigma, and cyclosporine, in the form of Neoral, was from Novartis. Anti-IL-4 was produced and purified from the 11B11 hybridoma using standard methods. Recombinant murine IL-4 was from R&D Systems.

Plasmids

pLKO.1 lentiviral constructs containing shRNA targeting FZD-8 ("shA": clone ID TRC N0000008348, and "shB": clone ID TRC N0000008351) and an shRNA to SFRS1 that is non-targeting ("negative control": clone ID TRC N0000010592) were purchased from Open Biosystems. Adenoviral 9xNFAT-luc reporter was obtained from Jeff Molkentin (Cincinnati Children's Hospital Medical Center). caFNATc1/GFP construct was obtained fron Anjana Rao (Harvard Medical School). Mouse stem cell virus (MSCV)-internal ribosome entry site (IRES)-green fluorescent protein (GFP) constructs expressing native Bcr-Abl, M351T, and E255K were obtained from Michael Deininger (Oregon Health & Science University). TOP-FLASH-luc reporter was obtained from Heide Ford (UC Denver).

Real-time PCR primers and probe

The following primer/probe sequences were used for FZD-8 detection. FWD: 5'-CAAGACAGGCCAGATCGCTAA - 3'; REV: 5'- GCTCGTCCTGGCTGAAAAAG - 3' and the probe 6FAM-CGCGCTGCCCTGCCACAAC -TAMRA. The TaqMan probe was purchased from Applied Biosystems 5'labeled with 6-carboxyfluorescein (FAM) and 3'-labeled with 6-caboxy-tetramethylrhodamine (TAMRA). Quantities of FZD8 in test samples were normalized to the corresponding 18s rRNA (PE ABI, P/N 4308310).

MTS assays

Primary mononuclear cells from newly diagnosed CML patients were plated ($5x10^4$/well) in quadruplicate in complete media (RPMI medium 1640 supplemented with 10% FCS and 100

μM β-mercaptoethanol) in the presence of cyclosporin A (1, 2.5, 5 μM) or imatinib (1 μM) either alone or in dual-combinations. Cells were incubated at 37 °C for 72 h and analyzed using a methanethiosulfonate-based viability assay (CellTiter96 Aqueous One solution reagent; Promega). Mean values for each treatment condition were normalized to that of untreated cells, and error bars +/- S.E.M. Samples were obtained with informed consent and under the approval of the OHSU Institutional Review Board.