Supplementary Information

β-Catenin is required for intrinsic but not extrinsic BCR-ABL1 kinase-independent resistance to tyrosine kinase inhibitors in chronic myeloid leukemia

Anna M. Eiring[1], Jamshid S. Khorashad[1], David J. Anderson[1], Fan Yu[1,2], Hannah M. Redwine[1], Clinton C. Mason[1], Kimberly R. Reynolds[1], Phillip M. Clair[1], Kevin C. Gantz[1], Tian Y. Zhang[1], Anthony D. Pomicter[1], Ira L. Kraft[1], Amber D. Bowler[1], Kara Johnson[3], Mary Mac Partlin[3], Thomas O'Hare[1,4], and Michael W. Deininger[1,4ξ]

[1]Huntsman Cancer Institute, The University of Utah, Salt Lake City, UT, U.S.A. [2]Beijing Tsinghua Chang Gung Hospital, Tsinghua University, Beijing, China. [3]Division of Hematology and Medical Oncology, Oregon Health & Science University Knight Cancer Institute, Portland, OR, U.S.A. [4]Department of Hematology and Hematologic Malignancies, The University of Utah, Salt Lake City, UT, U.S.A.

[ξ]Contact: Michael W. Deininger, MD, PhD; 2000 Circle of Hope, Room 4280, Salt Lake City, Utah 84112; Ph: 801-587-4077; E-Mail: michael.deininger@hci.utah.edu

RUNNING TITLE: Role of β-catenin in TKI resistance of CML

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Supplemental Figures

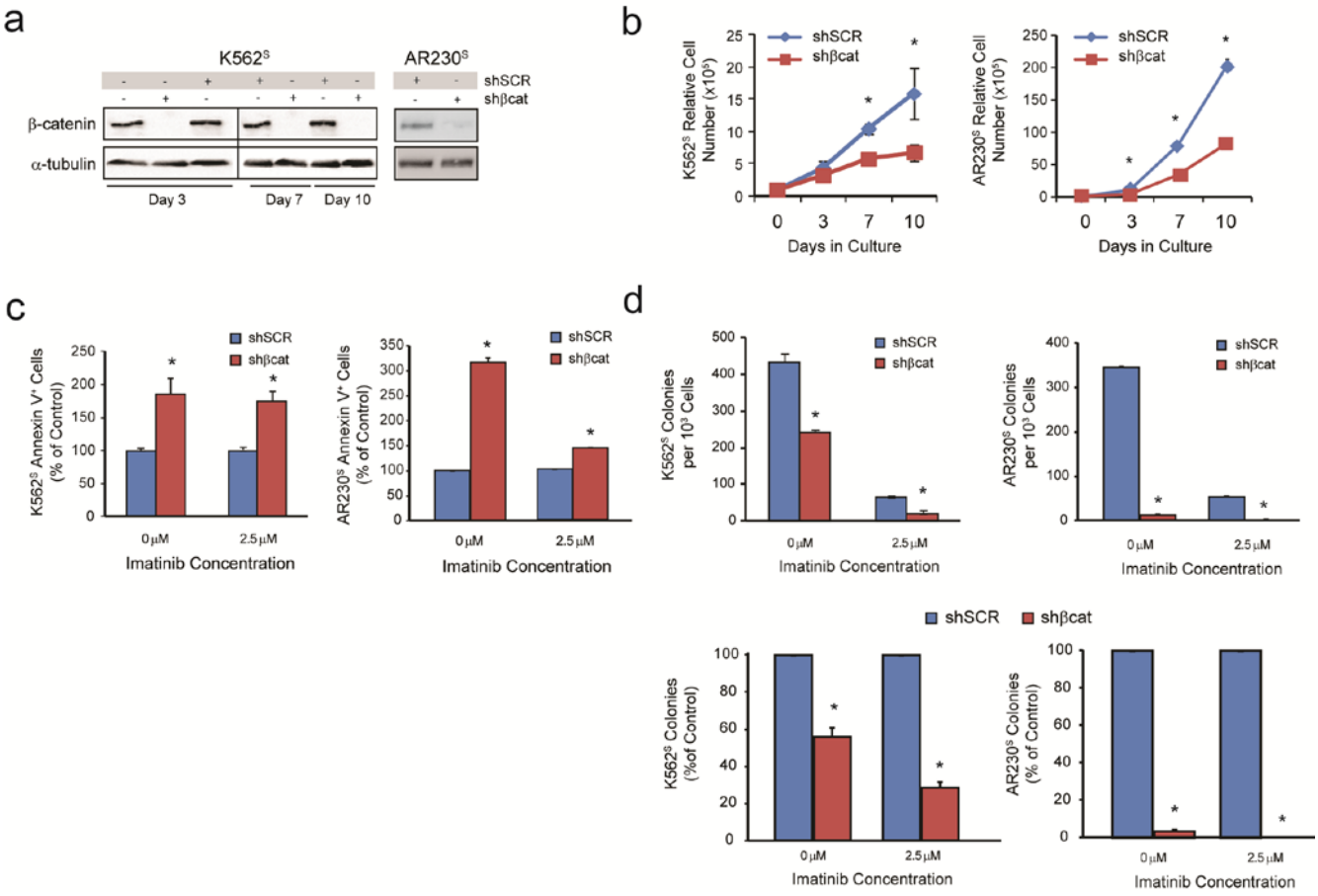

Figure S1. shRNA targeting β-catenin (shβcat) impairs survival of parental TKI-sensitive K562^S and AR230^S cells. a. Immunoblot analysis revealed >90% reduction of β-catenin protein levels upon lentiviral delivery of shβcat into parental TKI-sensitive K562^S (*left*) and AR230^S (*right*) cells compared to controls expressing shSCR (n=5). **b-d.** Reduced β-catenin protein levels resulted in reduced *in vitro* growth (**b**, n=4), increased apoptosis (**c**, n=3), and reduced colony formation in semisolid medium (**d**, n=4). In panel **d**, the top graphs represent absolute colony numbers, whereas the bottom graphs are the same data normalized to shSCR-expressing controls. Bars represent standard error of the mean (SEM). *p<0.05.

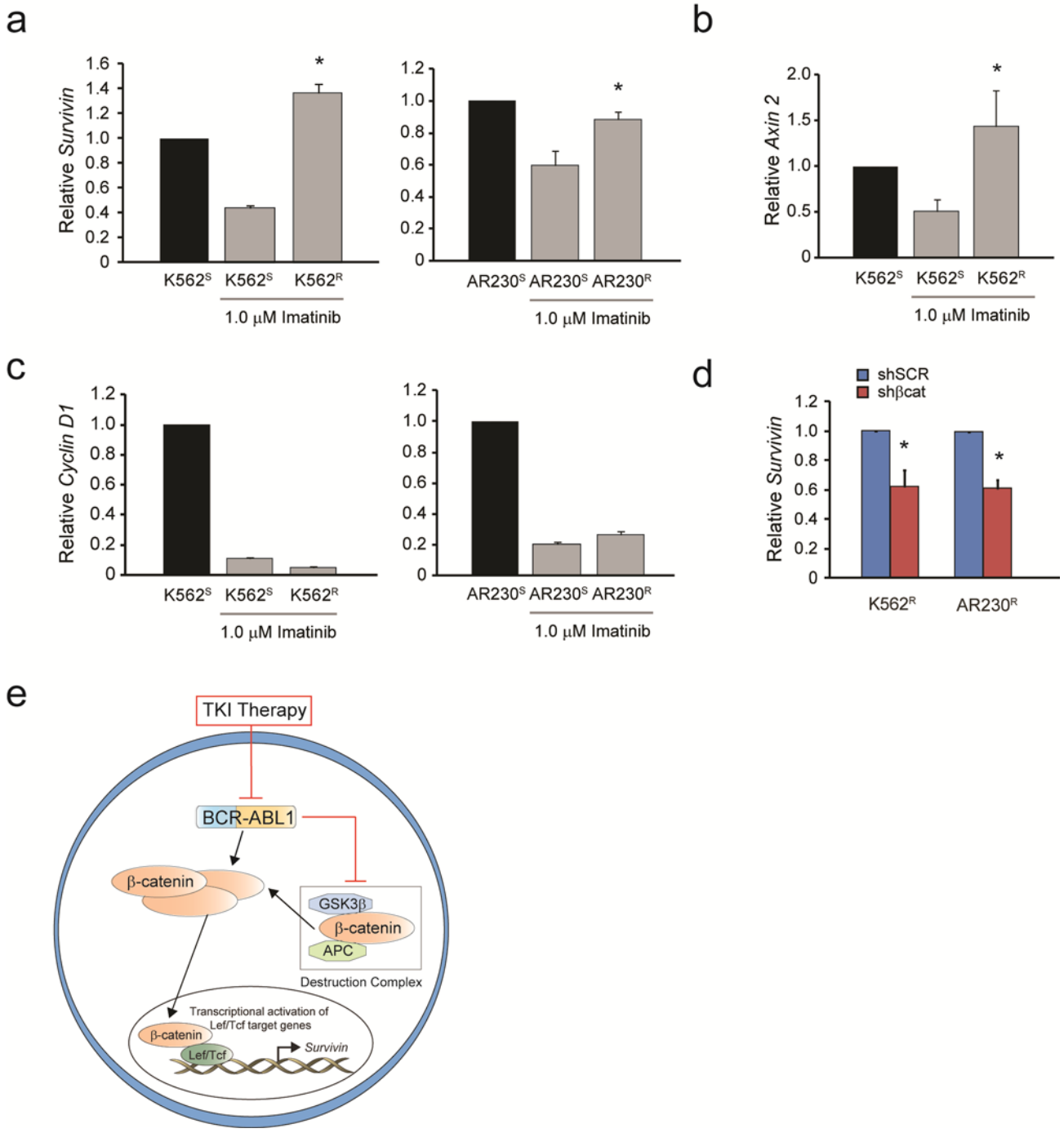

Figure S2. β-catenin transcriptional activity promotes intrinsic TKI resistance. a-b. qRT-PCR analyses revealed increased levels of canonical β-catenin target genes encoding survivin (**a**, n=3) and axin2 (**b**, n=3) in TKI-resistant K562^{R} and AR230^{R} cells compared to parental TKI-sensitive controls in the presence of imatinib. Axin2 was completely undetectable in TKI-sensitive AR230^{S} cells and was therefore not quantifiable (n=2). **c.** Levels of mRNA encoding cyclin D1 were not significantly different in TKI-resistant versus TKI-sensitive controls (n=3). **d.** Levels of mRNA encoding survivin were consistently downregulated in shβcat-expressing compared to shSCR-expressing K562^{R} and AR230^{R}

cells. Gene expression was not analyzed in TKI-resistant cells in the absence of imatinib due to cell death under these growth conditions (see Figures 2d-e and Eiring et al. *Leukemia* 2015;29(3):586-97). Bars represent SEM and data were normalized to expression of *GUS*. *p<0.05. **e.** In intrinsic TKI resistance, β-catenin expression is uncoupled from BCR-ABL1 kinase activity, allowing for β-catenin protein expression despite TKI-mediated BCR-ABL1 kinase inhibition. Our data suggest that intrinsic TKI resistance results in stabilization of β-catenin, likely from release by the destruction complex, followed by translocation into the nucleus and activation of Lef/Tcf target genes such as *survivin*.

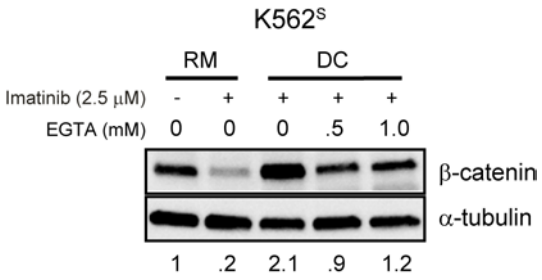

Figure S3. Direct contact-mediated β-catenin stabilization is partially blocked by treatment with EGTA. Addition of EGTA to HS-5 DC cultures partially blocks β-catenin stabilization (see Figure 3), indicating a Ca^{2+}-dependent process (n=2).

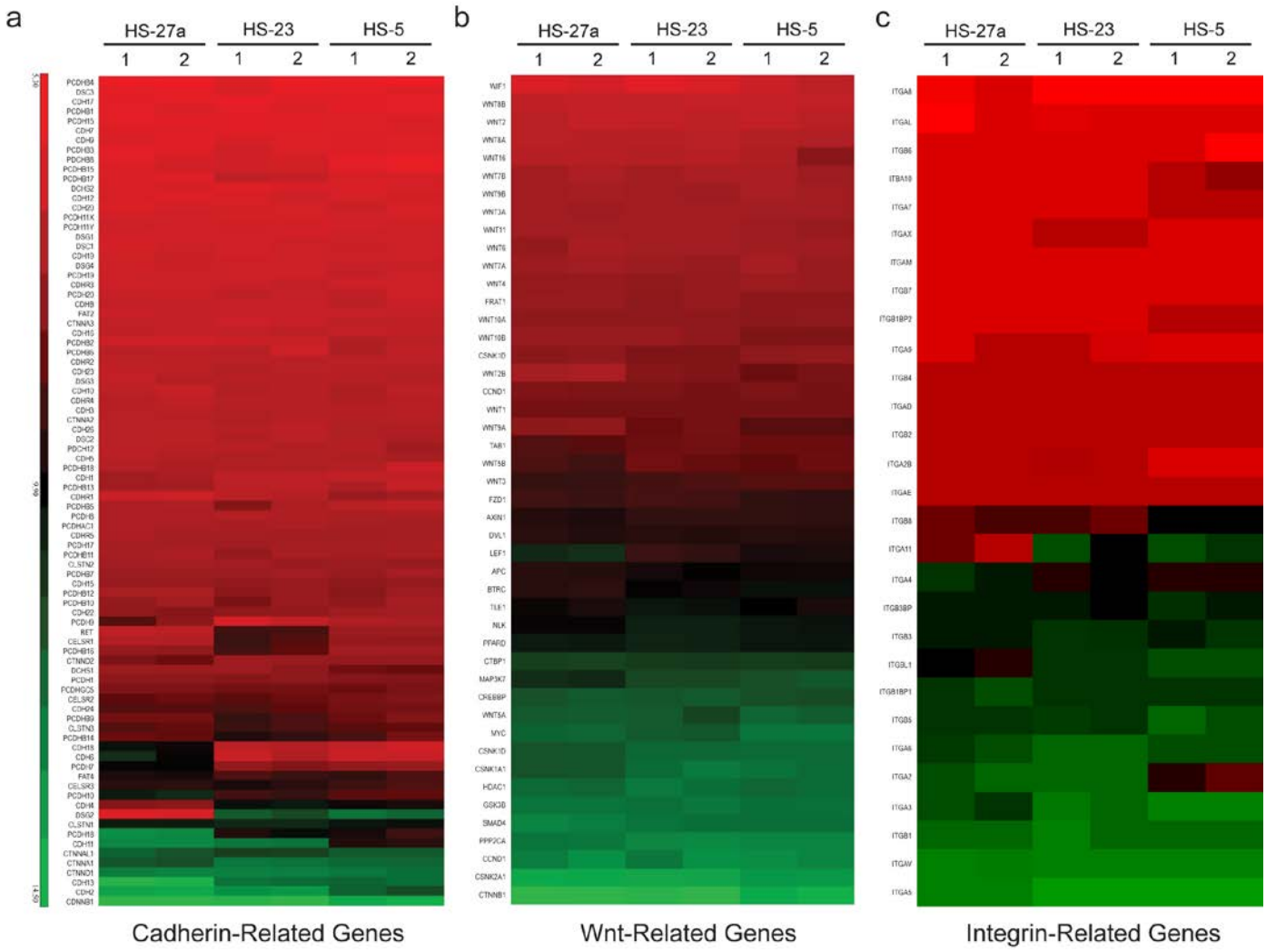

Figure S4. Microarray analysis of HS-5, HS-23, and HS-27a stromal cells reveals high levels of genes encoding N-cadherin, H-cadherin, WNT5A, and integrins. a-c. Human Gene 1.0 ST arrays revealed high levels of certain cadherin-related genes (**a**), Wnt-related genes (**b**), and integrin-related genes (**c**) in HS-5, HS-23, and HS-27a stromal cells (n=2). Among these are genes encoding N-cadherin, H-cadherin, and WNT5A, known regulators of β-catenin activity and function.

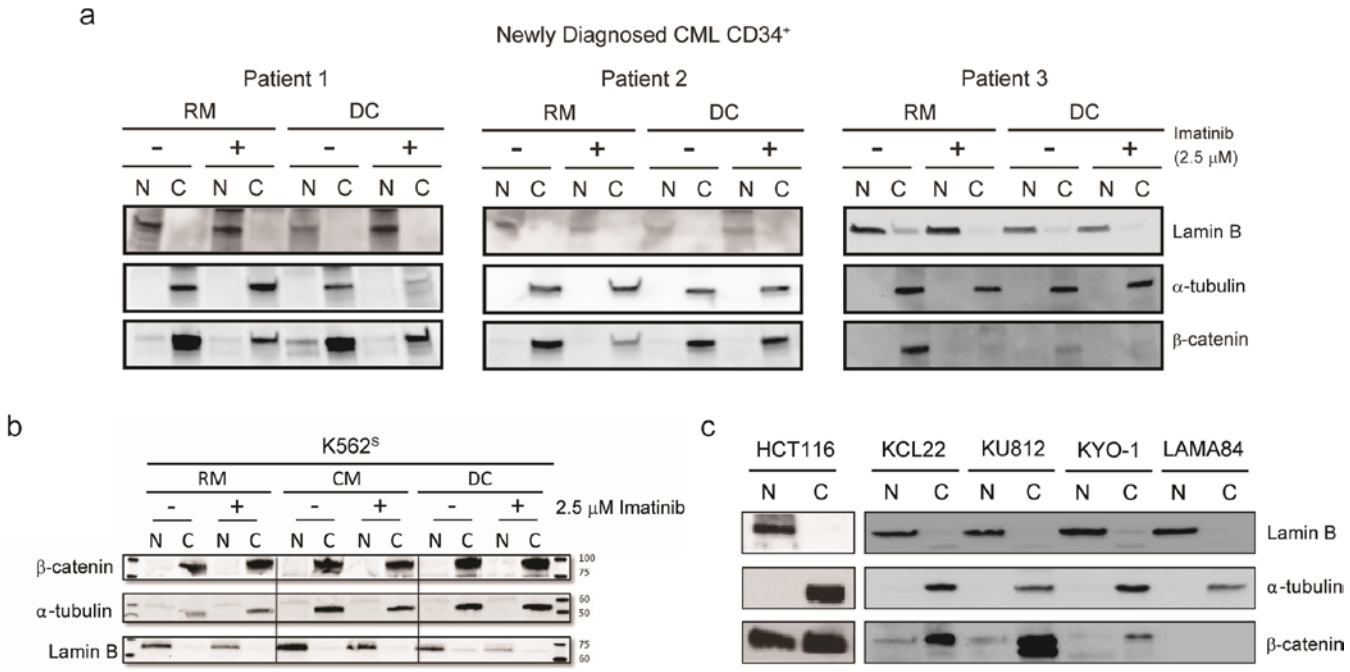

Figure S5. β-catenin is located almost exclusively in the cytoplasm of CML CD34$^+$ cells from newly diagnosed patients and CML cell lines regardless of culture conditions. a. Nucleocytoplasmic fractionation on three independent patient samples revealed that β-catenin was found almost exclusively within the cytoplasm of chronic phase CML CD34$^+$ cells cultured in RM or HS-5 DC. In one patient sample, low levels of nuclear β-catenin were detected in cells growing in RM that increased in DC, but this increase was abolished by imatinib treatment (Patient 1). **b.** Nucleocytoplasmic fractionation revealed cytoplasmic β-catenin in parental TKI-sensitive K562^S cells under all conditions tested. Densitometry confirmed that imatinib reduced β-catenin protein by 30% in K562^S cells cultured in RM. **c.** β-catenin protein was also primarily cytoplasmic in the KCL22, KU812, KYO-1, and LAMA84 CML cell lines (*right*). As expected, high levels of nuclear β-catenin were detected in the HCT116 human colon carcinoma cell line (*left*).

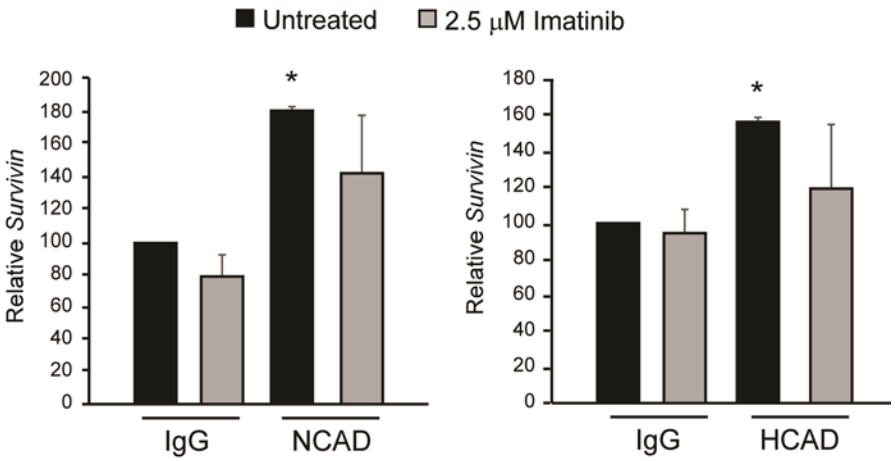

Figure S6. Antibody-mediated NCAD or HCAD inhibition increases mRNA encoding survivin in the absence but not presence of imatinib. Consistent with β-catenin luciferase reporter activity (see Figure 6d), treatment of co-cultures with NCAD (*left*) or HCAD (*right*) blocking antibodies increased the level of mRNA encoding survivin, a known β-catenin transcriptional target. This increase was abolished by treatment with the TKI, imatinib. Bars represent SEM and data were normalized to expression of *GUS*. *$p<0.05$.

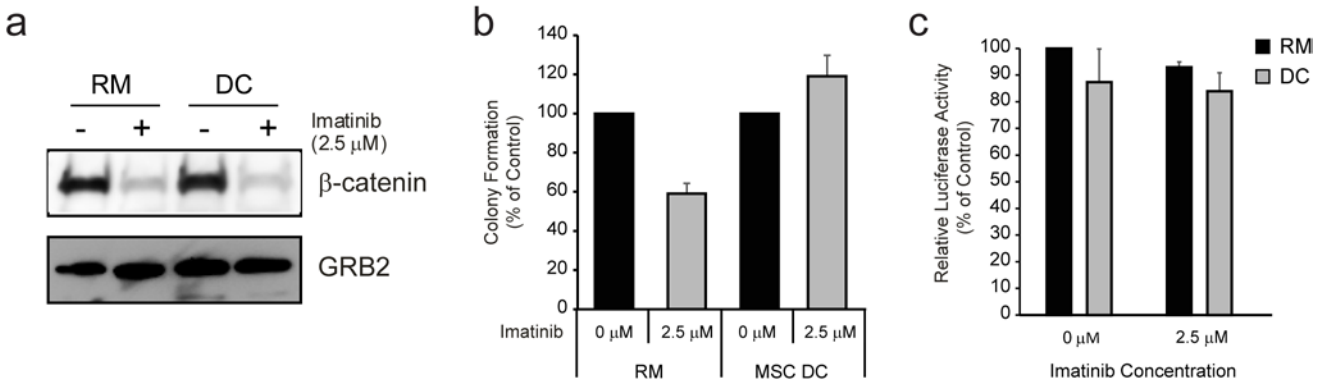

Figure S7. Co-culture with primary human MSCs does not stabilize β-catenin protein but protects chronic phase CML CD34$^+$ cells from imatinib treatment. a,b. CML CD34$^+$ cells from newly diagnosed patients were co-cultured with primary mesenchymal stromal cells (MSCs) harvested from healthy donors for allogeneic bone marrow transplantation (Tabe Y, et al. *Blood* 2004;103:1815-22). Contrary to observations with the HS-5 stromal cell line, culture of CML progenitors with primary MSCs did not stabilize β-catenin protein as observed by immunoblot analyses in five of six trials with three independent patient samples (**a**). Co-culture with primary MSCs still resulted in enhanced colony formation and survival in the presence of imatinib (**b**, n=4). **c.** Primary CML CD34$^+$ cells were transduced with pGF1-SCR or pGF1-LefTcf and analyzed for luciferase reporter activity following 24 h culture in RM or MSC DC. Co-culture with primary MSCs had no effect on luciferase reporter activity (n=3). Bars represent SEM.

Supplemental Materials and Methods

Stromal cell protection assays

$K562^S$ and $AR230^S$ cell lines ($1.5x10^5$ cells/mL) or CML^{CD34+} progenitors from newly diagnosed patients (10^6 cells/mL) were cultured ± the indicated inhibitors for 36 h, or as otherwise indicated, followed by plating of 10^3 viable cells in colony assays or analysis for apoptosis. CML cells cultured in DC with HS-5 cells or primary MSCs were harvested by washing twice with ice cold PBS. Integrity of the stromal cell monolayer and complete removal of CML cells from the co-culture was confirmed by microscopy.

Short hairpin RNA (shRNA) constructs

pLKO.1 lentiviral plasmids harboring shβcat or a control shSCR sequence were provided by Richard H. Goodman (Portland, OR)[1]. To improve infection efficiency for use in primary cells, shβcat and shSCR sequences were subcloned into the FUGW lentiviral expression vector[2] and confirmed by Sanger sequencing. A lentiviral plasmid (pRSIT16-U6Tet-(sh)-CMV-TetRep-2A-RFP-2A-Puro) harboring a doxycycline-inducible shRNA targeting human WNT5A (shWNT5A) was obtained from Cellecta (Mountain View, CA USA).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was extracted (RNeasy Mini Kit; Qiagen, Valencia, CA) and converted to cDNA (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA). Gene expression was measured by real-time PCR using SsoAdvanced™ SYBR® Green Supermix (Bio-Rad) in a CFX96 Real-Time PCR Detection System (Bio-Rad) using primers listed in Supplementary Table S2.

Apoptosis and proliferation assays

Following in vitro culture in RM or HS-5 DC, apoptosis was assayed using the Guava Nexin Annexin V Binding Assay (Millipore, Billerica, MA) or by staining with Annexin V and 7-aminoactinomycin D antibodies (BD Biosciences, San Jose, CA) followed by flow cytometric analyses. For proliferation assays, cells were plated at $5x10^4$ cells/mL and counted on the indicated days. Every other day, half of the medium was replaced, and this was accounted for in the final cell number calculations.

Clonogenic assays

Methylcellulose colony assays were performed by plating CML cell lines or patient samples in 0.9% MethoCult (H4230; StemCell Technologies, Vancouver, BC, Canada). For cell lines, 10^3 cells were plated in cytokine-free methylcellulose ± imatinib at the indicated concentrations. Where indicated, 10^3 viable cells were plated following *in vitro* culture for 36 h in RM, HS-5 CM, or HS-5 DC ± imatinib (2.5 μM). For patient samples, cells were treated for 36 h in RM, HS-5 CM, or HS-5 DC, without additional cytokines, ± imatinib (2.5 μM). Following culture, 10^3 viable $CD34^+$ cells were plated in methylcellulose with rhIL-3 (20 ng/mL), rhIL-6 (20 ng/mL), rhFlt-3 ligand (100 ng/mL), and rhSCF (100 ng/mL). CFU-GMs were scored following 7-14 days in culture, and input cell numbers were used to calculate the total number of clonogenic cells in the initial culture. Samples were blinded prior to scoring.

Gene expression microarrays

HS-5, HS-23 and HS-27a cells were grown to confluence. RNA was extracted using the RNeasy kit (Qiagen) and labeled target cDNA prepared from two independent RNA preparations. Samples were amplified and labeled using the Ambion Whole Transcript sense/Affymetrix terminal labeling protocol (Ambion Life Technologies, Grand Island, NY). Samples were randomized prior to processing.

Lef/Tcf Reporter Assay

Reporter plasmids were made by ligating the PCR-amplified sequences (including *Cla*I and *Spe*I restriction sites) into the pGF1 reporter plasmid (System Biosciences) and confirmed by Sanger sequencing. Stably infected cells were produced by lentiviral infection, then treated as indicated, and analyzed by detection of luciferase reporter activity on a GloMax-Multi Detection System Luminometer (Promega, E7041) using the OneGlo luciferase kit (Promega, Madison, WI, USA). The oligonucleotides used for cloning of the pGF1-LefTcf and pGF1-SCR reporter constructs were as follows:

LefTcf:
TTTATCGATAGATCAAAGGGGGTAAGATCAAAGGGGGTAAGATCAAAGGGGGTAAGATCA
AAGGGGGTAGAATTCAGATCAAAGGGGGTAAGATCAAAGGGGGTAAGATCAAAGGGGGTA
AGATCAAAGGGGGTAACTAGTTAG

SCR:
TTTATCGATAGAGTCCGAGGGGTAAGAGTCCGAGGGGTAAGAGTCCGAGGGGTAAGAGTCC
GAGGGGTAGAATTCAGAGTCCGAGGGGTAAGAGTCCGAGGGGTAAGAGTCCGAGGGGTAA
GAGTCCGAGGGGTAACTAGTTAG

SI Tables

Supplementary Table S1. Primary samples used for these studies

Category	Patient ID	CML Disease Phase	BCR-ABL1 Kinase Domain Mutations	Prior TKI Exposure	Type of Resistance	Assay(s)
Newly Diagnosed	1	CP	NONE	NONE	N/A	shβcat CA, WB
	2	CP	NONE	NONE	N/A	shβcat CA, WB
	3	CP	NONE	NONE	N/A	Anti-NCAD CA
	4	CP	NONE	NONE	N/A	Anti-NCAD CA
	5	CP	NONE	NONE	N/A	Anti-NCAD CA
	6	CP	NONE	NONE	N/A	Anti-NCAD CA, shWNT5A CA
	7	CP	NONE	NONE	N/A	shβcat CA, WB, Anti-NCAD CA Lef/Tcf Reporter
	8	CP	NONE	NONE	N/A	shβcat CA, Anti-NCAD CA, N/C Fractionation
	9	CP	NONE	NONE	N/A	Lef/Tcf Reporter N/C Fractionation
	10	CP	NONE	NONE	N/A	Lef/Tcf Reporter N/C Fractionation
	11	CP	NONE	NONE	N/A	Lef/Tcf Reporter
	12	CP	NONE	NONE	N/A	Lef/Tcf Reporter
	13	CP	NONE	NONE	N/A	Lef/Tcf Reporter
	14	CP	NONE	NONE	N/A	Anti-HCAD CA
	15	CP	NONE	NONE	N/A	Anti-HCAD CA, shWNT5A CA
	16	CP	NONE	NONE	N/A	shWNT5A CA
	17	CP	NONE	NONE	N/A	Anti-HCAD CA, shβcat CA, IF Anti-NCAD CA
	18	CP	NONE	NONE	N/A	Anti-HCAD CA, Anti-NCAD CA, IF
	19	CP	NONE	NONE	N/A	Anti-HCAD CA, Anti-NCAD CA, IF
	20	CP	NONE	NONE	N/A	CD38 Selection, IF
	21	CP	NONE	NONE	N/A	CD38 Selection, IF
	22	CP	NONE	NONE	N/A	CD38 Selection, IF
	23	CP	NONE	NONE	N/A	Anti-HCAD CA, Lef/Tcf Reporter, Primary MSC Co-Culture
	24	CP	NONE	NONE	N/A	Anti-HCAD CA, Lef/Tcf Reporter, Primary MSC Co-Culture

	25	CP	NONE	NONE	N/A	Anti-HCAD CA, Lef/Tcf Reporter, Primary MSC Co-Culture
	26	CP	NONE	NONE	N/A	Primary MSC Co-Culture
	27	CP	NONE	NONE	N/A	HS-5 Co-Culture, IF
	28	CP	NONE	NONE	N/A	HS-5 Co-Culture, IF
	29	CP	NONE	NONE	N/A	HS-5 Co-Culture, IF
TKI-Resistant	30	CP	NONE	I, D, N	Cytogenetic	shβcat CA
	31	CP	NONE	I, D, N	Cytogenetic	shβcat CA
	32	CP	NONE	I, D, N	Cytogenetic	shβcat CA

CML indicates chronic myeloid leukemia; TKI, tyrosine kinase inhibitor; ND, newly diagnosed; CP, chronic phase; I, imatinib; D, dasatinib; N, nilotinib; CA, colony assay; WB, western blot; NCAD, anti-N-cadherin antibody; N/C, nuclear-cytoplasmic; IF, immunofluorescence.

Supplementary Table S2. Primer sequences used for these studies

Gene Name	Forward Primer	Reverse Primer
β-catenin	5'–AAAATGGCAGTGCGTTTAG–3'	5'–TTTGAAGGCAGTCTGTCGTA–3'
WNT5A	5'–GAGTTCGTGCACGCCCGCGA–3'	5'–CCGCCAGGAACGTGCTTG–3'
Survivin	5'–CCTTCTTGGAGGGCTGCGCC–3'	5'–ATGGGGTCGTCATCTGGCTCC–3'
Cyclin D1	5'–ACGAAGGTCTGCGCGTGTT–3'	5'–CCGCTGGCCATGAACTACCT–3'
Axin 2	5'–CTGGCTCCAGAAGATCACAAAG–3'	5'–ATCTCCTCAAACACCGCTCCA–3'
GUS	5'–GAAAATATGTGGTTGGAGAGCTCATT–3'	5'–CCGAGTGAAGATCCCCTTTTTA–3'

Supplementary References

1. Yochum GS, Sherrick CM, Macpartlin M, Goodman RH. A beta-catenin/TCF-coordinated chromatin loop at MYC integrates 5' and 3' Wnt responsive enhancers. *Proc Natl Acad Sci U S A* 2010 Jan 5; **107**(1)**:** 145-150.

2. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 2002 Feb 1; **295**(5556)**:** 868-872.