β-Catenin is required for T cell leukemia initiation and MYC transcription downstream of Notch1

Christos Gekas[1], Teresa D'Altri[1], Rosa Aligué[2], Jessica González[1], Lluis Espinosa*[1], Anna Bigas*[1]

[1]Program in Cancer Research. Institut Hospital del Mar d'Investigacions Mèdiques (IMIM), Barcelona, 08003, Spain.

[2]Department of Cell Biology. Facultat de Medicina. Universitat de Barcelona. Barcelona 08036, Spain.

Running title: β-Catenin and Notch drive T-ALL

Corresponding author:

Anna Bigas, PhD.

Institut Hospital del Mar d'Investigacions Mèdiques (IMIM).

Dr. Aiguader 88 08003 Barcelona;

Phone: 34 933160440 ; Fax: 34 9321600410; e-mail:abigas@imim.es

Footnotes: * both authors contributed equally to this work.

26

27 **Summary**

28 Notch activation is instrumental in the development of most T-cell acute lymphoblastic leukemia 29 (T-ALL) cases, yet Notch mutations alone are not sufficient to recapitulate the full human 30 disease in animal models. We here found that Notch1 activation at the fetal liver stage 31 expanded the hematopoietic progenitor population and conferred it transplantable leukemic 32 initiating capacity. However, leukemogenesis and Leukemic Initiating Cell (LIC) capacity 33 induced by Notch1 was critically dependent on the levels of β-Catenin, in both fetal liver and 34 adult bone marrow contexts. In addition, inhibition of β-Catenin compromised survival and 35 proliferation of human T-ALL cell lines carrying activated Notch1. By transcriptome analyses, we 36 identified the MYC pathway as a crucial element downstream of β-Catenin in these T-ALL cells, 37 and demonstrate that the MYC 3´enhancer required β-Catenin and Notch1 recruitment to 38 induce transcription. Finally, PKF115-584 treatment prevented and partially reverted 39 leukemogenesis induced by active Notch1.

40

41 ## Introduction

42 T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological malignancy 43 characterized by the outgrowth of immature T-cells (1), with Notch1 mutations being the most 44 prominent genetic alteration in this disease (60% of cases) (2). In mice, constitutive Notch1 45 activation through the ectopic expression of the Notch1 intracellular fragment (N1IC) is sufficient 46 to induce T-cell leukemia (3). However, activating mutations found in human T-ALL samples are 47 not powerful enough to reproduce T-ALL in mice (4, 5), suggesting that Notch dose and/or other 48 signals are also required for cell transformation. Supporting this concept, inactivating mutations 49 of the E3 ubiquitin ligase FBXW7, which regulates N1IC stability, are commonly found in T-ALL 50 patients (6), as well as a diversity of genetic lesions including the cell cycle regulator CDKN2A 51 and the transcription factors TAL1/2, LYL1, LMO1/2, TLX/HOX, MYB and MYC (7). Moreover, 52 roles for PTEN/PI3K (8), Cyclin/CDK (9), RAS (5), IL7R (10) and NFκB (11) in Notch-dependent 53 T-cell leukemia have been demonstrated.

54 The Wnt/β-Catenin (CTNNB1; catenin (cadherin-associated protein), beta 1, 88kDa) pathway
55 is a prevalent modulator (either activating or repressing) of Notch-dependent cell decisions (12).
56 Moreover, β-Catenin is a constitutive element of the cellular adherens junctions and, similarly to
57 Notch, is involved in the integration of cell-to-cell signals. Under basal conditions, free β-Catenin
58 is efficiently degraded by the proteasome downstream of the APC/AXIN/GSK3β destruction
59 complex. In the presence of Wnt factors, the destruction complex is inhibited leading to the
60 accumulation of β-Catenin, which translocates to the nucleus to activate specific gene
61 transcription in association with the DNA-binding factors of the TCF/LEF family (13). Ectopic
62 activation of β-Catenin in the T-cell compartment induces a leukemic phenotype (14), which is
63 associated with an increased genomic instability (15).

64 β-Catenin-dependent transcription is essential for the maintenance of stem cell function in
65 different tissues (16, 17). To a lesser extent, β-Catenin also regulates stem cell self-renewal in
66 the hematopoietic system (18), and it maintains leukemic initiating cells (LICs) in CML and AML
67 (19). In T-ALL, LIC activity has been associated with cells that contain and require Notch (20),
68 myc (21) and more recently, β-Catenin and Hif1a (22). In addition, β-Catenin has been identified
69 as a crucial element for Notch-independent, PTEN null induced T-cell leukemia (23, 24). Taken
70 together, these findings underline the relevant role of Notch, β-Catenin and Myc in T-ALL.

71 We have here investigated the mechanism by which β-Catenin contributes to LIC regulation
72 in Notch-induced T-ALL murine models and its possible use as therapeutic target for leukemia.

73

74 **Materials and Methods**

75 **Mouse models**

76 Ef1a-Lox-Stop-Lox-Notch1IC (25), VavCre (26) and β-Catenin-fl/fl (27) mice were crossed as
77 indicated. Animal work adhered to the guidelines from Generalitat de Catalunya and the ethics
78 committee at Parc de Recerca Biomèdica de Barcelona.

79

80 **Antibodies, FACS analysis and sorting**

81 CD117 (c-kit; APC-Cy7 or APC-eFluor780), sca1 (PE-Cy7), CD150 (APC), CD48 (PE), Lineage
82 (CD3, Ter119, B220, Gr1; Biotin), streptavidin-PerCP-Cy5.5, CD4 (PE-Cy7), CD8a (PE),
83 CD45.1 (APC-Cy7) and CD45.2 (FITC) from antibodies from BD. DAPI (D1306, Invitrogen) was
84 used for viability. Cell-cycle was analyzed with Ki67-APC (558615, BD Pharmingen) in
85 permeabilizing buffer (GAS-003, Invitrogen) and DAPI. Apoptosis was analyzed with
86 AnnexinV/DAPI-kit (BD) FACS was performed on LSRII (BD), sorting on FACSAria (BD) and
87 data analyzed with FlowJo v.10 (TreeStar, Inc).
88

89 **Cell transductions**

90 Recombinant retro- or lentiviruses were produced according to standard protocols. Bone
91 marrow was lineage-depleted (CD3, B220, Mac-1, Gr-1, Ter-119, BD Pharmingen) with Midi-
92 MACS (Miltenyi). Lin- cells were cultured for 24 hours with 10%FCS, 1%
93 Penicillin/Streptomycin, 55μM β-Mercaptoethanol, SCF 50ng/ml, Flt3L 50 ng/ml, IL6 10 ng/ml,
94 IL7 10 ng/ml and then infected with pMIG-N1IC-GFP or pMIG-GFP. A short-hairpin RNA vector
95 against β-Catenin (shβCat; pLKO.1-Hygro, target sequence TCTAACCTCACTTGCAATAAT,
96 SIGMA Mission shRNA ID# TRCN0000314921) or scrambled control (shCtrl;
97 GTCACGATAAGACAATGAT) were previously described (28). Briefly, T-ALL cell lines were
98 infected, and selected for 3 days with hygromycin (400-800ug/mL; Hygromycin B Gold,
99 Invivogen).
100

101 **Transplantation experiments**

102 Donor cells (CD45.2) were transplanted together with 200,000 BM support cells (CD45.1) into
103 lethally irradiated (4+4Gy) recipients (CD45.1). Peripheral blood (PB) donor chimerism was
104 analyzed by FACS at 3-4 and 6-8weeks. In vivo PKF115-584 treatment was administered
105 through intraperitoneal injections (25-50mg/kg) every 2-3 days.
106

107 **Cell lysates, Western blotting and immunoprecipitation**

108 Cells were lysed in 20min at 4C in PBS plus 0.5% Triton X-100, 1mM EDTA, 100 mM Na-
109 orthovanadate and protease inhibitors (Roche). For cytoplasm/nuclear/chromatin separations,
110 10mM HEPES, 1.5mM MgCl2, 10mM KCl, 0.05%NP40 at pH 7.9, 10min on ice and centrifuged
111 at 13.000 rpm. Supernatants were recovered and pellets lysed in 5mM HEPES, 1.5mM MgCl2,
112 0.2 mM EDTA, 0.5mM DTT and 26% glycerol and sonicated three times. Lysates were
113 analyzed by western blot. β-Catenin (c2206, Sigma), cleaved Notch1 (#2421, Cell Signaling)
114 and Kaiso (ab12723, Abcam) antibodies were used.

115 For immunoprecipitations, cells were lysed 30min at 4ºC in 300µl PBS plus 0.5% Triton X-
116 100, 1mM EDTA, 100 mM Na-orthovanadate, 0.25 mM PMSF and complete protease inhibitor
117 cocktail (Roche). After centrifugation, supernatants were pre-cleared 2h with 1% of BSA, 1µg
118 IgGs and 50µL SPA beads. Precleared lysates were incubated O/N with 3µg of indicated
119 antibodies (Irrelevant IgG or anti-β-Catenin (BD Bioscience, catalog no. 610154) or cleaved
120 Notch1 (ab8925, Abcam)). Antibody-Protein complexes were captured with 30µL SPA beads for
121 2h. After washing, precipitates were analyzed by western blot.
122
123

124 **Chromatin immunoprecipitation**

125 Chromatin from crosslinked cells (0.5% formaldehyde, 10 min) was sonicated, incubated o/n
126 with N1IC (kindly provided by J. Aster) or β-Catenin (BD Bioscience, 610154) antibodies and
127 precipitated with protein G/A-Sepharose, reverse crosslinked and used for Q-PCR (primers in
128 Supplementary Table 1).
129

130 **Cell lines and drug treatments**

131 All cell lines were cultured in standard conditions (RPMI-1640 + 10% FBS, Invitrogen). Inhibitors
132 were resuspended in DMSO in various concentrations; PKF115-584 (a gift from Novartis) at
133 10nM – 1µM, DAPT (Calbiochem) at 5-50 µM, and Pyrvinium (Sigma) at 31nM - 1µM in different

134 experiments. Cell number and viability was assessed using DAPI (D1306, Invitrogen) by FACS
135 (LSRII, BD).
136
137 **Luciferase assays**
138 T-ALL cell lines were electroporated with the 3' myc reporter (N-Me) (29) plasmid at 250V,
139 1050µF with Gene Pulser® II (Bio-Rad) and treated with DMSO, 25µM DAPT, 310nM PKF115-
140 584 or DAPT+PKF115-584, or, were untreated in the case of hygromycin selected shRNA-
141 transduced T-ALL cells. DMSO/DAPT were added after electroporation and at 24h, medium
142 was replaced with new DAPT and/or PKF115-584. Luciferase activity was measured after 48hrs
143 (Luciferase Assay System, Promega). Values were normalized to β-Galactosidase (co-
144 electroporation of CMV-β-Gal plasmid) or total protein (Bradford Assay).
145
146 **Next generation sequencing analyses**
147 Libraries created from polyA+ mRNA from 4 T-ALL cell lines (+DMSO or +PKF115-584), were
148 sequenced on a Solexa HiSeq 2000 (Illumina) and yielded 20-35 million 50-nt single-end reads
149 per sample. Reads were mapped to the human GRCh38.77 genome assembly with TopHat and
150 RNASeq expression data were obtained using the Cufflinks suite.
151
152 **Statistics**
153 Data were plotted and statistics calculated using GraphPad Prism 6.07.
154
155 **RESULTS**
156 **Transgenic Notch1-IC drives the expansion of LSKCD150-CD48+ leukemic initiating cells**
157 **(LICs) in the fetal liver.**
158 To study the early stages of Notch1-driven T-ALL, we set up a transgenic mouse model utilizing
159 the Ef1a-Lox-Stop-Lox-Notch1IC ($N1IC^{LSL}$) mouse line (25) crossed to VavCre-deleter mice
160 (26). VavCre+$N1IC^{LSL}$ (here N1IC+) embryos were embryonic lethal after E14.5 (Supplementary
161 Figure 1A). Analysis of E14.5 N1IC+ embryos revealed a decrease in FL cellularity compared to

162 VavCre+ control (WT) littermates (Supplementary Figure 1B), whereas the frequency as well as
163 total number of phenotypically defined hematopoietic stem and progenitor cells (HS/PCs;
164 Lineage-Sca1+ckit+ (LSK)) were significantly increased in N1IC+ FL (Figure 1A-B and
165 Supplementary Figure 1C). The N1IC+ LSK population was predominantly CD48+CD150-,
166 resembling bone marrow lineage-restricted HSCs (30), and showed decreased levels of CD150
167 (Figure 1A and Supplementary Figure 1D-E).

168 To evaluate the leukemic initiating potential of FL N1IC+ cells, we performed competitive
169 transplantation assays of FACS-sorted LSK, Lin-Sca-Kit+, Lin-Sca-Kit- and Lin+ cells into
170 lethally irradiated adult recipients (Figure 1C). Analysis of peripheral blood (PB) at 3 weeks after
171 transplantation showed high levels of donor cell chimerism in the recipient mice transplanted
172 with 0.2-0.6 embryo equivalents (ee) of N1IC+ LSK cells, comparable to that obtained from
173 5,000 unfractionated N1IC+ FL cells (Figure 1D). Lower chimerism was obtained by Lin-Sca-
174 Kit+ cells (corresponding to the myelo-erythroid progenitors in WT mice), whereas no
175 engraftment was obtained by Lin-Sca-Kit- cells or by an excess (0.5-1 million) of Lin+ cells
176 (Figure 1D). Engraftment derived from N1IC+ LSK and Lin-Sca-Kit+ cells was characterized by
177 the presence of aberrant double positive (DP) CD4+CD8a+ T-cells in the blood at 3 weeks after
178 transplantation (Figure 1E and Supplementary Figure 1F) indicative of leukemic growth, and all
179 LSK and most Lin-Sca-Kit+ receiving recipients succumbed to T-ALL with a median survival of
180 ≈7 weeks (Figure 1F).

181 These data demonstrate that Notch1 activation in the fetal hematopoietic compartment leads
182 to the expansion of a phenotypically defined cell population with robust leukemic initiating cell
183 (LIC) activity upon transplantation, thus providing a good model for a mechanistic investigation
184 of T-ALL initiation by Notch.

185

186 **β-Catenin levels determine the leukemic initiation capacity of Notch1-induced leukemia.**

187 Since β-Catenin plays important roles in HSC generation and self-renewal as well as in many
188 types of cancer, we sought to investigate the status of β-Catenin in the FL LSK population.
189 Immunofluorescence of sorted LSK cells from E14.5 FL showed abundant cytoplasmic as well

as nuclear β-Catenin in both VavCre+ control (WT) and N1IC+ LSK cells, indicative of active Wnt signaling in HSCs and LICs (Figure 2A).

To investigate whether β-Catenin was functionally relevant in the N1IC+ LICs, we analyzed triple transgenic VavCre+N1IC+βCatKO embryos. Similar levels of E14.5 FL cellularity reduction, expansion of the FL LSK CD150-CD48+ population, and embryonic lethality were observed in N1IC+ embryos irrespective of β-Catenin status (Figure 2B-C, Supplementary Figure 2A-C). By QPCR analysis, we detected variable levels of residual β-Catenin expression among individual N1IC+βCat$^{fl/fl}$ samples, ranging from ≈ 2-5% (termed Δ/fl) to < 0.1% (termed Δ/Δ) compared to control N1IC+βCat$^{fl/+}$ samples (100%, termed Δ/+) (Figure 2D). We next performed transplantation experiments using 1000 (1k) or 5000 (5k) unfractionated FL cells from N1IC+βCat$^{\Delta/+}$, N1IC+βCat$^{\Delta/fl}$ and N1IC+βCat$^{\Delta/\Delta}$ embryos and analyzed donor chimerism and DP cells at 3 weeks by FACS (Figure 2E). We found that N1IC+βCat$^{\Delta/+}$ as well as N1IC+βCat$^{\Delta/fl}$ but not N1IC+βCat$^{\Delta/\Delta}$ transplanted animals yielded substantial levels of DP cells (Supplementary Figure 2D-E). Importantly, levels of residual β-Catenin expression in the donor cells significantly correlated with donor chimerism in the recipient mice (Figure 2F), and the number of functional LICs in the transplanted population (Figure 2G), Specifically, we estimated LIC frequencies of 1 in 600 FL cells for N1IC+βCat$^{\Delta/+}$; 1 in 2800 for N1IC+βCat$^{\Delta/fl}$ and 1 in 6000 for N1IC+βCat$^{\Delta/\Delta}$ (Figure 2G). Moreover, whereas all N1IC+βCat$^{\Delta/+}$ cell recipients succumbed to T-ALL within a period of 9 weeks, animals transplanted with N1IC+βCat$^{\Delta/fl}$ and to a higher extent with N1IC+βCat$^{\Delta/\Delta}$ cells displayed improved survival for up to 1 year (log-rank test for trend p=0.0068) (Figure 2H and Supplementary Figure 2F). These results demonstrate a functional requirement for β-Catenin in Notch-induced T-ALL LICs.

β-Catenin deletion in adult hematopoietic cells abrogates Notch-driven T-ALL.

To achieve a consistent complete β-Catenin deletion, we next utilized an independent model of Notch T-ALL. Briefly, lineage-depleted bone marrow cells (Lin- BM) from VavCre^{+}βCat$^{+/+}$ and VavCre^{+}βCat$^{fl/fl}$ mice were transduced with mock pMIG-GFP (+pMIG) or pMIG-N1IC-GFP (+N1IC) retroviral vectors, and transplanted into lethally irradiated recipients (Figure 3A). Mice

218 transplanted with βCat$^{+/+}$+N1IC cells developed T-ALL with marked accumulation of blood DP
219 cells within 3-4 weeks (Figure 3B-C), as previously described (3). In contrast, animals
220 transplanted with βCat$^{fl/fl}$+N1IC cells did not contain detectable levels of DP cells at any time
221 point analyzed (Figure 3B-C) despite comparable engraftment levels (%GFP+) at 3-4 weeks
222 (Figure 3D). In agreement with this, βCat$^{+/+}$+N1IC-derived chimerism increased between 3-4
223 and 6-8 weeks, while βCat$^{fl/fl}$+N1IC-derived chimerism remained constant, similarly to that
224 observed in the mock-infected (βCat$^{fl/fl}$+pMIG) controls (Figure 3D). Importantly, animals
225 receiving βCat$^{fl/fl}$+N1IC cells survived for the duration of the study (>6months) whereas
226 βCat$^{+/+}$+N1IC recipients invariably succumbed with a median survival of ≈9 weeks (Figure 3E).
227 Persistence of N1IC vector in the unfractionated and FACS-sorted CD4+CD8a+ DP thymic cell
228 population from both β-Catenin genotypes was confirmed at the study endpoint (Supplementary
229 Figure 3).
230

231 **β-Catenin inhibitor PKF115-584 induces apoptosis and reduces proliferation in human T-**
232 **ALL cells.**

233 We next investigated the status of β-Catenin in human T-ALL. By western blot (WB) analysis we
234 observed elevated levels of β-Catenin in T-ALL cell lines carrying cleaved Notch1 (N1IC),
235 compared with the relatively low β-Catenin levels found in myeloid and B-cell leukemic lines
236 (Supplementary Figure 4A). Further analysis of four T-ALL lines (RPMI8402, HPB-ALL, Jurkat,
237 CCRF-CEM, referred to as 4T-ALL) showed cytoplasmic and nuclear accumulation of both N1IC
238 and β-Catenin (Figure 4A), suggesting that both factors are active in T-ALL. To test whether β-
239 Catenin was functionally required in human T-ALL, we utilized a small molecule, PKF115-584,
240 previously shown to inhibit the transcriptional activity of β-Catenin (31). We found that PKF115-
241 584 rapidly (16-24 hours of treatment) and permanently affected the cell number kinetics of all
242 tested cell lines (Figure 4B and Supplementary Figure 4B), mainly as a result of increased cell
243 death and reduced proliferation, as determined by AnnexinV/DAPI and Ki67 staining,
244 respectively (Figure 4C-D). A comparable cytotoxic effect on T-ALL cells was observed after

245 treatment with pyrvinium, also known to inhibit β-Catenin activity in vivo (32) (Supplementary
246 Figure 4C).
247

248 **Myc is a direct target of Notch and β-catenin in T-ALL that is inhibited by PKF115-584 and**
249 **shRNA against β-Catenin.**

250 To further understand the mechanism underlying β-catenin function in T-ALL cells, we treated
251 four T-ALL cell lines (4T-ALL) with PKF115-584 or DMSO for 3 hours and performed RNA-
252 Sequencing (Figure 5A). GSEA analysis on the differentially expressed genes across all four
253 lines (FDR q-val<0.01, log2FC≥0.4, FPKM≥5) showed significant downregulation of Oxidative
254 phosphorylation and MYC target gene sets (Figure 5B-C and Supplementary Figure 5A-C). In
255 contrast, gene categories associated with apoptosis and stress response (e.g. p53 pathway,
256 NF-κB) were significantly upregulated (Figure 5B-C and Supplementary Figure 5D). Selected
257 genes from MYC targets or apoptosis categories were validated by QPCR (Figure 5D and
258 Supplementary Figure 5E). Notably, MYC mRNA and protein levels were rapidly (2-3hrs and
259 24hrs, respectively) downregulated in all four T-ALL lines in response to PKF115-584 (Figure
260 5D-E and Supplementary Figure 5E-F), while Notch activity, as estimated by Hes1 mRNA
261 expression or GSEA analysis against Notch-responsive gene sets, was unaffected (Figure 5E
262 and Supplementary Figure 5G). Pyrvinium was also efficient at downregulating Myc protein
263 levels, albeit while also broadly downregulating β-Catenin and Notch1-IC levels, in contrast to
264 PKF115-584 (Supplementary Figure 5F).

265 Because of its relevance in several cancer models, we studied the possible regulation of
266 MYC by Notch and β-Catenin in T-ALL. To confirm the role of β-Catenin, we utilized sh-control
267 (shCtrl) or sh-β-Catenin (shβCat) lentiviruses to efficiently target β-catenin at both mRNA and
268 protein levels in T-ALL cell lines (Figure 5F-G). We observed increased levels of cell death and
269 apoptosis while cell growth was differentially affected in the different T-ALL cell lines tested
270 (Supplementary Figure 6A-B and data not shown). Importantly, knockdown of β-Catenin led to a
271 decrease of MYC mRNA and protein levels (Figure 5F-G). Similarly to what was observed with
272 PKF115-584, Hes1 was not downregulated indicating that Notch activity was not affected

273 despite a decrease in protein levels of active Notch observed in shβCat-infected T-ALL cells
274 (Figure 5F-G).

275 By Chromatin IP (ChIP) using antibodies against β-Catenin, N1IC and RBPj in two different
276 T-ALL cell lines (Jurkat and CCRF-CEM), we did not detect any binding in the MYC promoter
277 (Figure 5H), which contain WRE/TCF/LEF binding sites that associate with β-Catenin in other
278 tissues (33). In contrast, we found that the recently identified 3' MYC Enhancer (MYC-Enh),
279 known to bind Notch1 (29, 34), also showed a significant enrichment for β-Catenin occupancy
280 (Figure 5F). Since we found, by co-immunoprecipitation in Jurkat and RPMI8402 cells, that
281 Notch1 physically interacted with β-Catenin (Figure 5I and Supplementary Figure 5H), we
282 hypothesized that β-Catenin may be recruited to this site by direct interaction with Notch1 as in
283 other double Notch/β-Catenin targets (35, 36). To test whether Notch1 and β-Catenin were
284 necessary for MYC enhancer activity, we transfected the 3' enhancer cloned in a luciferase
285 reporter vector (29) into Jurkat cells which were either untreated (DMSO) or treated overnight
286 with DAPT (a γ-secretase/Notch inhibitor), PKF115-584 or DAPT+PKF115-584. As previously
287 described (29), we observed a decrease of luciferase activity by DAPT (Figure 5J; left panel). Of
288 note, treatment of cells with PKF115-584 also significantly reduced the luciferase activity of the
289 Myc-enhancer construct, and the DAPT+PKF115-584 combination showed an almost complete
290 (≈98%) inhibition of enhancer activity (Figure 5J; left panel). In addition, a significant decrease in
291 the Myc enhancer transcriptional activity was measured in RPMI8402 cells previously
292 transduced with shβCat compared to shCtrl (Figure 5J; right panel).

293 These results indicate that the interaction between β-Catenin and Notch1 is important for
294 maintaining the oncogenic program in T-ALL, at least in part through co-regulation of MYC
295 transcription through its 3´ enhancer.

296

297 **PKF115-584 treatment inhibits survival of T-ALL leukemic cells in vivo.**

298 To test the possibility of using PKF115-584 to treat leukemia in vivo, we used our FL
299 transplantation model. E14.5 FL cells from N1IC+ embryos were transplanted together with
300 bone marrow competitor cells into lethally irradiated mice (Figure 6A). Recipient mice were

301 randomly grouped in three regimens: Animals treated with PKF115-584 by intraperitoneal
302 injection every 2 days, i) for 6 weeks starting at day 3 post-transplantation (PKF0-6), ii) 3 weeks
303 of treatment starting at week 3 (PKF3-6), or iii) animals were left untreated (Figure 6A). FACS
304 analysis of PB at 3 weeks showed a significant reduction in donor chimerism (≈50%, p=0.003)
305 in the cohort treated with PKF115-584 compared to untreated recipients (Figure 6B). In
306 contrast, PKF115-584 had no significant effect on wild-type total leukocyte counts (WBC),
307 indicating that the drug specifically targeted leukemic N1IC+ cells (Figure 6C). In support of the
308 requirement for β-Catenin activity in leukemia initiation, mice in the PKF0-6 cohort displayed
309 significantly improved overall survival, and a delay in leukemic death onset compared to
310 untreated mice (Figure 6D). Finally, although not statistically significant (p=0.22), PKF115-584
311 treatment for 3 weeks (cohort PKF3-6) prevented death for ≥40 weeks of 22% of mice with
312 confirmed initial CD4+CD8a+ DP leukemic burden (DP+) (Figure 6E and data not shown), in
313 contrast with DP+ untreated recipients that invariably succumbed to T-ALL by 9 weeks.
314 In summary, these data nominate β-Catenin as a promising therapeutic target in T-ALL
315 treatment.
316

317 **Discussion**

318 Despite increasing evidence for the roles of Notch and β-Catenin in tissue maintenance, stem
319 cell self-renewal and cancer, separate findings from different groups yield seemingly
320 contradictory results, with Wnt and Notch acting synergistically or antagonistically depending on
321 cellular/tissue context (reviewed in (37)). In this study, we found an essential crosstalk between
322 Notch1 and β-Catenin in T-ALL through direct regulation of MYC, consistent with the
323 association of these three proteins with leukemic initiating cell (LIC) activity in T-ALL (20-22). In
324 addition, mutations in other pathways such as PTEN may determine the levels of β-Catenin
325 activity (23), which would affect Notch-induced leukemogenic potential as shown in our murine
326 model, although we did not observe any correlation between PTEN mutations in T-ALL cell lines
327 and total β-catenin levels. However, more work should be done to specifically address this issue
328 and understand the relevance of PTEN in this connection.

329 The Wnt pathway has long been associated to T-cell development and T-cell leukemia (14,
330 38-40); however, the specific role of particular Wnt-pathway family members remains highly
331 controversial. For example, LEF1 and TCF7 (also known as TCF-1) nuclear factors regulate T-
332 cell differentiation but they can either act as leukemia/lymphoma inducers or suppressors in a
333 context-dependent manner. Published data demonstrated that TCF7 acts as tumor suppressor
334 by inhibiting LEF1, but in turn inactivating LEF1 mutations were also found to result in T-ALL
335 associated with MYC gene upregulation (38), with Notch1 activity being crucial for the leukemic
336 process driven by either TCF7 or LEF1 inactivation (39, 40). Whether leukemia development
337 requires β-Catenin in these LEF/TCF deficient systems remains unknown. Our analysis of the
338 MYC regulatory regions revealed that both Notch1 and β-Catenin associate in the same region
339 of 3' MYC enhancer, containing three well-defined RBPj consensus sites, whereas binding was
340 not observed for either factor in the MYC promoter containing a TCF/LEF binding motif. The fact
341 that β-Catenin physically interacted with cleaved Notch1 in T-ALL cells by co-
342 immunoprecipitation under conditions where DNA is not detected supports the possibility that β-
343 Catenin binds DNA through an RBPj/Notch1 complex. In agreement with this, we found that
344 both proteins positively regulate the 3' *MYC* enhancer, and *MYC* mRNA levels as well as MYC
345 targets were significantly reduced after PKF115-584 treatment in T-ALL cells. Further work
346 comparing the DNA binding activity of β-Catenin and Notch1 in a genome-wide approach will
347 help to elucidate the specific contribution of β-Catenin to Notch1-dependent gene transcription.

348 Consistent with a prominent role of β-Catenin in leukemogenesis, we have here shown that
349 PKF115-584 efficiently inhibited cell growth and induced apoptosis in T-ALL cells. In fact, in our
350 assay, PKF115-584 was a more potent inhibitor of MYC enhancer-dependent transcription than
351 the γ-secretase inhibitor DAPT. This was dependent on dose and time of incubation, although
352 we cannot exclude that PKF115-584 is targeting other proteins than just β-Catenin. In this
353 sense, our in vivo genetic studies, as well as our in vitro depletion of β-Catenin by a single
354 shRNA previously shown to efficiently inhibit β-Catenin (28), confirm that T-ALL cells strongly
355 rely on β-Catenin for MYC expression and survival. In vivo, mice transplanted with Notch1-
356 active T-ALL progenitors (LICs) that received PKF115-584 treatment 3 days after the initiation

357 of the experiment showed increased survival compared with control leukemic animals.
358 However, mice treated from week 3 after transplantation did not show a significantly different
359 survival, but 22% of the animals carrying double CD4+CD8a+ double positive cells at week 3
360 post-transplantation remained alive after 40 weeks, compared with 100% lethality in the control
361 group by week 9. These results suggest that PKF115-584 was affecting not only leukemia
362 initiation, as recently shown for β-catenin (22), but also maintenance, which is relevant from a
363 therapeutic point of view. Most importantly, normal hematopoietic cells were refractory to the in
364 vivo PKF115-584 treatment indicating a differential sensitivity of leukemic and normal cells
365 towards β-Catenin inhibition.

366 Together our data indicate that Notch depends on β-Catenin for the leukemogenic activity
367 associated to MYC upregulation and identifies β-Catenin as a promising therapeutical target for
368 T-ALL.

369

370 **Author Contributions**

371 CG, LE and AB designed experiments, analyzed data and wrote the manuscript; CG, TD, LE,
372 RA, JG conducted experiments.

373

374 **Acknowledgements:**

375 We would like to thank Carla L. Bello for RNA-Seq data mapping and analysis, Iannis Aifantis
376 and Apostolos Klinakis for EF1a-LSL-N1IC mice and retroviral constructs, Steve Blacklow and
377 Sarah Bray for helpful discussions, Jon Aster for anti-N1 antibody and Warren Pear for myc
378 enhancer constructs and helpful discussions. This research was funded by the Worldwide
379 Cancer Research (formerly AICR, 13-0064), Fundación AECC (Cancer infantil), Ministerio de
380 Economía y Competitividad (SAF2013-40922-R), Red Temática de Investigación Cooperativa
381 en Cáncer (RD12/0036/0054), Agència de Gestió d’Ajuds Universitaris i de Recerca (AGAUR)
382 (2014SGR-124) to AB.

383

384 **References**

385 1. Aifantis I, Raetz E, Buonamici S. Molecular pathogenesis of T-cell leukaemia and
386 lymphoma. Nat Rev Immunol. 2008 May;8(5):380-90.
387 2. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, et al.
388 Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004
389 Oct 8;306(5694):269-71.
390 3. Pear WS, Aster JC, Scott ML, Hasserjian RP, Soffer B, Sklar J, et al. Exclusive
391 development of T cell neoplasms in mice transplanted with bone marrow expressing activated
392 notch alleles. J Exp Med. 1996;183(5):2283-91.
393 4. Berquam-Vrieze KE, Swing DA, Tessarollo L, Dupuy AJ. Characterization of transgenic
394 mice expressing cancer-associated variants of human NOTCH1. Genesis. 2012 Feb;50(2):112-
395 8.
396 5. Chiang MY, Xu L, Shestova O, Histen G, L'Heureux S, Romany C, et al. Leukemia-
397 associated NOTCH1 alleles are weak tumor initiators but accelerate K-ras-initiated leukemia. J
398 Clin Invest. 2008 Sep;118(9):3181-94.
399 6. Thompson BJ, Buonamici S, Sulis ML, Palomero T, Vilimas T, Basso G, et al. The
400 SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia. J Exp Med. 2007
401 Aug 6;204(8):1825-35.
402 7. Koch U, Radtke F. Notch in T-ALL: new players in a complex disease. Trends Immunol.
403 2011 Sep;32(9):434-42.
404 8. Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M, et al. Mutational loss of
405 PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med. 2007
406 Oct;13(10):1203-10.
407 9. Sawai CM, Freund J, Oh P, Ndiaye-Lobry D, Bretz JC, Strikoudis A, et al. Therapeutic
408 targeting of the cyclin D3:CDK4/6 complex in T cell leukemia. Cancer Cell. 2012 Oct
409 16;22(4):452-65.
410 10. Gonzalez-Garcia S, Garcia-Peydro M, Martin-Gayo E, Ballestar E, Esteller M, Bornstein
411 R, et al. CSL-MAML-dependent Notch1 signaling controls T lineage-specific IL-7R{alpha} gene
412 expression in early human thymopoiesis and leukemia. J Exp Med. 2009 Apr 13;206(4):779-91.
413 11. Espinosa L, Cathelin S, D'Altri T, Trimarchi T, Statnikov A, Guiu J, et al. The Notch/Hes1
414 pathway sustains NF-kappaB activation through CYLD repression in T cell leukemia. Cancer
415 Cell. 2010 Sep 14;18(3):268-81.
416 12. Bigas A, Guiu J, Gama-Norton L. Notch and Wnt signaling in the emergence of
417 hematopoietic stem cells. Blood Cells Mol Dis. 2013 Dec;51(4):264-70.
418 13. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. Cell. 2012 Jun
419 8;149(6):1192-205.
420 14. Guo Z, Dose M, Kovalovsky D, Chang R, O'Neil J, Look AT, et al. Beta-catenin
421 stabilization stalls the transition from double-positive to single-positive stage and predisposes
422 thymocytes to malignant transformation. Blood. 2007 Jun 15;109(12):5463-72.
423 15. Dose M, Emmanuel AO, Chaumeil J, Zhang J, Sun T, Germar K, et al. beta-Catenin
424 induces T-cell transformation by promoting genomic instability. Proc Natl Acad Sci U S A. 2014
425 Jan 7;111(1):391-6.
426 16. Huelsken J, Vogel R, Erdmann B, Cotsarelis G, Birchmeier W. beta-Catenin controls hair
427 follicle morphogenesis and stem cell differentiation in the skin. Cell. 2001 May 18;105(4):533-
428 45.
429 17. Fevr T, Robine S, Louvard D, Huelsken J. Wnt/beta-catenin is essential for intestinal
430 homeostasis and maintenance of intestinal stem cells. Mol Cell Biol. 2007 Nov;27(21):7551-9.
431 18. Zhao C, Blum J, Chen A, Kwon HY, Jung SH, Cook JM, et al. Loss of beta-catenin
432 impairs the renewal of normal and CML stem cells in vivo. Cancer Cell. 2007 Dec;12(6):528-41.
433 19. Wang Y, Krivtsov AV, Sinha AU, North TE, Goessling W, Feng Z, et al. The Wnt/beta-
434 catenin pathway is required for the development of leukemia stem cells in AML. Science. 2010
435 Mar 26;327(5973):1650-3.

436 20. Armstrong F, Brunet de la Grange P, Gerby B, Rouyez MC, Calvo J, Fontenay M, et al.
437 NOTCH is a key regulator of human T-cell acute leukemia initiating cell activity. Blood. 2009
438 Feb 19;113(8):1730-40.
439 21. King B, Trimarchi T, Reavie L, Xu L, Mullenders J, Ntziachristos P, et al. The ubiquitin
440 ligase FBXW7 modulates leukemia-initiating cell activity by regulating MYC stability. Cell. 2013
441 Jun 20;153(7):1552-66.
442 22. Giambra V, Jenkins CE, Lam SH, Hoofd C, Belmonte M, Wang X, et al. Leukemia stem
443 cells in T-ALL require active Hif1alpha and Wnt signaling. Blood. 2015 Jun 18;125(25):3917-27.
444 23. Guo W, Lasky JL, Chang CJ, Mosessian S, Lewis X, Xiao Y, et al. Multi-genetic events
445 collaboratively contribute to Pten-null leukaemia stem-cell formation. Nature. 2008 May
446 22;453(7194):529-33.
447 24. Kaveri D, Kastner P, Dembele D, Nerlov C, Chan S, Kirstetter P. beta-Catenin activation
448 synergizes with Pten loss and Myc overexpression in Notch-independent T-ALL. Blood. 2013
449 Aug 1;122(5):694-704.
450 25. Buonamici S, Trimarchi T, Ruocco MG, Reavie L, Cathelin S, Mar BG, et al. CCR7
451 signalling as an essential regulator of CNS infiltration in T-cell leukaemia. Nature. 2009 Jun
452 18;459(7249):1000-4.
453 26. Stadtfeld M, Graf T. Assessing the role of hematopoietic plasticity for endothelial and
454 hepatocyte development by non-invasive lineage tracing. Development. 2005 Jan;132(1):203-
455 13.
456 27. Brault V, Moore R, Kutsch S, Ishibashi M, Rowitch DH, McMahon AP, et al. Inactivation
457 of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation
458 and failure of craniofacial development. Development. 2001 Apr;128(8):1253-64.
459 28. Aulicino F, Theka I, Ombrato L, Lluis F, Cosma MP. Temporal perturbation of the Wnt
460 signaling pathway in the control of cell reprogramming is modulated by TCF1. Stem Cell
461 Reports. [Research Support, Non-U.S. Gov't]. 2014 May 6;2(5):707-20.
462 29. Yashiro-Ohtani Y, Wang H, Zang C, Arnett KL, Bailis W, Ho Y, et al. Long-range
463 enhancer activity determines Myc sensitivity to Notch inhibitors in T cell leukemia. Proc Natl
464 Acad Sci U S A. 2014 Nov 18;111(46):E4946-53.
465 30. Wilson A, Laurenti E, Oser G, van der Wath RC, Blanco-Bose W, Jaworski M, et al.
466 Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis
467 and repair. Cell. [Research Support, Non-U.S. Gov't]. 2008 Dec 12;135(6):1118-29.
468 31. Lepourcelet M, Chen YN, France DS, Wang H, Crews P, Petersen F, et al. Small-
469 molecule antagonists of the oncogenic Tcf/beta-catenin protein complex. Cancer Cell. 2004
470 Jan;5(1):91-102.
471 32. Fong CY, Gilan O, Lam EY, Rubin AF, Ftouni S, Tyler D, et al. BET inhibitor resistance
472 emerges from leukaemia stem cells. Nature. 2015 Sep 24;525(7570):538-42.
473 33. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al. Identification of
474 c-MYC as a target of the APC pathway. Science. 1998 Sep 4;281(5382):1509-12.
475 34. Herranz D, Ambesi-Impiombato A, Palomero T, Schnell SA, Belver L, Wendorff AA, et al.
476 A NOTCH1-driven MYC enhancer promotes T cell development, transformation and acute
477 lymphoblastic leukemia. Nat Med. 2014 Oct;20(10):1130-7.
478 35. Lopez-Arribillaga E, Rodilla V, Pellegrinet L, Guiu J, Iglesias M, Roman AC, et al. Bmi1
479 regulates murine intestinal stem cell proliferation and self-renewal downstream of Notch.
480 Development. 2015 Jan 1;142(1):41-50.
481 36. Yamamizu K, Matsunaga T, Uosaki H, Fukushima H, Katayama S, Hiraoka-Kanie M, et
482 al. Convergence of Notch and beta-catenin signaling induces arterial fate in vascular
483 progenitors. J Cell Biol. 2010 Apr 19;189(2):325-38.
484 37. Collu GM, Hidalgo-Sastre A, Brennan K. Wnt-Notch signalling crosstalk in development
485 and disease. Cell Mol Life Sci. 2014 Sep;71(18):3553-67.
486 38. Gutierrez A, Sanda T, Ma W, Zhang J, Grebliunaite R, Dahlberg S, et al. Inactivation of
487 LEF1 in T-cell acute lymphoblastic leukemia. Blood. 2010 Apr 8;115(14):2845-51.
488 39. Tiemessen MM, Baert MR, Schonewille T, Brugman MH, Famili F, Salvatori DC, et al.
489 The nuclear effector of Wnt-signaling, Tcf1, functions as a T-cell-specific tumor suppressor for
490 development of lymphomas. PLoS Biol. 2012;10(11):e1001430.

40. Yu S, Zhou X, Steinke FC, Liu C, Chen SC, Zagorodna O, et al. The TCF-1 and LEF-1 transcription factors have cooperative and opposing roles in T cell development and malignancy. Immunity. 2012 Nov 16;37(5):813-26.

Figure Legends

Figure 1. N1IC causes expansion of LSKCD150-CD48+ Leukemic Initiating Cells in the fetal liver. (A) FACS analysis of FL from E14.5 wild-type (WT) or VavCre+N1IC^{LSL} (N1IC+). **(B)** Number of LSK per 5k E14.5 FL of the indicated genotypes. ***; p<0.001 by unpaired t-test. **(C)** Transplantation setup for cells FACS sorted as shown below. ee; embryo equivalents, L; Lineage, S; sca1, K; ckit. **(D)** Donor chimerism in peripheral blood (PB) of recipient mice 3 wks after transplantation determined by FACS. **(E)** Frequency of donor-derived (CD45.2+CD45.1-) CD4+CD8a+ double-positive (DP) cells in PB. 0 indicates no engraftment. **(F)** Survival curve with median survival (wks) following two independent transplantation experiments of each population. Number of recipients: LSK; 9, L-S-K+; 8, L-S-K-; 6, Lin+; 7.

Figure 2. β-Catenin levels determine the Notch1-dependent leukemogenic potential in a FL transplantation model. (A) Immunofluorescence of sorted WT or N1IC+ E14.5 FL LSK cells. **(B)** FACS analysis of E14.5 FL LSK cells from indicated genotypes. Numbers indicate %LSK frequency. **(C)** LSK number per 5k E14.5 FL cells, *** indicates unpaired t-test p<0.001 vs control genotypes (Cre- or Cre+N1IC-). **(D)** QPCR from E14.5 FL LSK cells, for β-Catenin floxed exon relative to *Gapdh*, normalized to N1IC+βCat$^{fl/+}$. Incompletely (2-5%) or totally (<0.1%) excised samples referred as Δ/fl or Δ/Δ. **(E)** Transplantation protocol for the different β-Catenin excised samples as defined in D. **(F)** XY scatter plot representing levels of unexcised β-Catenin mRNA in donor cells (X-axis) and PB donor chimerism after 3 weeks (Y-axis). Solid lines represent linear regression with 95% confidence intervals (dotted lines). **(G)** Estimated LIC frequency in E14.5 N1IC+ FL. **(H)** Survival curve after transplantation with 1000 (1k) cells from N1IC+βCat$^{\Delta/+}$ (n=9), N1IC+βCat$^{\Delta/fl}$; (n=14), N1IC+βCat$^{\Delta/\Delta}$; (n=3). Statistical analysis with the indicated test is shown: * p<0.05, ** p<0.01.

521 **Figure 3. Total β-Catenin deletion in adult BM cells inhibits Notch-induced T-ALL in vivo.**
522 **(A)** Transplantation protocol for VavCre+βCat$^{+/+}$ or βCat$^{fl/fl}$ lin- BM cells transduced with GFP
523 control (pMIG) or N1IC-IRES-GFP (N1IC) retroviruses. **(B)** Representative FACS plots of PB at
524 3 weeks. **(C)** Lineage distribution for CD4+, CD8a+, double positive (DP) or double negative
525 (Non T-cells) populations in whole blood (top) or donor-derived (GFP+) fraction (bottom) at 6-
526 8weeks. **(D)** Donor chimerism (%GFP+) at 3-4 and 6-8 weeks after transplantation. Bars
527 indicate mean and standard error (SEM) and dots show individual recipients. **(E)** Survival curve
528 with median survival in weeks (wks).
529
530 **Figure 4. Inhibition of β-Catenin activity by PKF115-584 compromises the survival and**
531 **proliferation of human T-ALL. (A)** WB of cytoplasmic and nuclear fractions of four human T-
532 ALL cell lines for cleaved Notch1 (N1IC) and β-Catenin. Lamin B and tubulin are used as purity
533 and loading controls. **(B)** Dose response assays after single treatment with the indicated doses
534 of PKF115-584. FACS counting of DAPI- cells normalized to DMSO control. Mean and SEM of
535 three independent experiments are represented. **(C)** AnnexinV binding and DAPI staining after
536 24h of PKF115-584 treatment (310nM). **(D)** Overlay FACS plots for Ki67 levels after 24h
537 treatment as indicated.
538
539 **Figure 5. MYC is a direct target of Notch and β-catenin in T-ALL (A)** Schematic
540 representation of RNA-Sequencing experiments. **(B)** GSEA analysis for MSigDB Hallmark gene
541 sets using up- or downregulated (DN) genes after PKF115-584 treatment. **(C)** GSEA plots of
542 selected top enriched gene sets. **(D)** Validation by Q-PCR in PKF115-584 treated RPMI8402
543 cells for selected genes. **(E)** Integrative Genomics Viewer (IGV) visualization of RNASeq tracks
544 for MYC and HES1 genes in DMSO (grey) or PKF115-584 treated (310nM; 3hrs, red) T-ALL
545 lines. Values indicate normalized tag count (expression level) and Y-axes are set to the highest
546 value within each cell line DMSO/PKF pair. **(F)** Q-PCR for the indicated genes in T-ALL cell
547 lines transduced with shCtrl or shβCat, 1-2 days after completion of hygromycin selection (n=3,
548 except HPB-ALL n=1). **(G)** Representative western blot analysis of the indicated proteins from

549 shRNA-transduced RPMI8402 1 day after completion of hygromycin selection**. (H)** ChIP with the
550 indicated antibodies analyzed by QPCR with MYC enhancer (Enh.1-2, flanking three RPBj
551 motifs) and MYC promoter (Prom.; flanking a TCF/LEF motif) primers. **(I)** Co-
552 immunoprecipitation experiment on Jurkat cells for β-Catenin and N1IC. **(J)** Luciferase assay for
553 Myc enhancer activity on transfected Jurkat cells treated with the indicated inhibitors (n≥3) (left);
554 or in shRNA transduced RPMI8402 cells (n=2) (right). Statistical significance is calculated by
555 unpaired t-test, *; p<0.05, **; p<0.01, ***; p<0.001.
556
557 **Figure 6. PKF115-584 treatment specifically inhibits the survival of T-ALL leukemic**
558 **cells in vivo. (A)** Schema for in vivo PKF treatment assay. **(B)** Donor (CD45.2+) chimerism
559 determined by FACS in recipient PB at 3 wks. T-test, **; p<0.01. **(C)** Total donor (CD45.2+)
560 and competitor (CD45.1+) derived leukocyte counts in total PB. NE indicates not engrafted
561 above threshold (WBC<0.01*10^{-9}/L). **; p<0.01 by unpaired t-test. **(D and E)** Survival curves
562 for cohorts untreated or treated with PKF for 6 (D) or 3 (E) weeks. In E only mice with
563 confirmed leukemic burden (≥1% CD4+CD8a+ DP cells in PB at 3 weeks) are included. Data
564 are from three independent experiments. *; p=0.0146 by Log-Rank (Mantel Cox) Test and
565 p=0.0059 by Gehan-Breslow-Wilcoxon test.

Figure 1

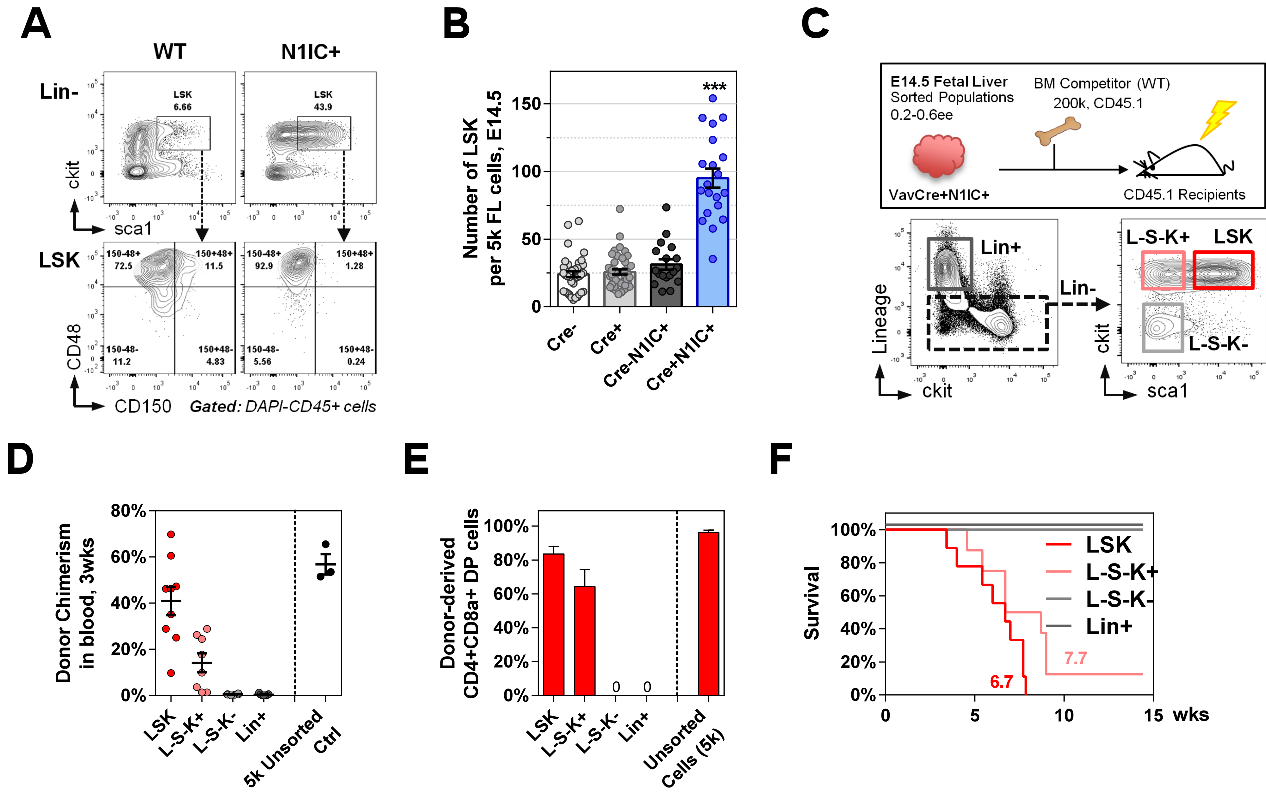


Figure 2

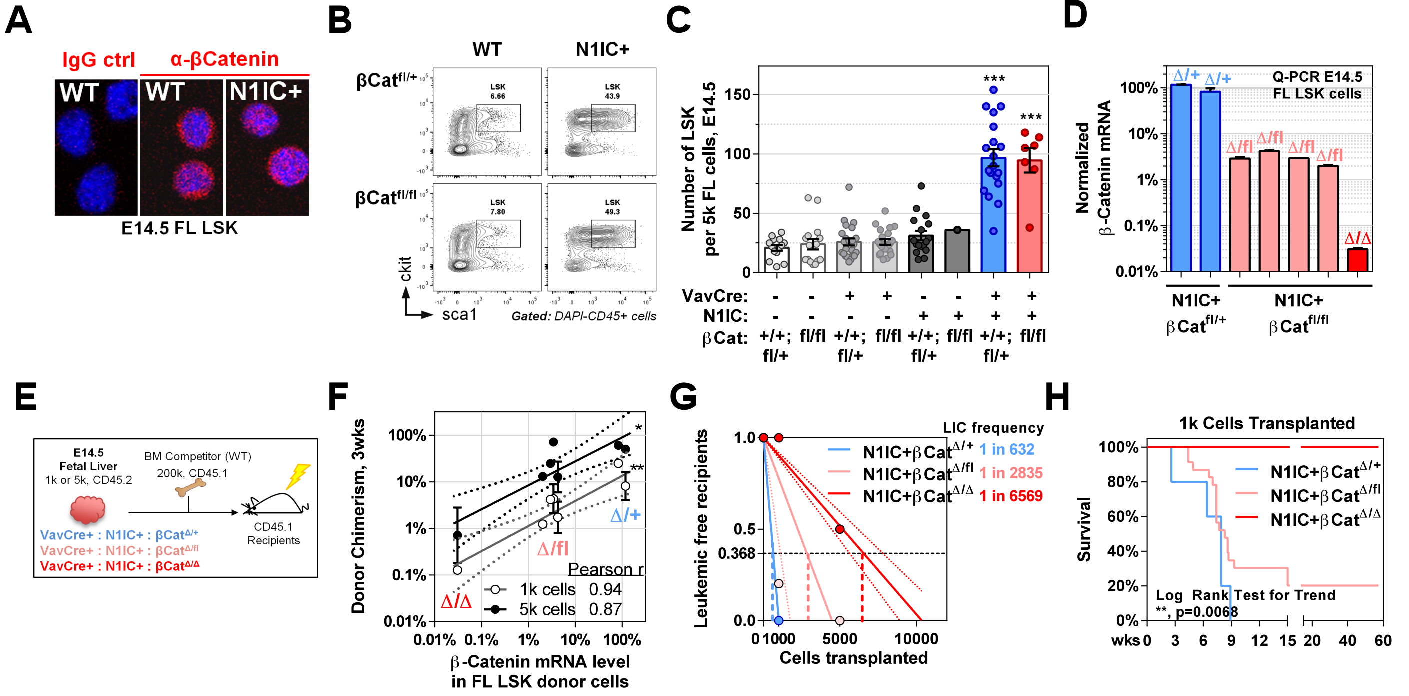
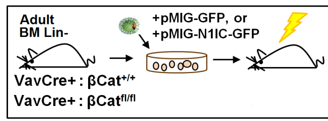
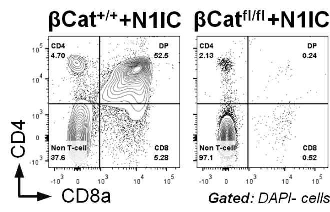


Figure 3

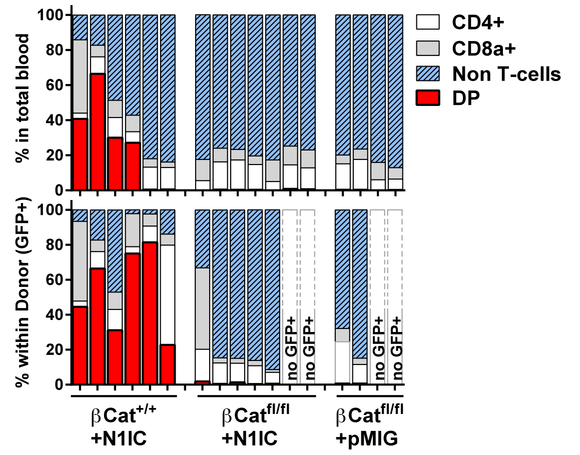
A



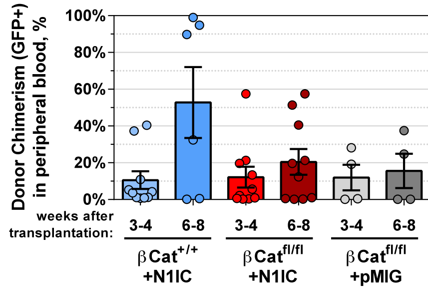
B



C



D



E

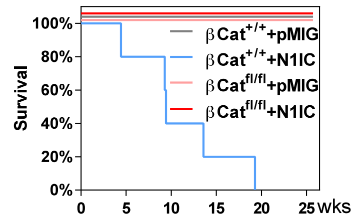
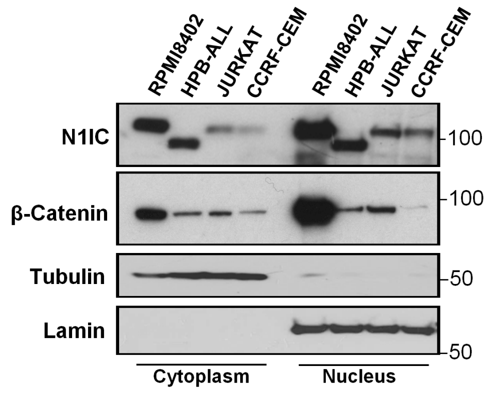
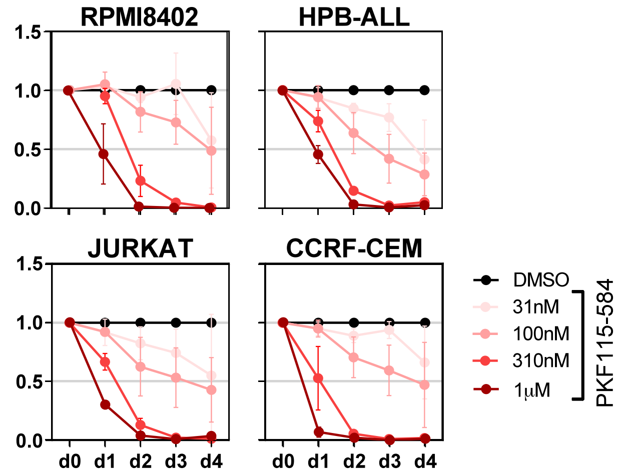


Figure 4

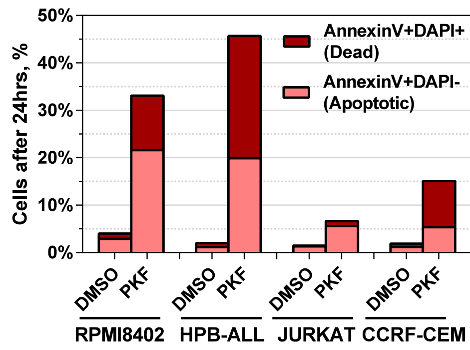
A



B



C



D

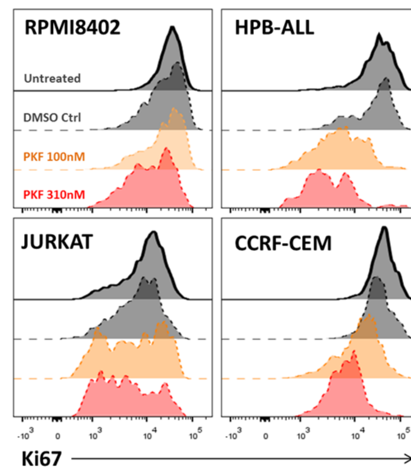


Figure 5

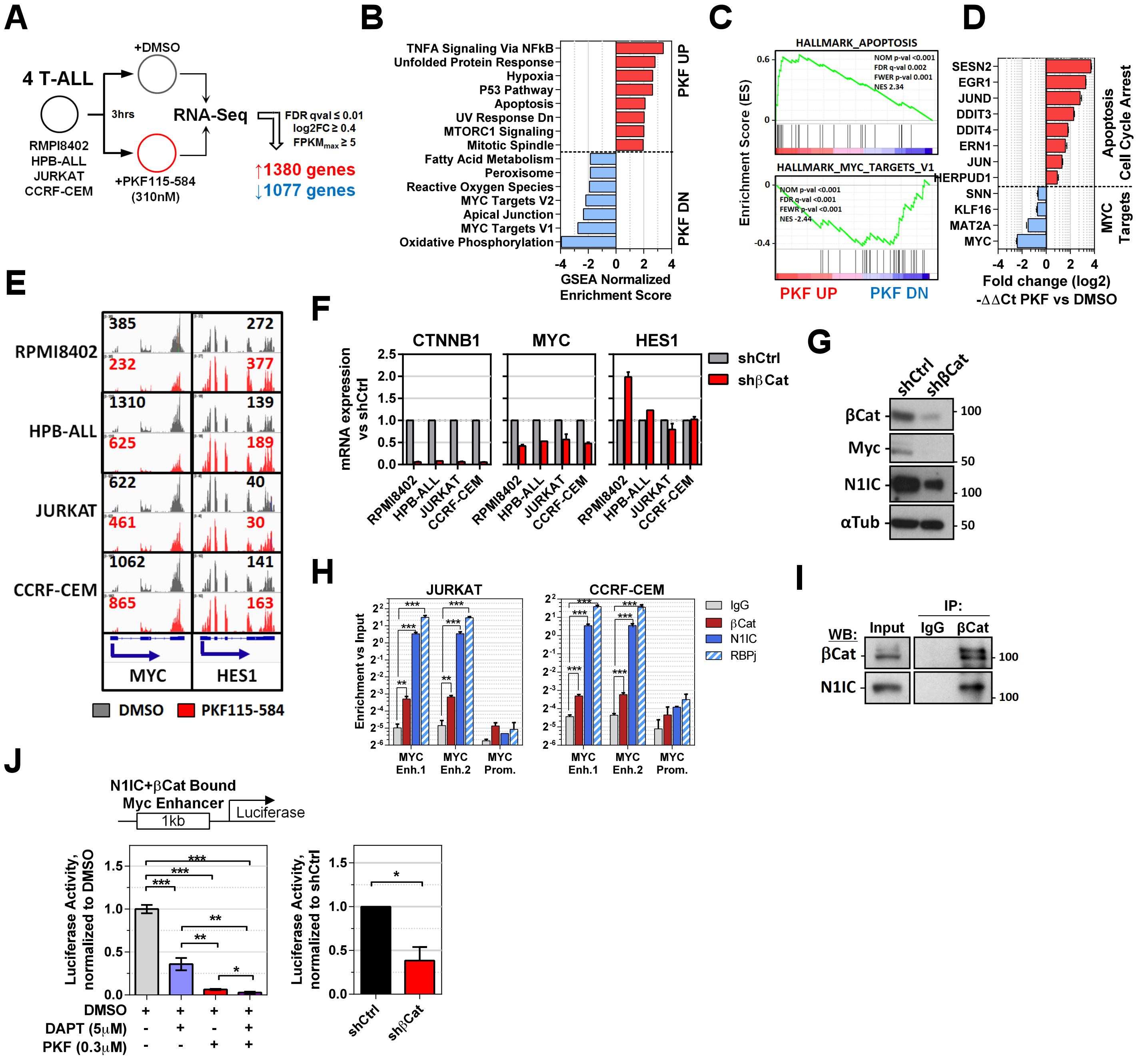


Figure 6