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

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

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Summary

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

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological malignancy characterized by the outgrowth of immature T-cells (1), with Notch1 mutations being the most prominent genetic alteration in this disease (60% of cases) (2). In mice, constitutive Notch1 activation through the ectopic expression of the Notch1 intracellular fragment (N1IC) is sufficient to induce T-cell leukemia (3). However, activating mutations found in human T-ALL samples are not powerful enough to reproduce T-ALL in mice (4, 5), suggesting that Notch dose and/or other signals are also required for cell transformation. Supporting this concept, inactivating mutations of the E3 ubiquitin ligase FBXW7, which regulates N1IC stability, are commonly found in T-ALL patients (6), as well as a diversity of genetic lesions including the cell cycle regulator CDKN2A and the transcription factors TAL1/2, LYL1, LMO1/2, TLX/HOX, MYB and MYC (7). Moreover, roles for PTEN/PI3K (8), Cyclin/CDK (9), RAS (5), IL7R (10) and NFκB (11) in Notch-dependent T-cell leukemia have been demonstrated.
The Wnt/β-Catenin (CTNNB1; catenin (cadherin-associated protein), beta 1, 88kDa) pathway is a prevalent modulator (either activating or repressing) of Notch-dependent cell decisions (12). Moreover, β-Catenin is a constitutive element of the cellular adherens junctions and, similarly to Notch, is involved in the integration of cell-to-cell signals. Under basal conditions, free β-Catenin is efficiently degraded by the proteasome downstream of the APC/AXIN/GSK3β destruction complex. In the presence of Wnt factors, the destruction complex is inhibited leading to the accumulation of β-Catenin, which translocates to the nucleus to activate specific gene transcription in association with the DNA-binding factors of the TCF/LEF family (13). Ectopic activation of β-Catenin in the T-cell compartment induces a leukemic phenotype (14), which is associated with an increased genomic instability (15).

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

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

Materials and Methods

Mouse models

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

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

**Cell transductions**

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

**Transplantation experiments**

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

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

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

Chromatin immunoprecipitation

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

Cell lines and drug treatments

All cell lines were cultured in standard conditions (RPMI-1640 + 10% FBS, Invitrogen). Inhibitors were resuspended in DMSO in various concentrations; PKF115-584 (a gift from Novartis) at 10nM – 1µM, DAPT (Calbiochem) at 5-50 µM, and Pyrvinium (Sigma) at 31nM - 1µM in different
experiments. Cell number and viability was assessed using DAPI (D1306, Invitrogen) by FACS (LSRII, BD).

Luciferase assays

T-ALL cell lines were electroporated with the 3′ myc reporter (N-Me) (29) plasmid at 250V, 1050µF with Gene Pulser® II (Bio-Rad) and treated with DMSO, 25µM DAPT, 310nM PKF115-584 or DAPT+PKF115-584, or, were untreated in the case of hygromycin selected shRNA-transduced T-ALL cells. DMSO/DAPT were added after electroporation and at 24h, medium was replaced with new DAPT and/or PKF115-584. Luciferase activity was measured after 48hrs (Luciferase Assay System, Promega). Values were normalized to β-Galactosidase (co-electroporation of CMV-β-Gal plasmid) or total protein (Bradford Assay).

Next generation sequencing analyses

Libraries created from polyA+ mRNA from 4 T-ALL cell lines (+DMSO or +PKF115-584), were sequenced on a Solexa HiSeq 2000 (Illumina) and yielded 20-35 million 50-nt single-end reads per sample. Reads were mapped to the human GRCh38.77 genome assembly with TopHat and RNASeq expression data were obtained using the Cufflinks suite.

Statistics

Data were plotted and statistics calculated using GraphPad Prism 6.07.

RESULTS

Transgenic Notch1-IC drives the expansion of LSKCD150-CD48+ leukemic initiating cells (LICs) in the fetal liver.

To study the early stages of Notch1-driven T-ALL, we set up a transgenic mouse model utilizing the Ef1a-Lox-Stop-Lox-Notch1IC (N1IC<sup>LSL</sup>) mouse line (25) crossed to VavCre-deleter mice (26). VavCre+N1IC<sup>LSL</sup> (here N1IC+) embryos were embryonic lethal after E14.5 (Supplementary Figure 1A). Analysis of E14.5 N1IC+ embryos revealed a decrease in FL cellularity compared to
VavCre+ control (WT) littermates (Supplementary Figure 1B), whereas the frequency as well as total number of phenotypically defined hematopoietic stem and progenitor cells (HS/PCs; Lineage-Sca1+ckit+ (LSK)) were significantly increased in N1IC+ FL (Figure 1A-B and Supplementary Figure 1C). The N1IC+ LSK population was predominantly CD48+CD150-, resembling bone marrow lineage-restricted HSCs (30), and showed decreased levels of CD150 (Figure 1A and Supplementary Figure 1D-E).

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

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

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

Since β-Catenin plays important roles in HSC generation and self-renewal as well as in many types of cancer, we sought to investigate the status of β-Catenin in the FL LSK population. 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+βCatfl/fl samples, ranging from ≈ 2-5% (termed Δ/fl) to < 0.1% (termed Δ/Δ) compared to control N1IC+βCatfl/+ samples (100%, termed Δ/+). (Figure 2D). We next performed transplantation experiments using 1000 (1k) or 5000 (5k) unfractionated FL cells from N1IC+βCat+, N1IC+βCat+/fl and N1IC+βCatΔΔ embryos and analyzed donor chimerism and DP cells at 3 weeks by FACS (Figure 2E). We found that N1IC+βCatΔ+ as well as N1IC+βCatΔ/fl but not N1IC+βCatΔΔ 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+βCatΔΔ; 1 in 2800 for N1IC+βCatΔfl and 1 in 6000 for N1IC+βCatΔΔ (Figure 2G). Moreover, whereas all N1IC+βCatΔ+ cell recipients succumbed to T-ALL within a period of 9 weeks, animals transplanted with N1IC+βCatΔfl and to a higher extent with N1IC+βCatΔΔ 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′βCatfl/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
transplanted with $\beta$Cat$^{+/+}$N1IC cells developed T-ALL with marked accumulation of blood DP cells within 3-4 weeks (Figure 3B-C), as previously described (3). In contrast, animals transplanted with $\beta$Cat$^{fl/fl}$+N1IC cells did not contain detectable levels of DP cells at any time point analyzed (Figure 3B-C) despite comparable engraftment levels (%GFP+) at 3-4 weeks (Figure 3D). In agreement with this, $\beta$Cat$^{+/+}$N1IC-derived chimerism increased between 3-4 and 6-8 weeks, while $\beta$Cat$^{fl/fl}$+N1IC-derived chimerism remained constant, similarly to that observed in the mock-infected ($\beta$Cat$^{fl/fl}$+pMIG) controls (Figure 3D). Importantly, animals receiving $\beta$Cat$^{fl/fl}$+N1IC cells survived for the duration of the study (>6months) whereas $\beta$Cat$^{+/+}$N1IC recipients invariably succumbed with a median survival of ≈9 weeks (Figure 3E).

Persistence of N1IC vector in the unfractionated and FACS-sorted CD4+CD8a+ DP thymic cell population from both $\beta$-Catenin genotypes was confirmed at the study endpoint (Supplementary Figure 3).

$\beta$-Catenin inhibitor PKF115-584 induces apoptosis and reduces proliferation in human T-ALL cells.

We next investigated the status of $\beta$-Catenin in human T-ALL. By western blot (WB) analysis we observed elevated levels of $\beta$-Catenin in T-ALL cell lines carrying cleaved Notch1 (N1IC), compared with the relatively low $\beta$-Catenin levels found in myeloid and B-cell leukemic lines (Supplementary Figure 4A). Further analysis of four T-ALL lines (RPMI8402, HPB-ALL, Jurkat, CCRF-CEM, referred to as 4T-ALL) showed cytoplasmic and nuclear accumulation of both N1IC and $\beta$-Catenin (Figure 4A), suggesting that both factors are active in T-ALL. To test whether $\beta$-Catenin was functionally required in human T-ALL, we utilized a small molecule, PKF115-584, previously shown to inhibit the transcriptional activity of $\beta$-Catenin (31). We found that PKF115-584 rapidly (16-24 hours of treatment) and permanently affected the cell number kinetics of all tested cell lines (Figure 4B and Supplementary Figure 4B), mainly as a result of increased cell death and reduced proliferation, as determined by AnnexinV/DAPI and Ki67 staining, respectively (Figure 4C-D). A comparable cytotoxic effect on T-ALL cells was observed after
treatment with pyrvinium, also known to inhibit β-Catenin activity in vivo (32) (Supplementary Figure 4C).

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

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

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

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

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

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

To test the possibility of using PKF115-584 to treat leukemia in vivo, we used our FL transplantation model. E14.5 FL cells from N1IC+ embryos were transplanted together with bone marrow competitor cells into lethally irradiated mice (Figure 6A). Recipient mice were
randomly grouped in three regimens: Animals treated with PKF115-584 by intraperitoneal injection every 2 days, i) for 6 weeks starting at day 3 post-transplantation (PKF0-6), ii) 3 weeks of treatment starting at week 3 (PKF3-6), or iii) animals were left untreated (Figure 6A). FACS analysis of PB at 3 weeks showed a significant reduction in donor chimerism (≈50%, p=0.003) in the cohort treated with PKF115-584 compared to untreated recipients (Figure 6B). In contrast, PKF115-584 had no significant effect on wild-type total leukocyte counts (WBC), indicating that the drug specifically targeted leukemic N1IC+ cells (Figure 6C). In support of the requirement for β-Catenin activity in leukemia initiation, mice in the PKF0-6 cohort displayed significantly improved overall survival, and a delay in leukemic death onset compared to untreated mice (Figure 6D). Finally, although not statistically significant (p=0.22), PKF115-584 treatment for 3 weeks (cohort PKF3-6) prevented death for ≥40 weeks of 22% of mice with confirmed initial CD4+CD8a+ DP leukemic burden (DP+) (Figure 6E and data not shown), in contrast with DP+ untreated recipients that invariably succumbed to T-ALL by 9 weeks.

In summary, these data nominate β-Catenin as a promising therapeutic target in T-ALL treatment.

Discussion

Despite increasing evidence for the roles of Notch and β-Catenin in tissue maintenance, stem cell self-renewal and cancer, separate findings from different groups yield seemingly contradictory results, with Wnt and Notch acting synergistically or antagonistically depending on cellular/tissue context (reviewed in (37)). In this study, we found an essential crosstalk between Notch1 and β-Catenin in T-ALL through direct regulation of MYC, consistent with the association of these three proteins with leukemic initiating cell (LIC) activity in T-ALL (20-22). In addition, mutations in other pathways such as PTEN may determine the levels of β-Catenin activity (23), which would affect Notch-induced leukemogenic potential as shown in our murine model, although we did not observe any correlation between PTEN mutations in T-ALL cell lines and total β-catenin levels. However, more work should be done to specifically address this issue and understand the relevance of PTEN in this connection.
The Wnt pathway has long been associated to T-cell development and T-cell leukemia (14, 38-40); however, the specific role of particular Wnt-pathway family members remains highly controversial. For example, LEF1 and TCF7 (also known as TCF-1) nuclear factors regulate T-cell differentiation but they can either act as leukemia/lymphoma inducers or suppressors in a context-dependent manner. Published data demonstrated that TCF7 acts as tumor suppressor by inhibiting LEF1, but in turn inactivating LEF1 mutations were also found to result in T-ALL associated with MYC gene upregulation (38), with Notch1 activity being crucial for the leukemic process driven by either TCF7 or LEF1 inactivation (39, 40). Whether leukemia development requires β-Catenin in these LEF/TCF deficient systems remains unknown. Our analysis of the MYC regulatory regions revealed that both Notch1 and β-Catenin associate in the same region of 3' MYC enhancer, containing three well-defined RBPj consensus sites, whereas binding was not observed for either factor in the MYC promoter containing a TCF/LEF binding motif. The fact that β-Catenin physically interacted with cleaved Notch1 in T-ALL cells by co-immunoprecipitation under conditions where DNA is not detected supports the possibility that β-Catenin binds DNA through an RBPj/Notch1 complex. In agreement with this, we found that both proteins positively regulate the 3' MYC enhancer, and MYC mRNA levels as well as MYC targets were significantly reduced after PKF115-584 treatment in T-ALL cells. Further work comparing the DNA binding activity of β-Catenin and Notch1 in a genome-wide approach will help to elucidate the specific contribution of β-Catenin to Notch1-dependent gene transcription.

Consistent with a prominent role of β-Catenin in leukemogenesis, we have here shown that PKF115-584 efficiently inhibited cell growth and induced apoptosis in T-ALL cells. In fact, in our assay, PKF115-584 was a more potent inhibitor of MYC enhancer-dependent transcription than the γ-secretase inhibitor DAPT. This was dependent on dose and time of incubation, although we cannot exclude that PKF115-584 is targeting other proteins than just β-Catenin. In this sense, our in vivo genetic studies, as well as our in vitro depletion of β-Catenin by a single shRNA previously shown to efficiently inhibit β-Catenin (28), confirm that T-ALL cells strongly rely on β-Catenin for MYC expression and survival. In vivo, mice transplanted with Notch1-active T-ALL progenitors (LICs) that received PKF115-584 treatment 3 days after the initiation
of the experiment showed increased survival compared with control leukemic animals. However, mice treated from week 3 after transplantation did not show a significantly different survival, but 22% of the animals carrying double CD4+CD8a+ double positive cells at week 3 post-transplantation remained alive after 40 weeks, compared with 100% lethality in the control group by week 9. These results suggest that PKF115-584 was affecting not only leukemia initiation, as recently shown for β-catenin (22), but also maintenance, which is relevant from a therapeutic point of view. Most importantly, normal hematopoietic cells were refractory to the in vivo PKF115-584 treatment indicating a differential sensitivity of leukemic and normal cells towards β-Catenin inhibition.

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

Author Contributions

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

Acknowledgements:

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


**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^L/L_ (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^Δ/+ (n=9), N1IC+βCat^Δ/fl; (n=14), N1IC+βCat^Δ/Δ; (n=3). Statistical analysis with the indicated test is shown: * p<0.05, ** p<0.01.
Figure 3. Total β-Catenin deletion in adult BM cells inhibits Notch-induced T-ALL in vivo.

(A) Transplantation protocol for VavCre+βCat+/+ or βCatfl/fl lin- BM cells transduced with GFP control (pMIG) or N1IC-IRES-GFP (N1IC) retroviruses. (B) Representative FACS plots of PB at 3 weeks. (C) Lineage distribution for CD4+, CD8a+, double positive (DP) or double negative (Non T-cells) populations in whole blood (top) or donor-derived (GFP+) fraction (bottom) at 6-8 weeks. (D) Donor chimerism (%GFP+) at 3-4 and 6-8 weeks after transplantation. Bars indicate mean and standard error (SEM) and dots show individual recipients. (E) Survival curve with median survival in weeks (wks).

Figure 4. Inhibition of β-Catenin activity by PKF115-584 compromises the survival and proliferation of human T-ALL. (A) WB of cytoplasmic and nuclear fractions of four human T-ALL cell lines for cleaved Notch1 (N1IC) and β-Catenin. Lamin B and tubulin are used as purity and loading controls. (B) Dose response assays after single treatment with the indicated doses of PKF115-584. FACS counting of DAPI- cells normalized to DMSO control. Mean and SEM of three independent experiments are represented. (C) AnnexinV binding and DAPI staining after 24h of PKF115-584 treatment (310nM). (D) Overlay FACS plots for Ki67 levels after 24h treatment as indicated.

Figure 5. MYC is a direct target of Notch and β-catenin in T-ALL. (A) Schematic representation of RNA-sequencing experiments. (B) GSEA analysis for MSigDB Hallmark gene sets using up- or downregulated (DN) genes after PKF115-584 treatment. (C) GSEA plots of selected top enriched gene sets. (D) Validation by Q-PCR in PKF115-584 treated RPMI8402 cells for selected genes. (E) Integrative Genomics Viewer (IGV) visualization of RNA-Seq tracks for MYC and HES1 genes in DMSO (grey) or PKF115-584 treated (310nM; 3hrs, red) T-ALL lines. Values indicate normalized tag count (expression level) and Y-axes are set to the highest value within each cell line DMSO/PKF pair. (F) Q-PCR for the indicated genes in T-ALL cell lines transduced with shCtrl or shβCat, 1-2 days after completion of hygromycin selection (n=3, except HPB-ALL n=1). (G) Representative western blot analysis of the indicated proteins from...
shRNA-transduced RPMI8402 1 day after completion of hygromycin selection. (H) ChIP with the indicated antibodies analyzed by QPCR with MYC enhancer (Enh.1-2, flanking three RPBj motifs) and MYC promoter (Prom.; flanking a TCF/LEF motif) primers. (I) Co-immunoprecipitation experiment on Jurkat cells for β-Catenin and N1IC. (J) Luciferase assay for Myc enhancer activity on transfected Jurkat cells treated with the indicated inhibitors (n≥3) (left); or in shRNA transduced RPMI8402 cells (n=2) (right). Statistical significance is calculated by unpaired t-test, *; p<0.05, **; p<0.01, ***; p<0.001.

Figure 6. PKF115-584 treatment specifically inhibits the survival of T-ALL leukemic cells in vivo. (A) Schema for in vivo PKF treatment assay. (B) Donor (CD45.2+) chimerism determined by FACS in recipient PB at 3 wks. T-test, **; p<0.01. (C) Total donor (CD45.2+) and competitor (CD45.1+) derived leukocyte counts in total PB. NE indicates not engrafted above threshold (WBC<0.01*10^{-9}/L). **; p<0.01 by unpaired t-test. (D and E) Survival curves for cohorts untreated or treated with PKF for 6 (D) or 3 (E) weeks. In E only mice with confirmed leukemic burden (≥1% CD4+CD8a+ DP cells in PB at 3 weeks) are included. Data are from three independent experiments. *; p=0.0146 by Log-Rank (Mantel Cox) Test and p=0.0059 by Gehan-Breslow-Wilcoxon test.
Figure 2

A. IgG ctrl vs α-βCatenin

B. WT vs N1IC+

C. Number of LSK per 5x FL cells, E14.5

D. Normalized β-Catenin mRNA

E. E14.5 Fetal Liver

F. BM Competitor (WT)

G. Pearson

H. 1k Cells Transplanted
Figure 3

A

B

C

D

E

Donor Chimerism (GFP+)

in peripheral blood, %

weeks after transplantation:

βCat\textsuperscript{++}+N1IC

βCat\textsuperscript{+/−}+N1IC

βCat\textsuperscript{−/−}+N1IC

βCat\textsuperscript{−/−}+pMIG

Survival

% within Donor (GFP+)

βCat\textsuperscript{++}+pMIG

βCat\textsuperscript{+/−}+N1IC

βCat\textsuperscript{−/−}+pMIG

βCat\textsuperscript{−/−}+N1IC

CD4+

CD8a+

Non T-cells

DP

100%

80%

60%

40%

20%

0%
Figure 4

A

B

C

D
Figure 6

A

B

C

D

E

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**Footnotes and References**

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**Image Descriptions**

A: Diagram showing the experimental setup.

B: Graph showing the percentage of donor chimerism in blood at 3 weeks.

C: Graph comparing WBC counts between WT donors and competitors.

D: Survival curve showing the effect of treatment over weeks after transplantation.

E: Survival curve comparing untreated versus treated conditions.

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**Legend**

- **BM Competitor**
- **CD34.1**
- **Unr.**
- **+PKF**
- **N11C+**
- **WT**
- **vscCre**
- ***; p = 0.0146**
- **8/17 (47%)**
- **2/13 (15%)**
- **0/11 (0%)**
- **p=0.22**