

Interleukin-3-mediated regulation of β -catenin in myeloid transformation and acute myeloid leukemia

Teresa Sadras,^{*,†,‡,§,1} Michelle Perugini,^{*,†,||,1} Chung H. Kok,^{*,†,||} Diana G. Iarossi,^{*,†,§}
Susan L. Heatley,^{*,||} Gabriela Brumatti,[#] Michael S. Samuel,^{*,†,||} Luen B. To,^{*,||}
Ian D. Lewis,^{*,†,||} Angel F. Lopez,^{*,||,||} Paul G. Ekert,[#] Hayley S. Ramshaw,^{*,||}
and Richard J. D'Andrea^{*,†,‡,§,||,2}

*Centre for Cancer Biology and Departments of [†]Haematology and [‡]Immunology, SA Pathology, Adelaide, South Australia, Australia; [§]School of Molecular and Biomedical Science and Centre for Stem Cell Research and ^{||}School of Medicine, The University of Adelaide, Adelaide, South Australia, Australia; [#]Division of Cell Signalling and Cell Death, Walter and Eliza Hall Institute, Parkville, Victoria, Australia; and [§]Department of Pharmacy and Medical Sciences, The University of South Australia, Adelaide, South Australia, Australia

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ABSTRACT

Aberrant activation of β -catenin is a common event in AML and is an independent predictor of poor prognosis. Although increased β -catenin signaling in AML has been associated with oncogenic translocation products and activating mutations in the FLT3R, the mechanisms that activate β -catenin in AML more broadly are still unclear. Here, we describe a novel link between IL-3 signaling and the regulation of β -catenin in myeloid transformation and AML. In a murine model of HoxB8 and IL-3 cooperation, we show that β -catenin protein levels are modulated by IL-3 and that Cre-induced deletion of β -catenin abolishes IL-3-dependent growth and colony formation. In IL-3-dependent leukemic TF-1.8 cells, we observed increased β -catenin protein levels and nuclear localization in response to IL-3, and this correlated with transcriptional induction of β -catenin target genes. Furthermore, IL-3 promoted β -catenin accumulation in a subset of AML patient samples, and gene-

expression profiling of these cells revealed induction of WNT/ β -catenin and TCF4 gene signatures in an IL-3-dependent manner. This study is the first to link β -catenin activation to IL-3 and suggests that targeting IL-3 signaling may be an effective approach for the inhibition of β -catenin activity in some patients with AML. *J. Leukoc. Biol.* **96**: 83–91; 2014.

Introduction

AML is a heterogeneous disease with large variability in the genetic and molecular events underlying its development and progression, as well as significant variations in prognosis and response to therapy. Ultimately, the molecular, cytogenetic, and/or epigenetic lesions, which arise during the leukemic transformation, cooperate to drive the increased proliferation and survival and blocked differentiation, which is characteristic of AML blasts. Whereas significant progress has been made into the classification and pathology of AML, the overall 5-year survival rates remain considerably low (40–50%) and as low as 10–20% for some subtypes [1]. A more-thorough understanding of the molecular mechanisms underlying myeloid leukemogenesis is critical for the development of novel, targeted therapies for the treatment of AML.

Aberrant activation of the β -catenin signaling pathway occurs in a large proportion of AML patients, is associated with increased blast clonogenicity, and has been documented as an independent predictor of poor prognosis [2–4]. The stabilization and activation of β -catenin in AML have been associated previously with activating ITD mutations in the FLT3R and oncogenic chromosomal translocations, including promyelocytic

Abbreviations: β c= β -chain, ChEA=chromatin immunoprecipitation enrichment analysis, ChIP=chromatin immunoprecipitation, CISH=cytokine-inducible Src homology 2-containing protein, FANCC=Fanconi anemia, complementation group C, FDM=factor-dependent myeloid cell, FDR=false discovery rate, FLT3=FMS-like tyrosine kinase 3, FRA-1=Fos-related antigen 1, GMP=granulocyte macrophage progenitor, GSEA= gene-set enrichment analysis, h=human, HEK=human embryonic kidney, Hox=homeobox, HSC=hematopoietic stem cell, ITD=internal tandem duplication, LJMMA=linear model for microarray analysis, LSC=leukemic stem cell, m=murine, Meis1=Meis-homeobox 1, MIG=murine stem cell virus-IRES-GFP, MIG- β catS33A=murine stem cell virus-IRES-GFP- β -catenin-S33A, MIG-Cre=murine stem cell virus-IRES-GFP-tagged Cre retrovirus, MLL=mixed-lineage leukemia, MSigDB=Molecular Signature Database, MYC=myelocytomatosis viral oncogene, QPCR=quantitative PCR, PIM1/2=proviral integrations of Moloney virus 1/2, PTGS2=PG-endoperoxide synthase 2, TCF4=T-cell factor 4, UPP1=uridine phosphorylase 1, WNT=wingless

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1. These authors contributed equally to this work.

2. Correspondence: Dept. of Haematology, Centre for Cancer Biology and SA Pathology, Frome Rd., Adelaide, South Australia, Australia 5000. E-mail: richard.dandrea@unisa.edu.au

leukemia-retinoic acid receptor α and AML with t(8; 21)(q22; q22) [5, 6]. In addition, abnormal promoter methylation of specific WNT/ β -catenin inhibitors has been reported in myeloid malignancies [7, 8]. It is likely that several pathways contribute to the accumulation of β -catenin in AML, and identification of additional mechanisms may be beneficial for targeted inhibition of β -catenin in AML, particularly in the absence of these other lesions.

Accumulating evidence indicates that aberrant activation of β -catenin also occurs in the highly specialized LSC population [9–12]. In vivo, β -catenin has been shown to be critical for the maintenance and drug-resistant properties of AML LSC in patients with MLL translocations, a subtype of AML that is characterized by increased expression of HOX genes and a particularly poor prognosis [11, 12]. In addition, β -catenin is required for the leukemic transformation of GMP by Meis1/HoxA9 or MLL-AF9, further supporting a pivotal function for this oncogene in the development of selected subtypes of AML.

Another defining feature of AML LSC is the increased expression of the α subunit of the IL-3R relative to normal HSCs [13, 14]. The overexpression of IL-3R α in AML has been associated with higher blast counts at diagnosis, lower complete remission rate, and reduced overall survival [14]. Several approaches to target IL-3R α , including neutralizing mAb and diphtheria-toxin fusions, have been developed and are currently being trialed for the treatment of AML [15–18]. IL-3R comprises the ligand-specific α -chain and a common β c, which is shared with the receptors for GM-CSF and IL-5. IL-3R α binds IL-3 with high specificity but with low affinity, and the interaction of IL-3R α with β c results in a high-affinity receptor complex with subsequent activation of intracellular signaling cascades, including activation of the JAK/STAT, MAPK and PI3K/AKT signaling pathways (reviewed in ref. [19]). Whereas activities of IL-3 overlap considerably with those of GM-CSF, IL-3 has the unique property of acting as a proliferation and/or survival factor in HSCs and has been reported to promote self-renewal [20, 21]. Importantly, retained responsiveness to IL-3 and other hematopoietic cytokines is observed in leukemic cells, and rIL-3 can promote the growth and clonogenicity of >80% of AMLs in vitro [22–24]. In vivo, enforced expression of IL-3 in hematopoietic cells induces a myeloproliferative disorder in mice and results in an aggressive, transplantable myeloid leukemia when coexpressed with HoxB8 [25]. Critically, given the recent link between Hox genes and β -catenin, we wished to explore the possibility that IL-3 may act, in part, through the induction of the β -catenin pathway.

In this study, we demonstrate a link between IL-3 signaling and β -catenin levels and activity in Hox-transformed myeloid cells, leukemic cells, and primary AML patient samples. Critically, given the important role of these factors in leukemogenesis, our findings suggest that targeting β -catenin through inhibition of IL-3 signaling may be an effective approach for disrupting core transformation events in a subset of AML patients.

MATERIALS AND METHODS

Cell lines and AML patient samples

FDM cells were maintained in DMEM (1000 mg/L glucose) with 10% FBS and 10 ng/mL mIL-3. TF-1.8 cells were maintained in RPMI-1640 medium with 10% FBS and 2 ng/mL hIL-3. Primary AML apheresis samples were collected with informed consent with approval from the relevant ethics committees, in accordance with the Declaration of Helsinki. Mononuclear cells, isolated from all samples, were cryopreserved for storage. Cells were thawed for use and recovered overnight in IMDM with 20% FBS and DNase (50 U/mL). AML cells were then cultured in IMDM with 10% FBS and stimulated with IL-3, as described in Results and Discussion.

Production of retrovirus and transduction of hematopoietic cells

The DNA constructs MIG, MIG-Cre, and MIG- β catS33A were transfected into HEK293T cells together with the ecotropic pEQ packaging plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). HEK293T medium was replaced 24 h post-transfection with complete medium relevant to FDM cells for collection of viral supernatant, which was harvested at 48 h and 72 h post-transfection. FDM cells were transduced with viral supernatant, supplemented with 4 μ g/mL polybrene/ 1×10^6 cells via spinoculation. Cells expressing GFP were sorted using a BD FACSAria flow cytometer/cell sorter.

Western immunoblot

Cells were lysed for 30 min on ice in modified RIPA lysis buffer (1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate, 100 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 50 mM Hepes, pH 7.4), containing protease and phosphatase inhibitors. For nuclear and cytosolic fractionation, lysates were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Rockford, IL, USA), according to the manufacturer's instructions. Forty–100 μ g of each sample was separated using 8% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti- β -catenin (BD Biosciences, San Jose, CA, USA), anti-actin (Sigma-Aldrich, St. Louis, MO, USA), anti-heat shock protein 90 (Cell Signaling Technology, Beverly, MA, USA), anti-histone H3 (Abcam, Cambridge, UK), or antiphospho-Y694/699-Stat5a/b (Millipore, Billerica, MA, USA). Antibody binding was detected with corresponding HRP-conjugated secondary antibody (Pierce, Rockford, IL, USA), followed by chemiluminescence detection and visualized using an ImageQuant LAS 4000 digital imaging system (GE Healthcare, Pittsburgh, PA, USA).

Cellular assays

Proliferation assay. Cells were seeded in triplicate in a 96-well plate for each time-point. Readings were taken every 24 h for a total of 4 days. At each time-point, 25 μ l CellTiter 96 Aqueous One Solution (Promega, Madison, WI, USA) was added to each well and the plates incubated at 37°C for 4 h. Plates were read using the Opsys MR (Dynex Technologies, Chantilly, VA, USA) plate reader with a wavelength of 490 nm.

³H-Thymidine incorporation assay. AML mononuclear cells were seeded in triplicate in 96-well plates in IMDM + 10% FBS with a titration of IL-3 for 48 h at 37°C. ³H-Thymidine was added for the final 24 h at a concentration of 0.5 μ Ci. Cells were deposited onto glass fiber paper and counted using a Top Count (PerkinElmer, Waltham, MA, USA).

Colony-formation assay. Equal numbers of FDM or bone marrow cells, transduced with MIG-Cre or the MIG control, were seeded in semisolid methylcellulose (Stemcell Technologies, Vancouver, BC, Canada) with 10 ng/mL mIL-3, 3 days after sorting for GFP. Colonies were counted in triplicate plates after 7 days in methylcellulose culture at 37°C.

Cell viability. Cells were diluted 1:1 with trypan blue (Invitrogen), and viable and nonviable cells were enumerated based on trypan blue exclusion.

Immunofluorescence staining

Cells (5×10^4) were cytospun onto glass microscope slides and fixed with 1 C fixation buffer (eBioscience, San Diego, CA, USA). Cells were permeabil-

ized with $1\times$ permeabilization buffer (eBioscience). Anti- β -catenin (BD Biosciences) was used at 1:500 in $1\times$ permeabilization buffer overnight at 4°C . Slides were washed and subsequently incubated with 1:100 goat anti-mouse Alexa Fluor 488 (Invitrogen) for 1 h at room temperature. Washed slides were mounted with ProLong Gold reagent containing DAPI (Invitrogen) and viewed/photographed using a Bio-Rad Radiance 2100 confocal microscope with LaserSharp software version 5.

Real-time QPCR

RNA was purified using the Qiagen miRNeasy mini kit. Reverse transcription was performed using QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. QPCR reactions were carried out using the Roche FastStart Taq system (Roche Applied Systems, Indianapolis, IN, USA). Primer sequences are available upon request.

Microarray of primary AML patient samples

Total RNA was extracted using Qiagen miRNeasy kit. Gene-expression analysis was performed using Affymetrix Human Gene 1.0 ST arrays. The raw data were normalized using RMA, as implemented in the *aroma.affymetrix* software package (Affymetrix, Santa Clara, CA, USA). Affymetrix expression raw data are available from the Gene Expression Omnibus under Accession Number GSE51402.

Statistical analysis of microarray and experimental data

The Empirical Bayes and moderated *t*-statistic LIMMA were performed to determine differential gene expression induced by IL-3. Differential gene expression was adjusted for multiple testing to control the FDR using the Benjamini-Hochberg method. Instances of differential gene expression ex-

hibiting a FDR of $P < 0.05$ were considered statistically significant. All statistical analyses were performed using R statistical software. Probability and statistical significance of experimental data were analyzed using the two-tailed Student's *t*-test with a confidence interval of 95% ($P < 0.05$) or 99% ($P < 0.01$). Error bars represent the SEM.

GSEA and ChEA

GSEA (Broad Institute, Cambridge, MA, USA; <http://www.broadinstitute.org/gsea/index.jsp>), as described in ref. [26], was performed against the Broad Institute MSigDB (v4.0) to determine statistically significant enrichment between IL-3 differential gene expression and curated gene sets. GSEA was performed on preranked list, based on LIMMA *t*-statistics. Gene sets were ranked on their Normalized Enrichment Score and claimed as significant if exhibiting FDR $P \leq 0.05$. ChEA (graphical user interface version) was performed using significantly differentially expressed genes ($P \leq 0.05$) with fold change > 1.5 against data from 197 TCFs contained in a database derived from 212 publications.

RESULTS AND DISCUSSION

IL-3 mediates stabilization of β -catenin in a myeloid model of Hox transformation

We used a previously described IL-3-dependent model of myeloid transformation to investigate the link between IL-3 signaling and the activation of β -catenin in hematopoietic cells. For this purpose, FDM cell lines were generated by the transduction of E14.5 murine fetal liver progenitors with HoxB8

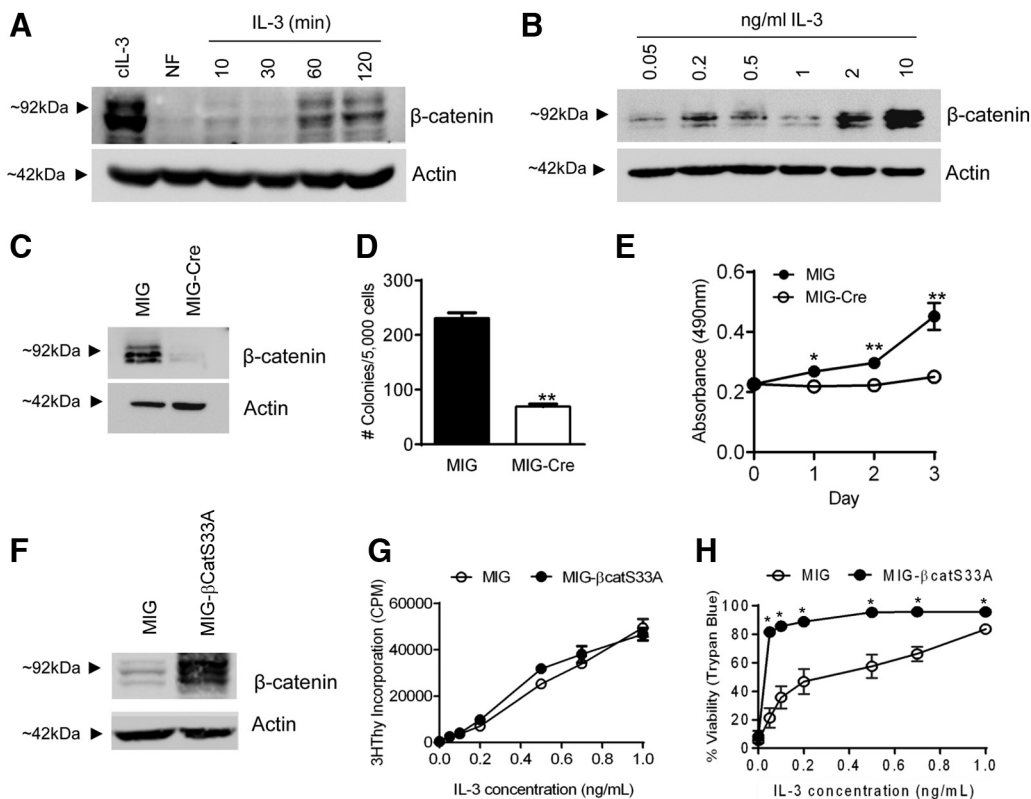


Figure 1. Modulation of β -catenin levels by IL-3 in Hox-transformed myeloid cells. (A) FDM cells were grown continuously in IL-3 (cIL-3), starved of IL-3 for 16 h [no factor (NF)], or starved and then stimulated with 10 ng/mL mIL-3 for 10, 30, 60, or 120 min. Lysates were analyzed by Western blotting for β -catenin protein levels. (B) FDM cells were cultured in a titration of mIL-3 for 72 h, and lysates were analyzed by Western blotting for β -catenin protein levels. (C) Western blot analysis of β -catenin in FDM cells derived from C57BL/6 mice containing a floxed β -catenin gene retrovirally transduced with the MIG vector control or MIG-Cre vectors to assess deletion of β -catenin. FDM cells transduced with MIG or MIG-Cre were analyzed for (D) colony formation in semisolid methylcellulose in the presence of 10 ng/mL mIL-3, 7 days after seeding, and (E) cell growth in liquid culture in the presence of mIL-3 over 3 consecutive days. (F) Overexpression of β -catenin was confirmed by Western blotting in FDM MIG- β -catS33A

cells. FDM MIG- β -catS33A and MIG cells were washed in PBS three times to remove traces of factor and cultured in a range of mIL-3 concentrations for 3 days. Cells were then analyzed for (G) proliferation by ^3H -thymidine (3HThy) incorporation at a concentration of $0.5 \mu\text{Ci}/\text{well}$; radioactive uptake was measured on a scintillation counter, 16 h after the addition of 3HThy. Percentage viability was determined using trypan blue exclusion (H). Error bars represent SEM ($n=3$). $*P \leq 0.05$; $**P \leq 0.01$.

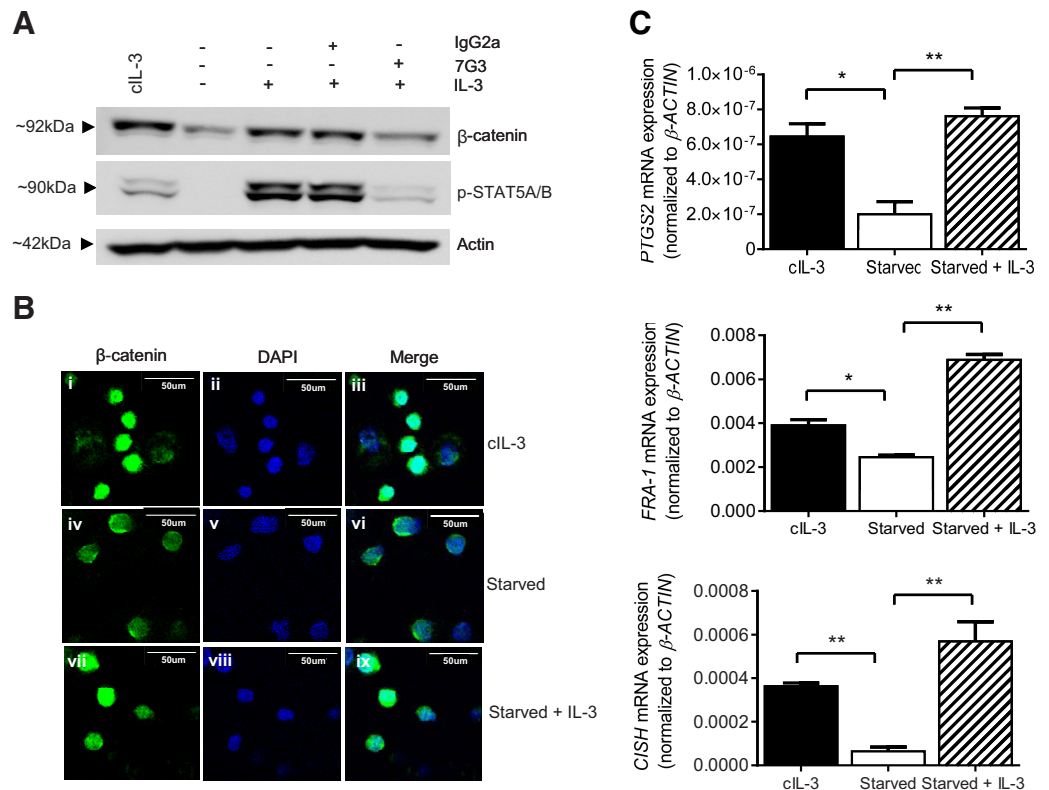
retrovirus in the presence of saturating levels of IL-3 (as described in ref. [27]). Withdrawal of IL-3 from FDM cells for 16 h resulted in a reduction of total β -catenin protein levels, and restoration of IL-3 caused a time-dependent accumulation of β -catenin (Fig. 1A). The accumulation of β -catenin was also dependent on the dose of IL-3 (Fig. 1B). Given that β -catenin and IL-3 are key mediators of growth and survival, we next investigated whether there was a functional requirement for β -catenin in IL-3 regulation of cell growth and survival in FDM cells. For this, we generated FDM cell lines from C57BL/6 mice containing a floxed β -catenin gene, allowing inducible ablation of β -catenin expression following transduction with a MIG-Cre (Fig. 1C). β -Catenin deficiency led to a 70% reduction in colony-forming potential (Fig. 1D) and blocked IL-3-mediated cell expansion in liquid culture (Fig. 1E), suggesting that there is a nonredundant requirement for β -catenin in the IL-3-mediated growth response. Colony formation was also reduced significantly following MIG-Cre expression in an independent pool of β -catenin^{flox/flox} FDM cells (Supplemental Fig. 1A) and in primary mouse bone marrow cells from β -catenin^{flox/flox} adult mice (Supplemental Fig. 1B). The marked reduction in growth upon β -catenin deletion was associated with a small but significant increase in cells in the G0/G1-phase of the cell cycle and a concomitant decrease in cells in S-phase (Supplemental Fig. 1C). We did not observe any significant changes in cell viability (Supplemental Fig. 1D) or

altered expression of leukocyte differentiation antigens (Gr-1, F4/80, or Ter119; Supplemental Fig. 1E).

To complement this gene ablation approach, we used retroviral transduction to generate FDM populations expressing a constitutively active form of β -catenin (or GFP-vector control). The constitutively active mutant β -catenin, β -CatS33A, contains 4-aa substitution mutations (S33A, S37A, T41A, and S45A) that prevent its phosphorylation by casein kinase-1 and GSK3 β and its subsequent proteasome-mediated degradation [28, 29]. FDM cells transduced with an expression vector encoding constitutively active β -catenin (MIG- β -catS33A) were expanded in 10 ng/mL mIL-3 and the elevated expression of β -catenin confirmed by immunoblotting (Fig. 1F). Surprisingly, cells expressing MIG- β -catS33A showed no difference in proliferation after 72 h in a range of IL-3 concentrations when compared with cells transduced with the GFP vector control (MIG; Fig. 1G), suggesting that whereas β -catenin is required for IL-3-mediated growth of FDM cells, the endogenous level of β -catenin in these cells may be sufficient for maximal cell growth in the conditions studied here. Of interest, however, there was a significant increase in viability of the β -catS33A-expressing FDM cells at low concentrations of IL-3 after 72 h when compared with cells containing the MIG vector alone (Fig. 1H), suggesting that β -catenin may be amplifying an IL-3 signal at suboptimal cytokine concentrations.

Figure 2. IL-3 promotes β -catenin stabilization in TF-1.8 leukemic cells.

(A) Cell lysates were prepared from TF-1.8 cells grown continuously for 16 h \pm 2 ng/mL hIL-3 (cIL-3), starved for 16 h, or starved and then stimulated with 10 ng/mL hIL-3 for 6 h \pm a 20-min pretreatment with 100 nM of the IL-3-neutralizing antibody 7G3 (or IgG2a isotype control; BM4). Protein expression of β -catenin and phospho-STAT5A/B (p-STAT5A/B; Y694/699) were determined by Western blotting. (B) TF-1.8 cells grown in cIL-3, starved for 16 h, or starved and then stimulated with 10 ng/mL hIL-3 for 1 h were stained with an anti- β -catenin mAb, followed by an Alexa 488 secondary antibody (green, i, iv, and vii) and mounted with anti-fade fluorescence medium containing DAPI (blue, ii, v, and viii). Overlay figures are shown (iii, vi, and ix). Slides were viewed using the Bio-Rad Radiance 2100 confocal microscope. (C) Expression of β -catenin target genes *PTGS2* and *FRA-1* were measured in RNA, prepared from TF-1.8 cells grown for 16 h \pm 2 ng/mL hIL-3 or restimulated with 10 ng/mL hIL-3 for 6 h by QPCR and normalized to β -actin expression. mRNA expression of the known IL-3 target gene *CISH* was measured as a control. Error bars represent SEM ($n=2$). * $P \leq 0.05$; ** $P \leq 0.01$.



IL-3 mediates stabilization of β -catenin in TF1.8 cells and in primary AML patient samples

We next investigated whether IL-3 modulates β -catenin levels and subcellular localization in human leukemic cells using the IL-3-dependent erythroleukemic cell line, TF-1.8 [30]. Basal β -catenin levels were reduced in TF-1.8 cells following IL-3 withdrawal for 16 h in low serum and increased again following restoration of IL-3 for 6 h (Fig. 2A). This increase in β -catenin was blocked by pretreatment of starved cells with the IL-3R-neutralizing mAb 7G3 [31] (Fig. 2A). Phosphorylation of STAT5A/B (Y694/699) was also induced by IL-3 stimulation and blocked by 7G3 treatment, consistent with the well-described activation of STAT5 downstream of IL-3 signaling [32] (Fig. 2A). To determine the localization of β -catenin following IL-3 treatment, we performed immunofluorescence analysis of

TF-1.8 cells cultured continuously in IL-3, following IL-3 withdrawal and IL-3 restoration for 1 h. As shown in Fig. 2B and Supplemental Fig. 2A, β -catenin was predominantly localized in the nucleus of TF-1.8 cells cultured continuously in IL-3. Withdrawal of IL-3 for 16 h in low serum resulted in cytoplasmic localization of β -catenin, which then redistributed into the nucleus upon readdition of IL-3. Consistent with this, Western blot analysis of fractionated nuclear and cytoplasmic lysates showed a reduction in nuclear β -catenin expression in the absence of IL-3 compared with cells grown continuously in IL-3 or starved and stimulated with cytokine (Supplemental Fig. 2B).

We also observed increased mRNA levels of the β -catenin target genes *FRA-1* and *PTGS2*, as well as a well-established IL-3 target gene, *CISH*, in TF-1.8 cells continuously maintained in IL-3 and also following IL-3 stimulation compared with cells

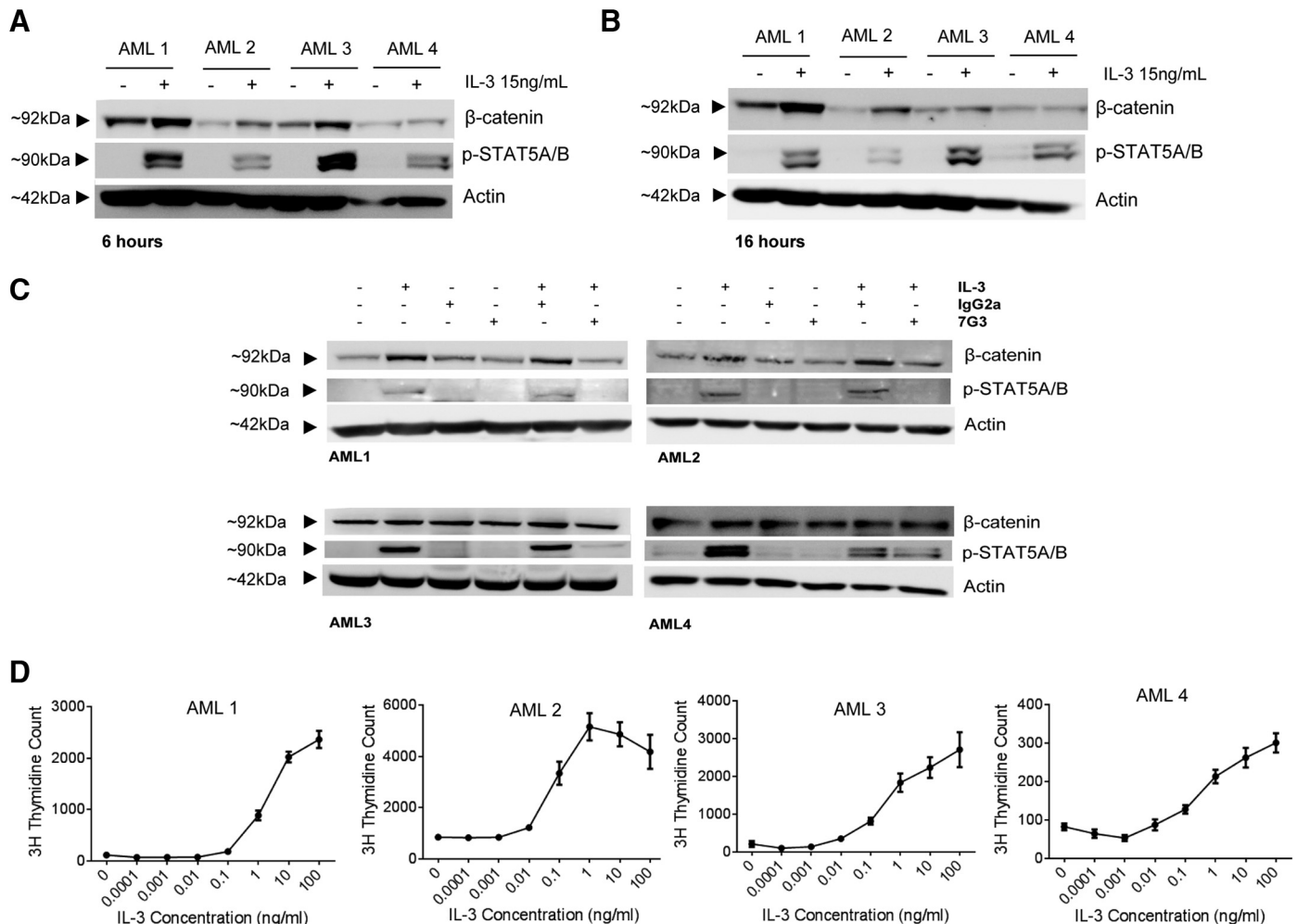


Figure 3. IL-3 regulates β -catenin levels in primary AML cells. Mononuclear cells from four AML patient samples (AML1–4) were thawed and cultured overnight in high serum media (IMDM+20% FBS). Cells were purified by Ficoll centrifugation and recultured in IMDM + 10% FBS \pm 15 ng/mL hIL-3 for (A) 6 h or (B) 16 h. Phospho-STAT5A/B (Y694/699) and β -catenin protein levels were determined by Western blotting. (C) Mononuclear cells from AML1–4 were treated \pm 100 nM of the IL-3-neutralizing antibody 7G3 or an IgG2a isotype control (Clone BM4) for 20 min on ice. Samples were subsequently cultured \pm 15 ng/mL hIL-3 for 3 h at 37°C. Cell lysates were analyzed for β -catenin and phospho-STAT5A/B (Tyr694/699) expression by Western blotting. (D) Mononuclear cells from AML patient samples 1–4 were incubated in IMDM + 10% heat-inactivated FBS with a titration of IL-3 for 48 h at 37°C. Proliferation was measured by the addition of ^3H -thymidine for the final 24 h at a concentration of 0.5 μCi /well. Data shown represent the mean \pm SEM of six replicates/patient.

starved of IL-3 (Fig. 2C). There was no significant change in expression of a non- β -catenin target gene, *PTGS1* (Supplemental Fig. 2C). The β -catenin targets were selected from the publicly available compilation of verified β -catenin targets (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes) and were confirmed to be expressed in TF-1.8 cells in the microarray analysis performed in our laboratory (unpublished data). Taken together, these results are consistent with activation of β -catenin-mediated transcription by IL-3 in leukemic cells.

Next, to determine whether IL-3 can induce the stabilization of β -catenin in primary AML cells, we analyzed the response of four AML patient apheresis samples cultured in the presence or absence of IL-3. Patient characteristics are summarized in

Supplemental Table 1. All patient samples showed induction of STAT5A/B tyrosine phosphorylation (Y694/699) following culture with IL-3 for 6 h or 16 h, consistent with activation of canonical IL-3 signaling (Fig. 3A and B). Importantly, in patient samples AML1, AML2, and AML3, we also observed increased β -catenin protein levels following treatment with IL-3 (Fig. 3A and B). Despite variability in the levels of basal β -catenin expression, the IL-3-induced change in β -catenin was reduced by pretreatment with 7G3 in these patients (Fig. 3C). These results are supportive of our cell line data and demonstrate that β -catenin levels can be induced by IL-3 stimulation in at least one subset of AML patients. We did not observe any changes in β -catenin levels in response to IL-3 in AML4, despite detectable stimulation of phospho-STAT5A/B. Of inter-

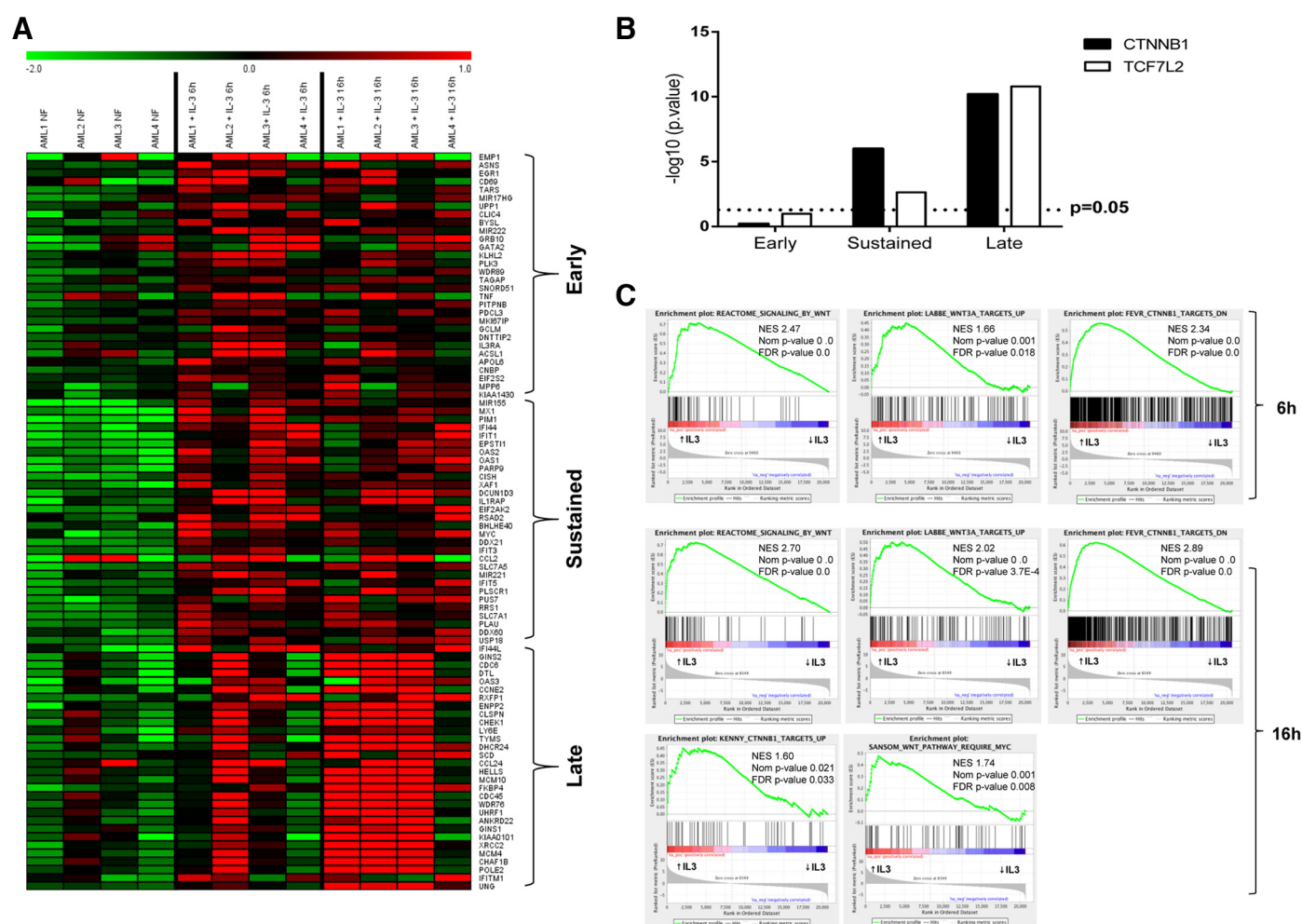


Figure 4. Gene-expression changes reveal enrichment of β -catenin target genes and gene signatures upon IL-3 treatment of AML patient cells. (A) Differential gene expression induced by IL-3 in AML1–4 was determined using a paired analysis of four primary AML patient samples, and significant genes were classified as Early (only modulated at 6 h), Sustained (modulated at both 6 h and 16 h), and Late (only modulated at 16 h) genes. Represented in the heat map are the top 30 genes from each category, displaying significant expression differences induced by IL-3 treatment (selected by their FDR P value and fold change). The heat map was generated using the MultiExperimental Viewer software v4.8. (B) The Early, Sustained, and Late gene signatures were used to perform ChEA on β -catenin and TCF7L2 ChIP targets. A Fisher's exact test with the Bonferroni correction, as implemented in ChEA, was used to compute the statistical enrichment between the IL-3 signature and indicated TCF ChIP target genes. The dotted horizontal line represents a P value cutoff of 0.05. (C) The data set of gene-expression differences resulting from the 6- and 16-h IL-3 stimulation of primary AML cells was used for GSEA. Analysis identified significant enrichment with Wnt and β -catenin [catenin (cadherin-associated protein), β 1, 88kDa (CTNNB1)] signatures at both 6 h and 16 h.

est, AML4 was the only patient harboring a FLT3-ITD mutation, a lesion that has been associated previously with the regulation of β -catenin expression in AML [6]. Furthermore, whereas the four patient samples displayed dose-dependent proliferation in response to IL-3, the proliferation of AML4 was considerably lower than the other patient samples (Fig. 3D), suggesting that in addition to the activation of STAT5, stabilization of β -catenin may play a critical role in amplifying growth signals induced by IL-3.

Gene-expression responses in primary AML cells treated with IL-3 are associated with β -catenin and TCF4 transcriptional signatures

To investigate whether the accumulation of β -catenin in primary AML cells in response to IL-3 corresponds with activation of β -catenin transcriptional activity, we used Affymetrix Human Gene 1.0 ST microarrays to assess global gene-expression changes in AML1–4, treated in the presence or absence of 15 ng/mL IL-3 for 6 h or 16 h. Differential gene expression was determined using a paired analysis (LIMMA method) of the four patient samples, as described in Materials and Methods. We found 160 probe sets significantly regulated (FDR, $P < 0.05$) only at 6 h (Early genes), 504 gene probe sets regulated at both 6 h and 16 h (Sustained genes), and 2646 gene probe sets only regulated at 16 h (Late genes). The top 30 genes in each group (Early, Sustained, and Late genes), selected by FDR P value and ranked by fold change, are shown as a heat map in Fig. 4A and listed in Supplemental Table 2. Several known cytokine-responsive genes, including *PIM1*, *PIM2*, and *CISH*, exhibited differential expression upon IL-3 treatment, consistent with a functional IL-3 response.

We used ChEA to test whether the gene sets regulated by IL-3 in primary AML cells were enriched for targets of TCF4 or β -catenin, based on publicly available data from ChIP-sequeing experiments. With the use of the Early, Sustained, and Late IL-3 gene signatures to query the ChEA database, we found significant enrichment of published β -catenin and TCF7L2 (TCF4) ChIP targets with the Sustained and Late IL-3 gene signatures ($P < 0.01$; Fig. 4B). GSEA, using the Broad Institute MSigDB curated gene sets, also demonstrated a signifi-

cant positive enrichment of genes modulated by IL-3 with gene signatures associated with WNT and β -catenin signaling (Fig. 4C). Finally, the expression of three β -catenin/TCF4 target genes (*MYC*, *UPP1*, and *FANCC*) was measured by QPCR in AML patient samples 1–4, treated \pm IL-3 for 6 h or 16 h. Importantly, two known IL-3 effector genes, *PIM1* and *CISH* [33, 34], were confirmed to be up-regulated significantly at both time-points following treatment with IL-3 (Supplemental Fig. 3A). The β -catenin targets *MYC* and *UPP1* were also up-regulated at both 6 h and 16 h (Supplemental Fig. 3B). Whereas *MYC* is an established functional target of β -catenin [35], *UPP1* is a novel, putative β -catenin ChIP target that we have identified using ChEA. The TCF4 target gene *FANCC* also displayed significant up-regulation at 16 h following treatment with IL-3 (Supplemental Fig. 3C). Together with the biochemical and cell biological data presented above, our gene-expression analysis supports a role for IL-3 in the accumulation and activation of β -catenin in primary AML cells. The results presented in this report also provide evidence for a nonredundant role of β -catenin in growth of transformed myeloid cells using a murine model of IL-3 and Hox cooperation (Fig. 5).

Whereas further studies are required to determine the mechanism by which IL-3 regulates β -catenin, JAK2 inhibition has been found previously to reduce β -catenin levels in leukemic cell lines [38] and may provide a link between IL-3 signaling and β -catenin. In addition, the inhibitory phosphorylation of GSK3 β , a key regulator of β -catenin stability, has been linked previously to IL-3R signaling through the activation of the PI3K/AKT pathway [39, 40]. Nonetheless, direct targeting of IL-3R with neutralizing mAb and diphtheria-toxin fusion proteins has shown promising results in mouse models and has been developed further for clinical trials in AML [15–18]. Importantly, given the selective up-regulation of IL-3R in LSC, we propose that it may be possible to reduce β -catenin activity and target LSC in selected AMLs through inhibition IL-3 signaling.

Lastly, our analysis of a small number of primary AML samples indicates heterogeneity with regard to the β -catenin response to IL-3, and this may be an important factor contributing to disease development and patient outcome. Indeed, re-

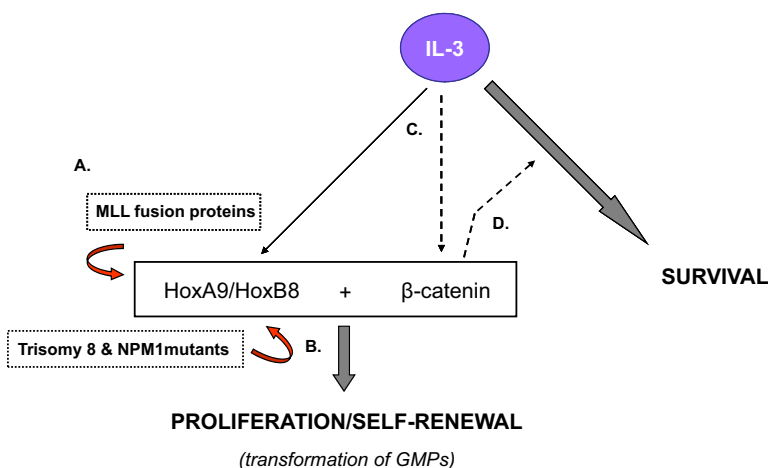


Figure 5. Working model for cooperation between β -catenin and Hox genes in myeloid transformation: a critical role for IL-3 signaling. (A) Previous studies have demonstrated that β -catenin is essential in the development and maintenance of AML stem cells with elevated Hox gene expression associated with MLL-AML [11, 12]. (B) Expression of β -catenin in combination with Meis1/HoxA9 expression is sufficient to induce leukemic transformation of GMPs in vivo [11]. Given this link, β -catenin may also be important in other AML subtypes associated with increased Hox expression, including Trisomy-8 [36] and nucleophosmin 1 (NPM1) mutant AML [37]. (C) Importantly, Hox genes have also been shown to cooperate with IL-3 signaling to generate a transplantable myeloid leukemia in mice [25], and the results from our investigation suggest that IL-3 may cooperate with Hox genes through the activation of β -catenin. (D) In addition, our data suggest that β -catenin overexpression may act further by amplifying survival-specific signals downstream of IL-3.

cent studies suggest that in combination with the basal levels of signaling proteins, the cytokine-induced signaling profiles of AML cells provide critical information for patient diagnosis and predicted response to therapeutic agents [41–43]. Further studies investigating the link between IL-3 and β -catenin function in a larger set of primary AML samples and in ex vivo models of AML will be necessary to establish the potential therapeutic and prognostic value of these findings.

AUTHORSHIP

T.S., M.P., and R.J.D. wrote the manuscript. T.S. performed the experiments, analyzed data, and interpreted the results. D.G.I. provided experimental assistance and review of the manuscript. C.H.K. analyzed the microarray data. S.L.H. and G.B. provided experimental assistance. H.S.R. was involved in data interpretation and critical review of the manuscript. M.S.S. and P.G.E. were involved in writing the manuscript. L.B.T. and I.D.L. provided patient material and clinical information. A.F.L. provided interpretation of signaling experiments and contributed to discussion of results.

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DISCLOSURES

The authors declare no conflicts of interest.

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