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Upregulation of homeodomain genes, DLX1/2, by FLT3 signaling

Running title: FLT3, DLX1/2 and TGF β interaction

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Abstract

Background. Activating mutations in FLT3 are frequent in acute myeloid leukemia and represent both a poor prognostic feature and therapeutic target. We have identified a previously unrecognized downstream effect of FLT3 activation, namely upregulation of the homeodomain genes, DLX1 and DLX2.

Design and Methods. MV4;11 cells with FLT3/ITD mutation, RS4;11 cells with wild-type FLT3 and AML patient blasts were used to pursue the relation between FLT3, DLX1/2 and TGF β . Quantitative RT-PCR, Western blot and reverse-phase protein array were performed to detect changes in gene and protein expression. RNA interference and MTS assay studied the interaction of PKC412, FLT3 inhibitor and TGF β 1.

Results. A direct relationship between FLT3 activity and DLX1/2 expression was revealed by both inhibition and upregulation of FLT3 signaling in MV4;11 and RS4;11 cell lines, respectively, in isolated blast cells from patients with acute myeloid leukemia, and in reverse-phase protein array from patient samples with acute myeloid leukemia. Mechanistically, the link between FLT3 and DLX1 expression appears to involve MAPK signaling through the ERK and JNK pathways. To determine whether elevated DLX1 had a functional consequence, we explored the reported inhibition by DLX1 on TGF β /Smad signaling. Indeed, TGF β responses were blunted by FLT3 activation in a DLX1-dependent manner and FLT3 inhibition resulted in a time-dependent increase in nuclear phospho-Smad2.

Conclusions. These findings suggest that alterations in DLX1/2 contribute to the biologic consequences of FLT3 activation.

Introduction

Homeodomain genes encode transcription factors that affect pattern formation, differentiation and proliferation during development. In humans, four clusters of homeodomain genes are distributed on chromosomes 7p15 (HOXA), 17q21 (HOXB), 12q13 (HOXC) and 2q31 (HOXD). Nonclustered homeodomain genes are distributed throughout the genome (1). For simplicity, all homeodomain-containing genes, clustered and nonclustered, will hereafter be referred to as HOX.

HOX deregulation is involved in the development of leukemia, as evidenced by recurrent chromosomal translocations that fuse the nucleoporin gene, NUP98, with HOXA9, HOXA11, HOXA13, PMX1, HOXC11, HOXC13, HOXD11 or HOXD13 (2-5). Rearrangements of MLL, which normally functions to maintain proper HOX levels (6), result in overexpression of HOXA9 and MEIS1 (7). In addition, overexpression of other HOX genes such as HOXA5 (8), HOXB3 (9) and HOXB4 (10, 11) have been shown to affect myeloid proliferation and differentiation, suggesting that their deregulation may contribute to the leukemogenic process or phenotype. Other examples of Hox involvement include murine BXH-2 leukemia, where HoxA7 and HoxA9 are frequent targets of retroviral integration (12), and are necessary for the development of leukemia in mice carrying a *MLL-ENL* rearrangement (13, 14).

We previously reported that HOX expression patterns correlated with major cytogenetic subtypes in human acute myeloid leukemia (AML) (15) and that AMLs with the highest levels of HOX expression represented a subset of intermediate cytogenetic cases with elevated levels of FLT3 (fms-like tyrosine kinase-3), a higher rate of FLT3 mutations, and a lower incidence of CEBP α mutations (16). More recently, we identified a common HOX expression signature in favorable AMLs (17), which may influence their biologic behavior.

FLT3 is a tyrosine kinase expressed in early hematopoiesis (18). The FLT3 pathway affects proliferation, differentiation and apoptosis of hematopoietic cells. FLT3 signaling leads to activation of RAS, STAT5 and PI3K (19, 20). It is constitutively activated in about 30% of AMLs (21) and the FLT3/ITD (internal tandem duplication) mutation is predictive of increased relapse rates and higher mortality (22-24). The

association between FLT3 mutations and certain patterns of HOX expression prompted us to ask whether there is a direct connection between FLT3 signaling and any particular HOX gene. As described here, we identified a novel interaction between FLT3 activity and regulation of the homeodomain transcription factors, DLX1 and DLX2, at both the mRNA and protein levels. The *DLX* genes are part of the *Drosophila distal-less* family that play a role in the control of craniofacial patterning and the differentiation and survival of inhibitory neurons in the forebrain (25). Moreover, the resulting changes in DLX1 expression appear to be functionally significant, as evidenced by effects on TGF β signaling pathway.

Design and methods

Cell lines and reagents

We used the following cell lines obtained from ATCC (American Type Culture Collection, VA, USA): MV4;11, derived from an acute monocytic leukemia with a FLT3/ITD mutation and RS4;11, an acute lymphoid leukemia with wild-type FLT3. We verified the cell lines by polymorphic microsatellite genome profiling (not shown). We performed *in vitro* treatments with the following agents: PKC412 (Novartis, Switzerland), imatinib mesylate (Novartis, Switzerland), staurosporine (Sigma, St. Louis, MO, USA), human recombinant TGF β 1, human recombinant FLT ligand (R&D Biosystems, Minneapolis, MN, USA), U0126, SP600125, SB203580 and LY294002 (Sigma, St. Louis, MO). Antibodies used for Western blot were: DLX1 monoclonal (M01, clone 2H3; Abnova), β -actin monoclonal (Sigma, St. Louis, MO), phospho-FLT3 (Tyr 591, Cell Signaling, Danvers, MA), CREB-1 monoclonal (X-12; Santa Cruz), GAPDH monoclonal (Ambion), phospho-Smad2 polyclonal (Ser465/467), Smad2 monoclonal (L16D3) (Cell Signaling, Danvers, MA, USA).

Treatment of leukemic cell lines with different compounds

To measure expression of HOX and TGFβ1 target genes, MV4;11 and RS4;11 cells (5×10^5 cells per ml) were treated 2, 5 and 24 hours with PKC412 (0.1 μM), TGFβ1 (1, 2, 3, or 5 ng/ml), FLT ligand (100 ng/ml), or with the following kinase inhibitors as described in the text: U0126 (MEK1/2 inhibitor, SP600125 (JNK inhibitor), LY294002 (PI3K inhibitor) and SB203580 (p38 inhibitor), all at 10 μM. Cells were seeded in 6-well plates containing RPMI 1640 (HyClone, UT), 10% FCS (HyClone, UT), 100 U/ml penicillin/streptomycin (HyClone, UT) 24 hours before the drug was added. Cells were harvested by centrifugation and washed with PBS (HyClone, UT) prior to preparation of protein lysates and RNA using standard methods. All experiments were performed in independent triplicates.

To measure changes in phospho-Smad2, cells were first incubated in serum-free RPMI 1640 medium for 7 hours. PKC412 (0.1 μM) was added for 5 hours (to achieve a decrease in DLX1), and cells were then incubated with two different low concentrations of TGFβ1 (1.2 and 0.6 ng/ml) for 2 hours. Control samples were treated with TGFβ1 only (in serum-free media).

Patient samples

Eighteen RNA samples from AML patients at diagnosis were used for DLX1 analysis. Patients were divided into two groups according FLT3 mutation. FLT3/ITD mutation was detected by PCR amplification and DNA agarose gel electrophoresis. Difference in blast count of diagnostic samples did not correlate with DLX1 expression. 576 samples from patients with AML were analysed by RPPA method. All experiments were conducted according to the principles expressed in the Declaration of Helsinki. Ethical committee and Institutional review board approved the project and all samples were analyzed with written informed consent of subjects or their guardians.

Treatment of patient sample ex vivo with PKC412

AML blast cells isolated from patient samples containing a FLT3/ITD mutation (n=3) or FLT3 wild type (n=1) were incubated *ex vivo* in RPMI 1640, 10% FBS enriched with a cytokine cocktail (10 ug/ml FLT3, 10 ug/ml IL-6 and 10 ug/ml SCF-1) for 24 hours, then PKC412 was added and the cells incubated another 24 hours prior to harvest.

Quantitative real-time PCR

Total RNA was isolated with RNeasy Plus mini kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer instructions. Intact RNA was verified by 1% agarose gel electrophoresis and cDNA was prepared using RT SuperScript III (Invitrogen, Carlsbad, CA, USA) and random-hexamer priming. Gene expression was measured on an ABI 7500 Fast real-time PCR machine using a 2X Power SybrGreen PCR Master kit (ABI, Foster City, CA, USA). The panel of HOX genes has been previously described and the assays validated (17). All genes were analyzed in the same PCR conditions, i.e., 95°C x 10 min. followed by 35 cycles of: 95°C x 15s then 60°C x 1 min. Primer sequences of the genes utilized are provided in the supplementary material (Table S3). Raw data were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), that is, $\Delta C_t = C_{t \text{ specific gene}} - C_{t \text{ GAPDH}}$. Expression data are reported as the number of transcripts per thousand copies of GAPDH.

Western blotting, nuclear and cytoplasmic fraction

Cells for protein extraction were washed with PBS and the cell pellets kept at -80°C before use. Whole-cell protein lysates were prepared in 50 mM Tris-HCl pH 7.5, 150 mM sodium chloride, 0.5% NP-40, 1 mM DTT, 2 mM MgCl₂ together with 1 mM sodium fluoride, 1 mM PMSF, 1 mM orthosodium vanadate, 10 µg/ml pepstatin A, 10 µg/ml aprotinin and 10 µg/ml leupeptin, followed by sonication for 15s and centrifugation for 4000 rpm/5 min. Protein concentrations were measured by the Bradford assay using a plate reader Synergy 2 (BioTek Instruments, VT). Expression of DLX1, phospho-FLT3 (Tyr 591) and Smad2 was measured in whole lysates. β-actin was used as a loading control.

NePer kit (Pierce, Rockford, IL, USA) was used according manufacturer instructions for nuclear-cytoplasmic fractionations. Protein concentration of nuclear and cytoplasmic extracts was adjusted to the same ratio. Phospho-Smad2 was measured in both fractions; CREB-1 was used as a loading control for the nuclear fractions and GAPDH for the cytoplasmic fractions.

Cell cycle analysis

DNA analysis was performed using BD FACSCalibur cytometer. Cells were washed in PBS and digested with 0.3% saponin, 50 µg/ml PI (Sigma, St. Louis, MO), 0.1 mM EDTA, RNase A (Sigma, St. Louis, MO) in 1x DPBS (HyClone, UT) and left overnight at 4°C in the dark before analysis.

Transfection and RNA interference

MV4;11 and RS4;11 cells were transfected by electroporation using an EPI2500 instrument (Dr. L. Fischer, Heidelberg, Germany) and 0.4 cm gap Gene Pulser/MicroPulser Cuvettes (Bio-Rad, Hercules, CA, USA) under the following conditions: 1 million cells/100µl of culture medium, 500 nM siRNA, and 1 pulse of 15ms, 250V and 1200µF. The siRNA transfection efficiency was monitored by flow cytometry. DLX1 targeting ON-TARGETplus SMARTpool (siDLX1) was used for the DLX1 knock-down in MV4;11 and RS4;11 cells with ON-TARGETplus Non-targeting Pool (sinonT; Dharmacon) as a negative control.

MTS assay

MTS is a colorimetric mitochondrial-based cell proliferation assay based on measurement of soluble formazan, which is proportional to the number of live cells. MV4;11 cells were seeded at 8×10^3 cells per well 24 hours before treatment containing either PKC412 (i.e., 25, 12.5, 6.25 nM), TGFβ1 (i.e., 0.3125, 0.625, 1.25 ng/ml), or the combination. After 96 hours, 14 µl of MTS reagent (Promega, Madison, WI) was added to cells and incubated for 3 hours. Absorbance at 490 nm was measured in a Synergy 2 plate-reader (BioTek Instruments, VT, USA).

RPPA analysis

RPPA allows measurement of protein expression levels in a large number of biological samples simultaneously in a quantitative manner (26). A RPPA dataset (576 samples printed in replicate with 5 serial 1:2 dilutions in 48 rows and 144 columns) was generated by using blood or marrow samples from patients with AML. Samples had been acquired during routine diagnostic assessments in accordance with the regulations and protocols approved by the MDACC Investigational Review Board (IRB). Informed consent had been obtained in accordance with the Declaration of Helsinki. We compared 383 negative and 76 positive FLT3/ITD patients at diagnosis which were analyzed for DLX1 and 187 other proteins. The samples were normalized to a concentration of 1×10^4 cells/ μ L and a whole-cell lysate prepared. Briefly, patient samples were printed in 5 serial dilutions onto slides along with normalization and expression controls. Slides were probed with a strictly validated primary antibody against ARC (Imgenex, San Diego, CA) and a secondary antibody to amplify the signal, and finally a stable dye was precipitated. The stained slides were analyzed using MicroVigene® software (Vigene Tech, Carlisle, MA) to produce quantified data. The RPPA data has been properly normalized. ANOVA analysis of protein levels was used to assess the association of protein level to categorical clinical variables.

Statistical analysis

Nonparametric Mann-Whitney test was performed to assess difference of gene expression measured by quantitative RT-PCR.

Results

Changes in DLX1/2 expression by FLT3 signaling. To search for possible interactions between FLT3 signaling and HOX gene expression, we first used MV4;11 cells, which were derived from a monocytic leukemia containing a FLT3/ITD gain of function mutation. These cells were treated with PKC412, a FLT3 inhibitor, and real-time quantitative RT-PCR was used to examine HOX expression (see Methods). Although we expected to find downregulation of the HOXA or HOXB genes, only *DLX1* and *DLX2*

expression were reproducibly affected by FLT3 inhibition ($\Delta\Delta C_t$ values are shown in Table S1). Other DLX genes were not expressed in these cell lines. At 24 hours, *DLX1* and *DLX2* mRNA levels were decreased by 4.8 and 3.3-fold (Figure 1A). The mRNA changes were mirrored at the protein level and reduced DLX1 protein expression could be observed as early as at 5 hrs after addition of PKC412 (Figure 1A).

To demonstrate that the change in DLX1 was unrelated to non-specific toxicity, we analyzed MV4;11 cells by flow cytometry following treatment with PKC412. No effects on cell-cycle parameters were evident after 2 or 5 hrs (Supplementary Figure S1A), although by 24 hours the cells were arrested in G0/G1. Furthermore, there was no evidence of a sub-G1 peak (i.e., DNA fragmentation), nor did we see any significant change in viability as assessed by trypan blue exclusion. To further test for non-specific effects, we treated MV4;11 cells with 0.1 μ M imatinib mesylate, an ABL inhibitor; no significant change in *DLX1* expression was observed after 24 hours (Supplementary Figure S1B). In contrast, treatment of cells with Staurosporine, a general tyrosine kinase inhibitor, led to widespread changes in most HOX genes examined (data not shown).

To confirm that FLT3 signaling affects DLX1 levels, we used the lymphoid leukemic cell line, RS4;11, which contains the same t(4;11) MLL/AF4 fusion gene as MV4;11 cells but has wild-type FLT3 and low *DLX1* levels. FLT3 signaling was activated by the addition of FLT ligand (100 ng/ml) for 2, 5 and 24 hours and DLX1/2 expression was examined. Increased DLX1 protein was evident within 5 hours; corresponding *DLX1* mRNA levels were elevated 2.6-fold and *DLX2* expression was increased by 2.4-fold (Figure 1B).

To demonstrate that FLT3 activation affects DLX1 levels in patients, we first examined 469 AML patients for DLX1 protein levels by reverse phase protein array (RPPA). The group of 383 patients negative for FLT3/ITD had significantly lower expression of DLX1 than 76 patients positive for FLT3/ITD ($p=0.0004$; Figure 2A). At the mRNA level, we examined *DLX1* expression in diagnostic samples from 18 additional AML patients with or without FLT3/ITD mutations (patient characteristics are shown in Supplementary Table S2). Patients with FLT3/ITD mutations had significantly higher expression of *DLX1* compared to patients with wild-type FLT3 (Figure 2B). Lastly, we isolated bone marrow cells from patients with AML containing a FLT3 wild

type or FLT3/ITD alteration. After 24 hours of *ex vivo* treatment with PKC412, *DLX1* was decreased only in FLT3/ITD positive patient blasts approximately by 3.1-fold. (Figure 2C). Thus, we conclude that FLT3 signaling affects expression of DLX1 and 2 in both cell lines and patient samples.

Regulation of DLX1 downstream of FLT3 signaling

FLT3 mediates signaling through three known pathways (RAS, PI3K and STAT5). We used specific inhibitors affecting ERK1/2 (U0126, a MEK1/2 inhibitor), JNK1/2 (SP600125), p38 MAPK (SB203580) and PI3K (LY294002) to look for effects on DLX expression in the RS4;11 and MV4;11 cell lines. For RS4;11, the cells were first incubated with the various inhibitors for 1 hour, then treated with FLT ligand for up to 24 hours with measurement of mRNA levels at 2, 5 and 24 hour time-points. FLT ligand treatment of RS4;11 cells caused an approximate 2.5-fold induction of *DLX1*, whereas U0126 and SP600125 reduced expression back to basal levels (Figure 1C). In contrast, neither the p38 or PI3K inhibitors had any significant effect. For MV4;11 (with constitutively activated FLT3), DLX1 expression was monitored at 2, 5 and 24 hours after addition of the inhibitors. Similar to the results in RS4;11 cells, blocking ERK1/2 and JNK1/2 activation with U0126 and SP600125 inhibited DLX1 expression and, moreover, the degree of inhibition was similar to that following FLT3 inhibition with PKC412 (i.e., 4-fold change; Figure 1D). LY294002 also inhibited DLX1 expression, although not to the same degree as the ERK1/2 and JNK1/2 inhibitors, and only at 5 the hour time-point. Blocking p38 had no effect on DLX1. These results demonstrate that both ERK1/2 and JNK1/2 participate in the regulation of DLX1 mRNA expression in both RS4;11 and MV4;11 cell lines. For *DLX2*, only U0126 inhibited expression consistently in both cell lines (data not shown).

To search for an ERK/JNK responsive transcription factor binding site in DLX1, we used multiple-sequence alignment analysis, i.e., Mulan (<http://mulan.dcode.org/Mulan>), which identifies local sequence conservation for the detection of evolutionarily conserved transcription factor binding sites. Activation of JNK and ERK pathways results in upregulation of the c-Jun and c-Fos gene products, which together comprise the AP-1 transcription factor. Of note, a conserved AP-1 dimer binding site, TGAGTCA, was found at 205 bp upstream from the DLX1 transcription start site (Supplementary Figure S2).

Repressive effect of DLX1 on TGF β signaling. It was previously shown that DLX1 is able to bind Smad4 and block TGF β signaling (27). To determine whether FLT3 inhibition with PKC412 and the downregulation of DLX1 affected TGF β target genes, we treated MV4;11 cells with threshold levels of TGF β 1 (1-5 ng/ml) and PKC412 (0.1 μ M) separately, or in combination, along with an analysis of selected TGF β target gene expression (*p15*, *CUTL-1*, *p21*, *Id-2* and *PAI-1*) using qRT-PCR. TGF β 1 alone induced *p15* by 2-fold, *CUTL-1* by 1.7-fold, and caused a 30-fold increase in *PAI-1*. PKC412 modestly increased the expression of *p15* and *CUTL-1* by 2.4 and 2.0-fold, respectively, but had no effect on *PAI-1*. The combination of PKC412 and TGF β 1 led to a further modest increase in target gene expression, i.e., *PAI-1* (37-fold induction), *p15* (3.5-fold induction) and *CUTL-1* (2.2-fold induction, Figure 3A). In addition, we isolated bone marrow cells from a patient with AML containing a FLT3/ITD and treated these with PKC412 (Figure 2B). *p15* levels were upregulated by 2.5-fold, as were levels of *CUTL-1* (1.7-fold) and *Id2* (1.7-fold).

Although the effects of PKC412 on TGF β gene expression were reproducible, this agent is not specific for FLT3. To confirm that DLX1 levels could be responsible for the effects observed with PKC412, we used RNA interference to inhibit DLX1 and treated MV4;11 cells with threshold levels of TGF β 1 (5 ng/ml for 24 hours). The siRNA against DLX1 reduced its level by 3-fold (Figure 3B) and caused a significant enhancement in the expression of *p15*, *CUTL-1*, *PAI-1* and *Id2* compared to TGF β 1

alone. Moreover, these changes were quite similar to the effects observed with PKC412 (Figure 3A).

We then asked whether FLT3 stimulation would have the opposite effect in RS4;11 cells, which are otherwise responsive to TGF β 1 as evidenced by increased levels of *CUTL-1*, *PAI-1* and *Id2*. As anticipated, the addition of FLT ligand reduced the expression of *CUTL-1* (2-fold), *PAI-1* (1.5-fold) and *Id2* (1.5-fold) at 24 hours (Figure 4A), whereas *p15* was not expressed in this cell line. Importantly, silencing DLX1 abrogated the effect of FLT ligand and brought TGF β target genes back to their basal level (Figure 4B).

Since TGF β can induce a G1/G0 cell-cycle and growth arrest, we used MTS assays to monitor the growth of MV4;11 cells following treatment with three different concentrations of TGF β 1, either alone (based on the half-maximal inhibitory concentration (IC₅₀) or in combination with varying concentrations of PKC412 (6.25 to 100 nM). At higher concentrations, TGF β 1 and PKC412 alone were able to cause a cytostatic effect on the leukemic cells. However, at threshold concentrations of TGF β 1 (0.3 and 0.6 ng/ml), we observed an enhanced cytostatic effect when combined with a low concentration (6.25 nM) of PKC412 (Figure 5).

PKC412 affects levels of nuclear phospho-Smad2

TGF β pathway activation results from ligand binding to the type II receptor and phosphorylation of a regulatory (R) Smad (e.g., Smad2, Smad3) by the type I receptor. The phosphorylated R-Smad then associates with Smad4 and the complex translocates to the nucleus to regulate gene expression (28). We examined the phosphorylation and nuclear translocation of Smad2/Smad3 in MV4;11 cells treated with TGF β 1 and PKC412. The addition of TGF β 1 (1.2 and 0.6 ng/ml) in serum-starved conditions led to increased nuclear Smad2 phosphorylation, as expected. Although PKC412 as a single agent did not increase nuclear phospho-Smad2, it reproducibly enhanced its levels at 2 hours after stimulation with TGF β 1 (Figure 6A), while total Smad2 remained unchanged (Figure 6B). These results are consistent with the observed changes in TGF β target gene expression following inhibition (or stimulation). Whether the changes in nuclear

phospho-Smad2 result directly from the reported interaction between DLX1 and Smad4 or involve another mechanism is unknown.

Discussion

We describe here a novel and specific relationship between the homeodomain genes, DLX1 and DLX2, and FLT3 kinase activity. This relationship was observed both in leukemic cell lines and in patient samples. Furthermore, at least *in vitro*, this relationship appears to have functional consequences on TGF β target gene expression and signaling (Figure S3).

We initiated this study based on previous results in patient samples suggesting that the expression of certain HOX genes correlated with FLT3 mRNA levels (16). To pursue this, we utilized two t(4;11) translocation containing cell lines, MV4;11 and RS4;11, which express constitutively active and wild-type FLT3 receptors, respectively. Among many homeodomain genes examined by qRT-PCR following FLT3 manipulation, only *DLX1* and *DLX2* mRNAs were consistently affected. Short-term inhibition of mutated FLT3 in MV4;11 cells by PKC412 caused downregulation of DLX1/2, while exposure of RS4;11 cells to FLT3 ligand resulted in DLX upregulation. Although the survival of MV4;11 cells is dependent on FLT3 signaling (29), downregulation of DLX1/2 preceded any measurable evidence of PKC412-mediated toxicity using trypan blue exclusion and sub-G1 DNA content as indices. While these indices might have been insufficiently sensitive, the corroborative results from FLT3 stimulation in RS4;11 cells strongly support the proposed regulatory interaction. Moreover, DLX1 levels were also downregulated by PKC412 in blast cells isolated from patients with FLT3/ITD positive AML, and our reverse phase arrays demonstrated a highly significant correlation between activating the presence of FLT3 mutations (ITDs) and DLX1 protein expression. Mechanistically, a link between DLX1 regulation and FLT3 signaling appears to involve the ERK and JNK pathways, which are known to affect AP-1 levels, and a conserved AP-1 binding site was identified in the promoter region of DLX1.

The DLX genes were originally identified in the forebrain of the developing mouse embryo. Although individual *DLX1* and *DLX2* knockouts do not cause forebrain deformities, the double knockout exhibits abnormalities in facial structures and is neonatally fatal (30, 31). In addition, DLX1 was shown to be a negative regulator of definitive erythropoiesis (27). Aberrant expression of DLX family members has also been associated with breast, ovarian and lung cancer progression / invasiveness (32-34).

Previous studies have demonstrated that DLX1 negatively affects TGF β signaling by direct interactions with Smad4 (27), a key downstream effector of TGF β /BMP signaling. Interactions between Smads and HOX genes were also shown in other studies (35, 36). We observed that the decrease in DLX expression by either PKC412 treatment or following siRNA-mediated knockdown of DLX1 was functionally significant, as evidenced by the upregulation *p15*, *CUTL-1* and *Id2*, known TGF β target genes (37-39). In contrast, FLT3 stimulation in RS4;11 cells led to a repression of *CUTL-1*, *Id2* and *PAI-1* mRNAs. This effect was blocked by siRNA-mediated DLX1 knockdown, thus confirming its direct role in the regulation. The *p15*, *CUTL-1*, *Id2* and *PAI-1* genes were not affected equally; however, the changes were reproducible and in the expected direction (upregulation vs. downregulation). There are several possible explanations for the lack of response in *PAI-1* expression after PKC412 treatment in MV4;11 cells, as well as in patient blasts. The transcriptional effects of SMADs are known to be dependent on the presence of other co-activators or co-repressors (40). PKC412 is also not specific for FLT3 signaling and potentially could affect other pathways that interfere with *PAI-1* activation. In addition, a novel type of ITD mutation not localized in juxtamembrane region of the FLT3 receptor was recently discovered by Breitenbuecher et al (41). This mutation was detected in about 29% of FLT3/ITD positive patients and was associated with persistence of phosphorylated-ERK1/2 despite PKC412 treatment.

In addition, TGF β signaling is known to inhibit proliferation of hematopoietic cells, and mutations in this pathway contribute to the growth of AML blasts (28, 42). We found that the combination of threshold levels of both TGF β and PKC412 caused more growth inhibition than either agent alone, which is consistent with an enhanced TGF β response, although other interpretations are not excluded.

Recent data indicate that Smads may repress gene expression by interacting with co-repressor complexes associated with TGIF, HOXC8 and ATF2 (43-45). Using nuclear-cytoplasmic fractionation and Western blots, we found that PKC412 reproducibly enhanced the level of nuclear phospho-Smad2 induced by TGF β 1 at 2 hours, while total Smad2 was unchanged. Mechanistically, because DLX1 has been reported to bind and sequester Smad4, reducing the level of DLX1 might result in more translocation of a p-Smad2/p-Smad3/Smad4 complex to the nucleus. In addition, it has been reported that ERK-mediated phosphorylation of Smad1-3 impairs nuclear transport and signaling activity of these proteins (40-42). Thus, inhibition of FLT3, which is known to signal in part by phospho-ERK1/2, would be predicted to enhance nuclear transport of phosphorylated Smad2. However, uncovering this mechanism will require further investigation.

In summary, our results demonstrate that FLT3 signaling leads to the specific upregulation in DLX1/2 expression at both the RNA and protein levels in leukemic cell lines and patient samples. Although the full consequences of this upregulation remained undetermined, we suggest that effects on TGF β target gene expression and cell growth may be involved. Thus, FLT3 signaling in leukemia may function in part through DLX1/2 to overcome the growth inhibitory effects of TGF β . Given the frequency of FLT3 alterations in AML, more investigation into the role of DLX1/2 in the disease process seems warranted.

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Authorship and Disclosures

HD was the principal investigator and co-ordinated the study. JS performed the laboratory work for this study and wrote the paper. SG performed screening by qRT-PCR on cell lines. SK and YQ co-ordinated RPPA study on patient samples. NZ participated in the statistical analysis. IH worked on ex vivo experiments.

The authors have no conflicts of interests and no competing financial interests to disclose.

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Figure 1. Effect of PKC412 on *DLX1* and *DLX2* gene expression (A) Y axis shows the fold-change of gene expression of *DLX1* and *DLX2* in samples treated 0, 2, 5 and 24 hours with kinase inhibitors. Western blot shows effectiveness of PKC412 drug to p-FLT3 inhibition and DLX1 protein expression in MV4;11 cell line. (B) Fold-change of *DLX1* and *DLX2* expression in RS4;11 cells treated 2, 5 and 24 hours with FLT ligand (100ng/ml) and *DLX1* and *DLX2* mRNA levels were detected. Western blot shows DLX1 protein expression in RS4;11 cell line. (C) Normalized DLX1 expression in RS4;11 (5 hours) and (D) MV4;11 (5 hours) cell lines after the treatment with ERK1/2 (U0126, 10μM), JNK1/2 (SP600125, 10μM), PI3K (LY294002, 10μM) and p38 (SB203580, 10μM) inhibitors in FLT3 activated pathway. Asterisks correspond to statistically significant change, ***p<0.0001.

Figure 2. *DLX1* expression in samples from AML patients and effect of PKC42 ex vivo (A) Box plots showing results of RPPA analysis in AML patients characterized according FLT3 status (NEG – FLT3wt; POS – FLT3/ITD) by using ANOVA test (B) Box plot describing results of Mann-Whitney statistical test in diagnostic samples of AML patients with (FLT3/ITDpos) or without FLT3/ITD (FLT3/ITDneg) of *DLX1* expression (C) Relative expression of *DLX1* presented as fold-change was detected in leukemic blasts isolated from bone marrow cells of AML patients with FLT3 wild-type or FLT3/ITD mutation. Moreover, the expression of *DLX2*, *CUTL-1*, *p15* and *Id2* was studied in leukemic blasts isolated from bone marrow of AML patient with FLT3/ITD and treated with PKC412 for 24 hours. Asterisks correspond to statistically significant changes, *p≤0.02, **p≤0.001.

Figure 3. Expression of *p15*, *CUTL-1* and *PAI-1* genes (A) Graphs describe fold-change of *p15*, *CUTL-1* and *PAI-1* genes in MV4;11 cells after PKC412 (0.1 μ M) and TGF β 1 (1, 2, 3, 5 ng/ml) treatment alone or in combination normalized to untreated control cells and effect of siDLX1 in the presence of TGF β 1 (5ng/ml). All measurements were performed 24 hours after treatment. All measurements were done in triplicates. (B) Downregulation of DLX1 in MV4;11 cells using siRNA after 24 hours. Asterisks correspond to statistically significant change, $^{**}p \leq 0.0008$.

Figure 4. Knockdown of DLX1 (siDLX1) alters response to TGF β 1 and FLT3 ligand. (A) Relative expression of *CUTL-1* and *PAI-1* in RS4;11 cells treated with TGF β 1 (5 ng/ml) or FLT ligand (100 ng/ml) for 24 hours compared to untreated control cells; (B) Downregulation of DLX1 in RS4;11 cells using siRNA after 24 hours blocks FLT3 ligand-mediated suppression of *CUTL-1* and *PAI-1*. Cells were transfected with either non-targeted siRNA (control) or siDLX1 and exposed to FLT3 (100 ng/ml for 24 hours). Asterisks correspond to statistically significant changes, $^{*}p < 0.05$; $^{**}p \leq 0.0004$.

Figure 5. Cytostatic effect of PKC412, TGF β 1 and the combination Results of MTS assay using threshold concentrations of TGF β 1 (T) and PKC412 (P) and the combination (T+P) were monitored 72 hours after incubation. Experiments were done in triplicate and the standard deviation was calculated. Asterisks correspond to statistically significant changes, $^{*}p \leq 0.03$; $^{**}p \leq 0.005$; $^{***}p < 0.0001$.

Figure 6. Effect of PKC412 on phosphorylation of Smad2 (A) Phospho-Smad2 detected by Western blot in MV4;11 cells treated with PKC412 (0.1 μ M) and TGF β 1 (0.6 and 1.2ng/ml) alone, or in combination, in nuclear and cytoplasmic fractions at 2 hours. CREB-1 (nuclear protein) and GAPDH (cytoplasmic protein) were used as controls. Values from densitometry are shown. (B) Total Smad2 was detected in whole lysates. All experiments were done in independent triplicates. Representative examples are shown.

Figure 1

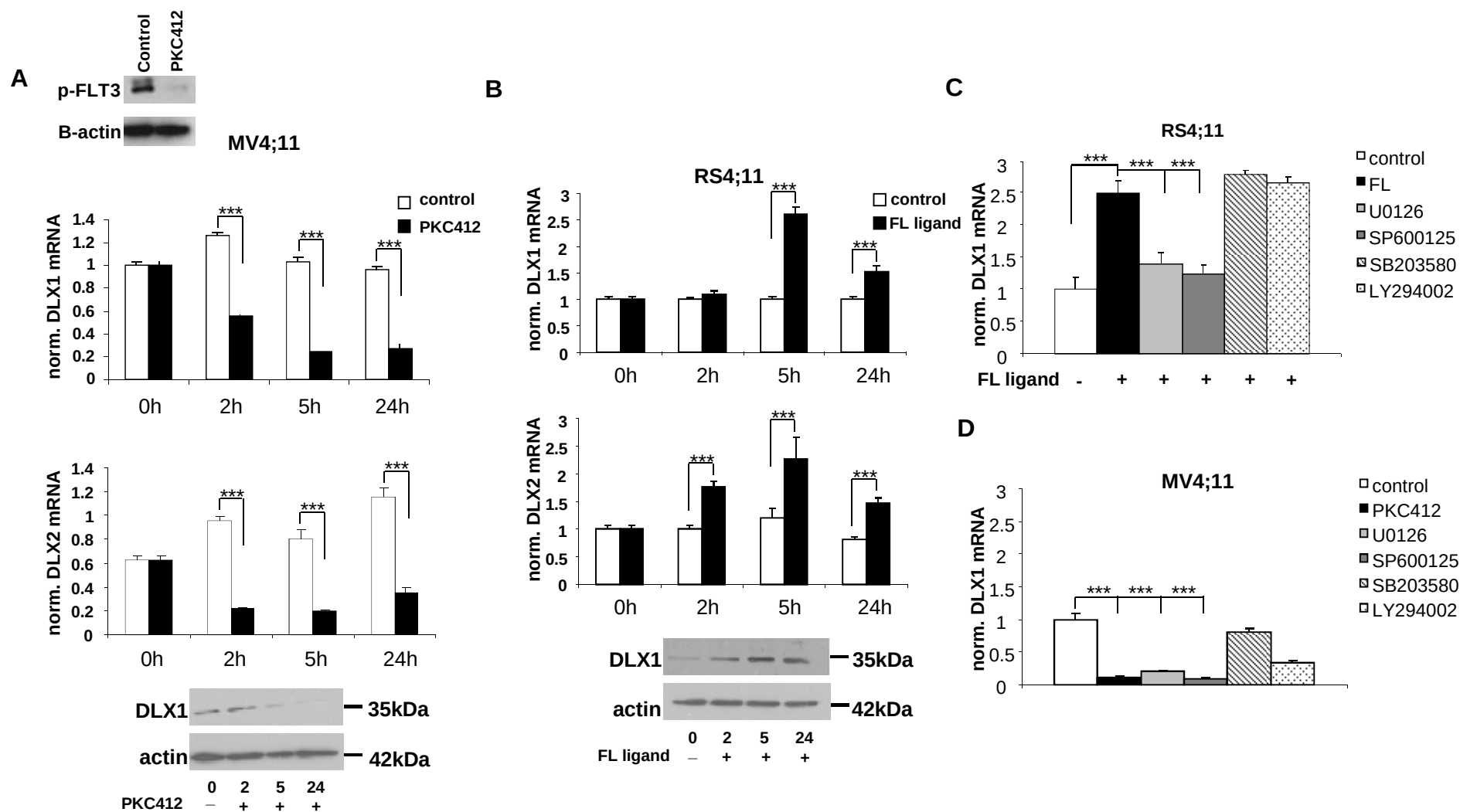


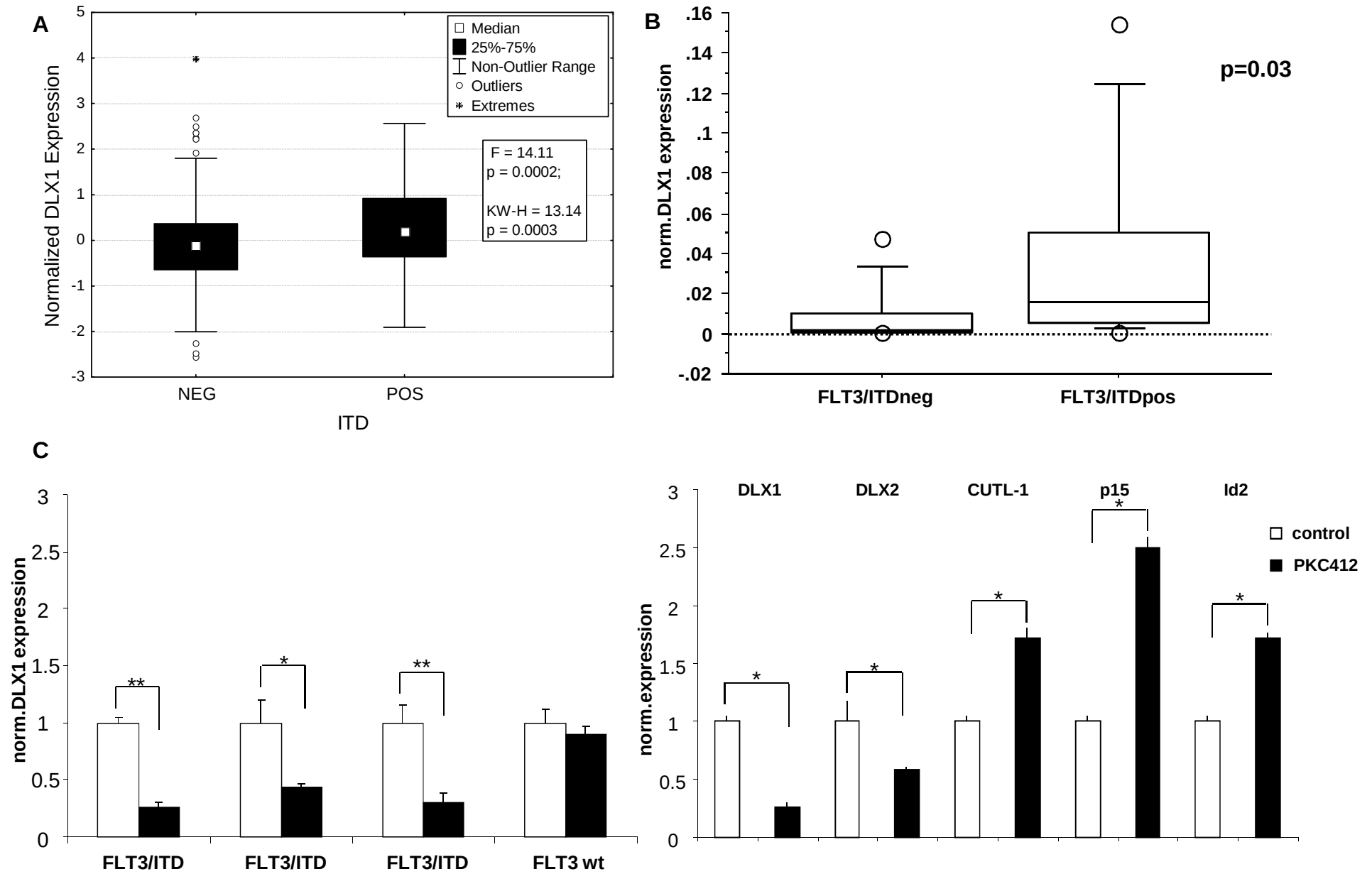
Figure 2

Figure 3

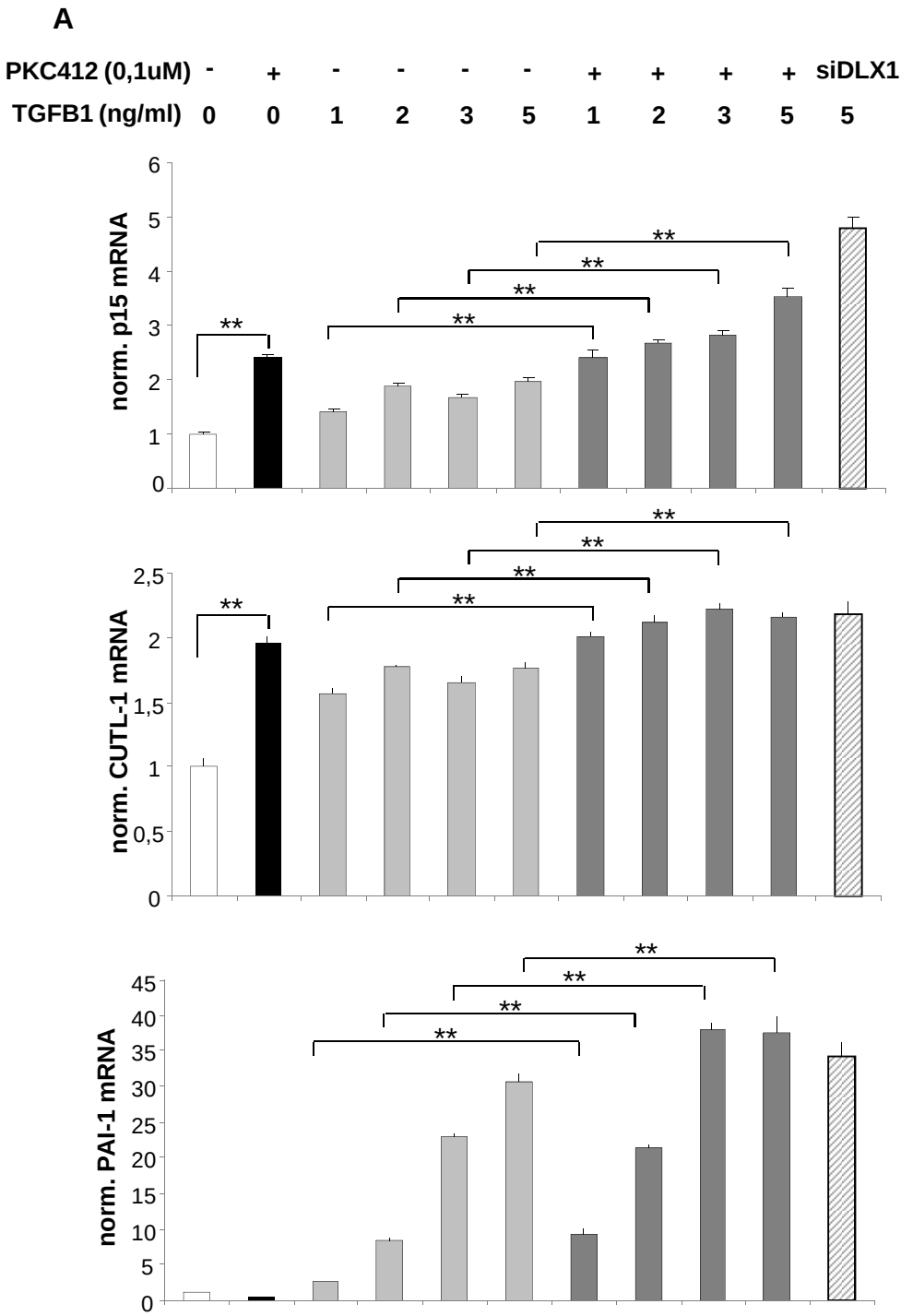


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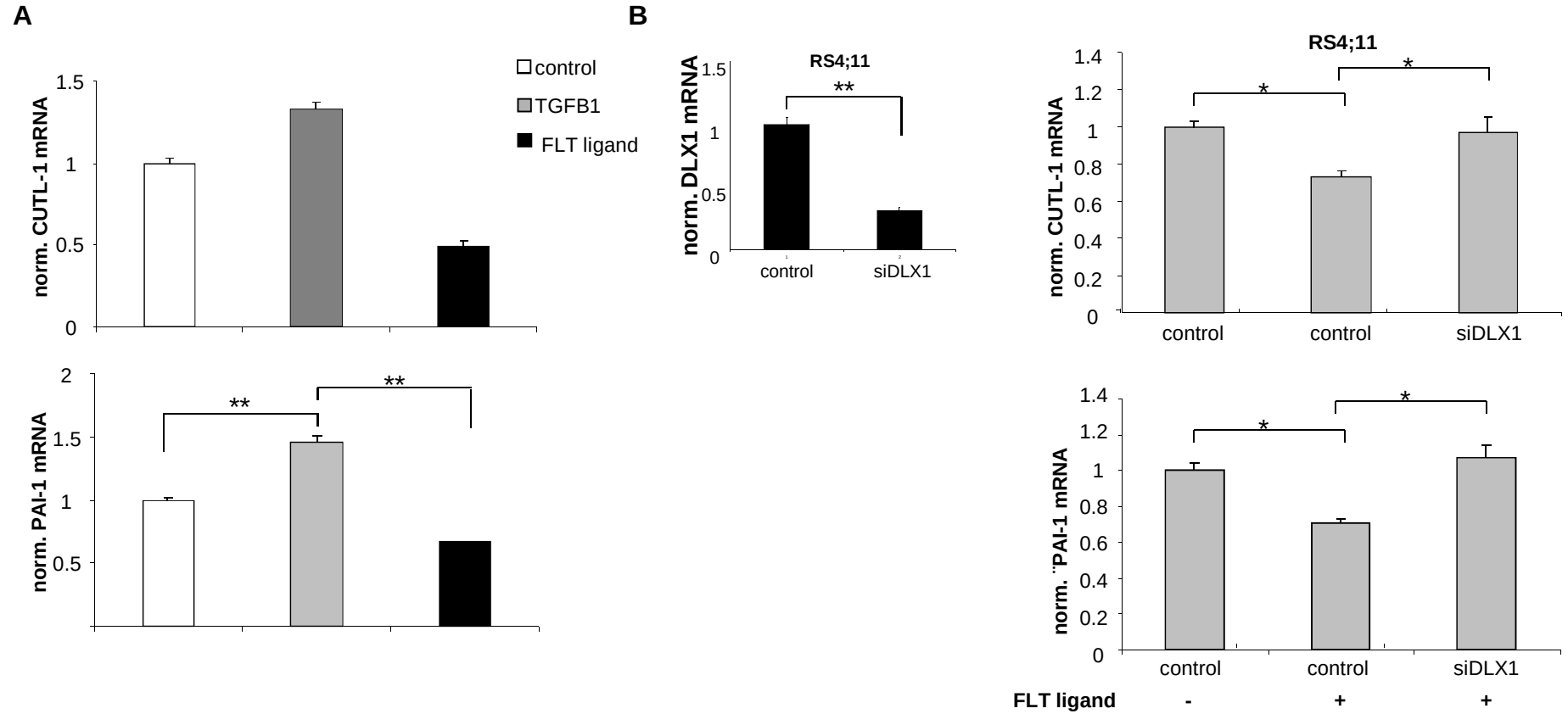


Figure 5

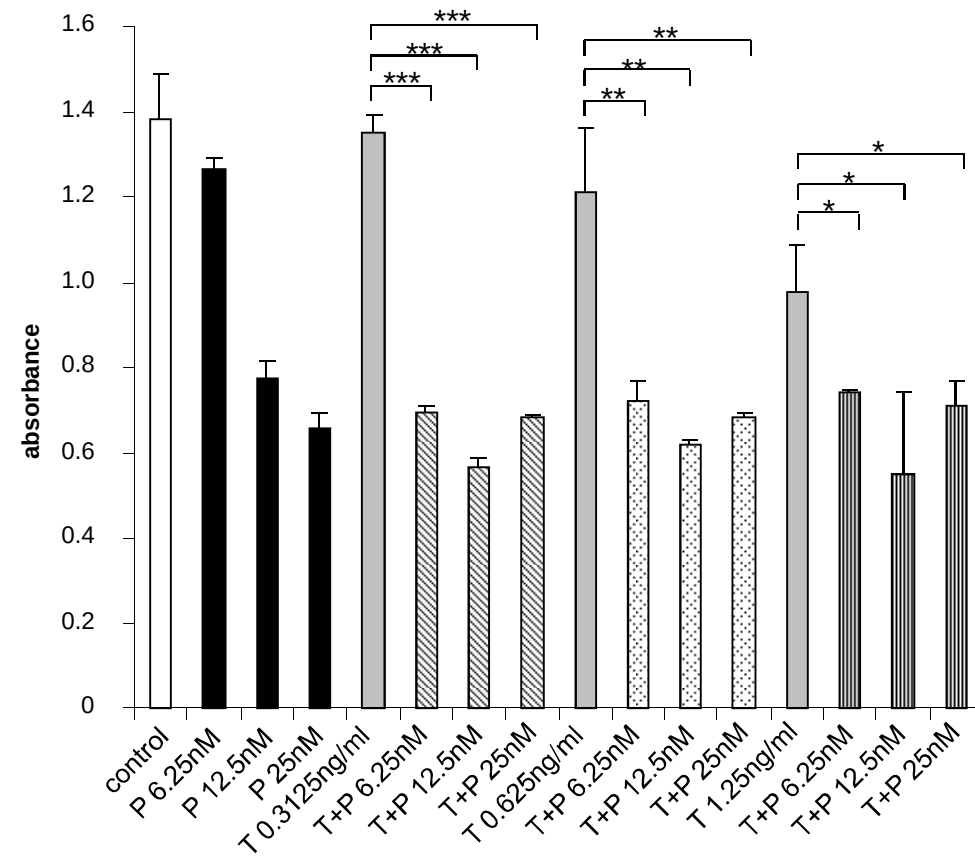


Figure 6

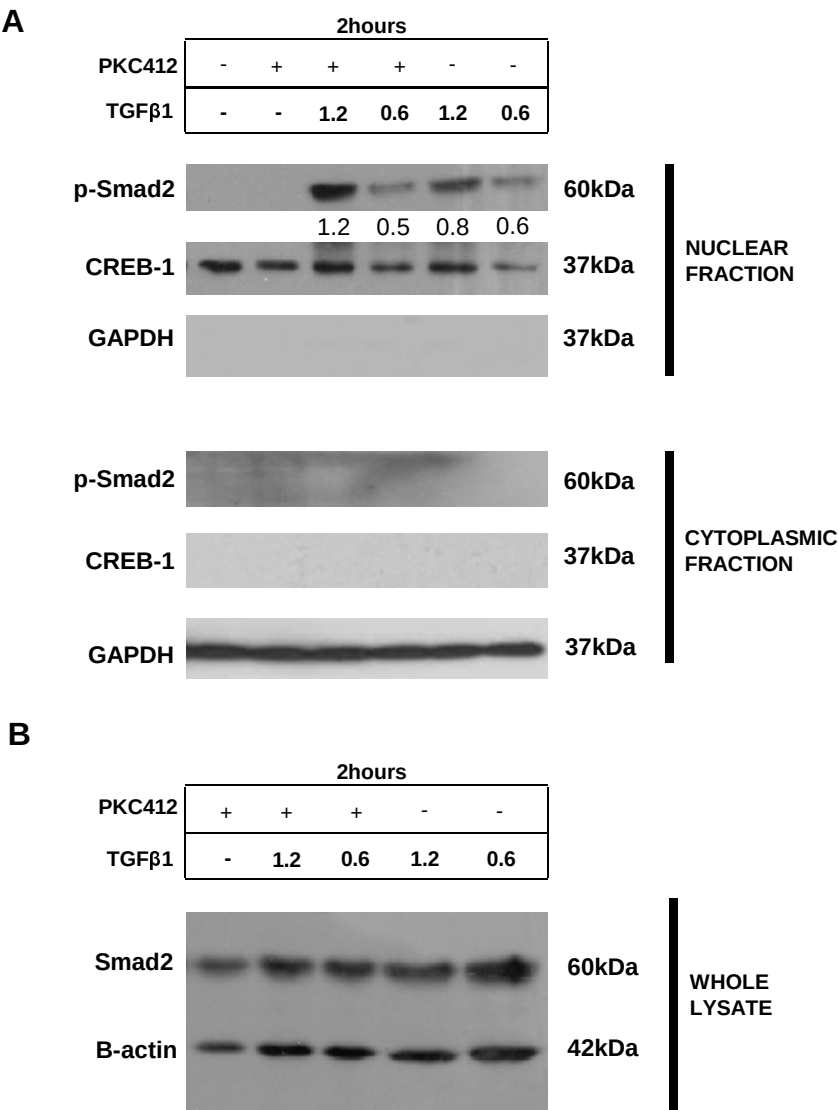
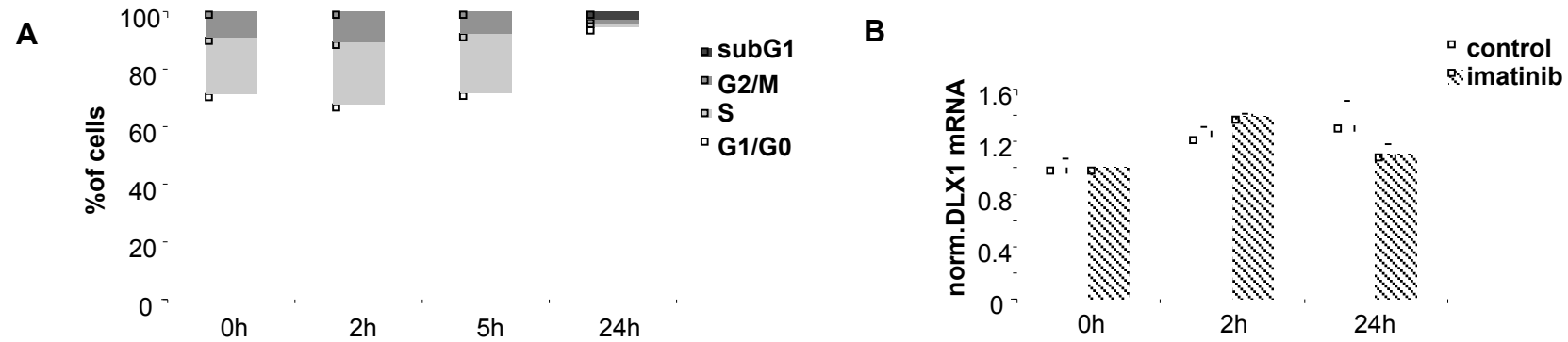


Figure S1



Specificity and non-toxicity of PKC412 in MV4;11 cells (A) Results from flow cytometry analysis are shown as a percentage of cells in subG1, G1-G0, S and G2-M phase of cell cycle after 0, 2, 5 and 24 hour treatment of MV4;11 cells with PKC412. (B) Graph shows fold-change of DLX1 gene expression in MV4;11 cells after imatinib treatment

Figure S2 AP1-binding site in DLX1 promoter

- **promoter** -

AGCGGAGCCCGAGCGCCTCGGACCAATCCCCAGTGATTATGCAAGACAGCGGACCAAT
 TCGCCTCGGGCTCGCGGAGCCTGGTTAGGGGTCACTAATACGTTCTGTCGCCTGGTTA

- **promoter** - **AP-1b.site** - **Exon 1** -

CAGCTCCGCCAGCTCATGAATATTTATGACCTTCGCT**TGAGTCAA**AGCTTTGAACCGAGT
 GTCGAGGCGGRCGAGTACTTATAAATACTGGAAGCGACTCAGTTTCGAAACTTGGCTCA

- **CDC start** -

TTGGGGAGCTCAGCAGCATC**ATG**CTTAGACTTTTCAAAGAGACAAACTCCATTTTCTTAT
 AACCCCTCGAGTCGTCGTAGTACGAATCTGAAAAGTTTCTCTGTTTGAGGTAAAAGAATA

Figure S3

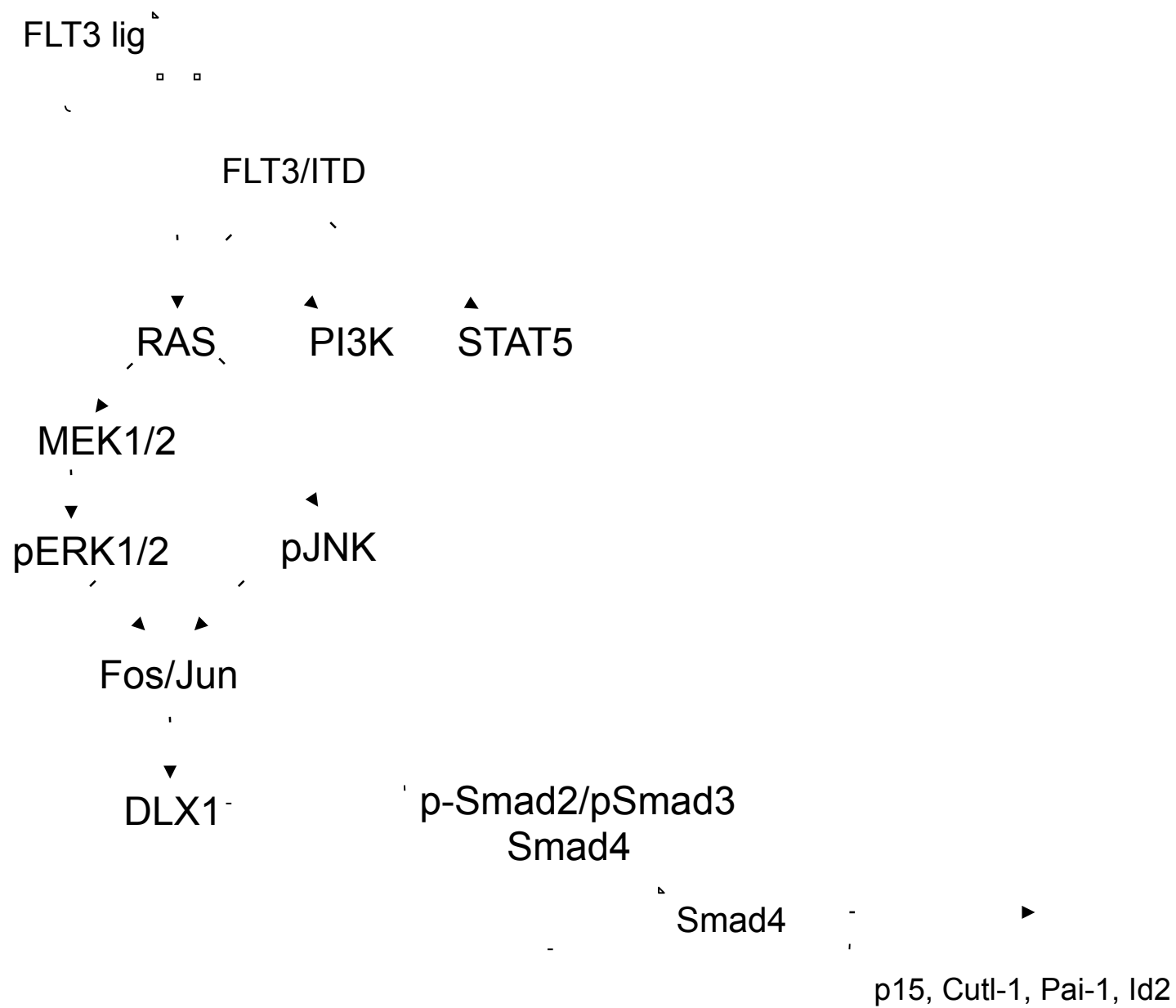


Table S1

	PKC412 (0.1µM)				
	0h	24h		0h	24h
HoxA1	11.69	11.715	Hox C5	ND	ND
HoxA2	13.78	11.7	Hox C6	9.84	8.7
HoxA3	13.94	12.06	Hox C8	ND	ND
Hox A4	10.98	10.47	Hox C9	10.95	9.66
HOXA5	11.88	10.91	Hox C10	11.27	10.53
HOXA6	12.47	13.06	Hox D3	ND	ND
HOXA7	ND	ND	Hox D4	ND	ND
HOXA9	6.05	5.19	Hox D8	ND	ND
Hox A10	5.81	5.49	Hox D9	ND	ND
Hox A11	ND	ND	Hox D10	ND	ND
Hox A13	ND	ND	Hox D13	ND	ND
Hox B2	ND	ND	PBX2	5.83	5.15
Hox B3	8.58	6.95	PBX3	6.2	5.81
Hox B4	7.52	6.5225	Meis1	5.01	4.13
Hox B5	12.79	10.38	Meis2	8.9	8.35
Hox B6	6.14	6.145	DLX1	5.28	7.5
Hox B7	11.48	10.36	DLX2	10.26	11.87
Hox B8	ND	ND	EN1	ND	ND
Hox B9	ND	ND	DLX6	ND	ND
Hox B13	ND	ND	GBX2	ND	ND
Hox C4	ND	ND			

Table S2

patient	diagnosis	Flt3 ITD	Flt3 D835	PML/RARa	AML1/ETO	CBFB/MYH11	other	blast count
M1	M4	+	-	-	-	-	-	87%
M53	M2	+	-	-	-	-	-	61%
M60	M4	+	-	-	-	-	-	67%
M73	M2	+	-	-	-	-	-	47%
M81	M3	+	-	+	-	-	-	88%
M82	M1	+	-	-	-	-	-	85%
M86	M3	+	-	+	-	-	-	73%
M312	M4	+	-	-	-	-	-	90%
M412	M4	+	-	-	-	-	-	93%
M16	M2	-	-	-	-	-	-	65%
M29	M2	-	-	-	-	-	-	68%
M36	M4	-	-	-	-	-	-	90%
M38	M2	-	-	-	-	-	-	37%
M87	M0	-	-	-	-	-	-	73%
M212	M4	-	-	-	-	-	-	20%
M281	M0	-	-	-	-	-	-	42%
M336	M5	-	-	-	-	-	-	80%
M337	M0	-	-	-	-	-	-	79%

Table S3

p15 fw 5`- GGCAGTCGATGCGTTCACTC

p15 rev 5`- TGCCATGCGCTCAAACATAAG

CUTL-1 fw 5`- CAGCGCCTGCACGATATTG

CUTL-1 rev 5`- AGAGCTATGGTTTCGGCTTGG

PAI-1 fw 5`- CCACAGACGCGATCTTCGTC

PAI-1 rev 5`- GTCCACTTGCTTGACCGTGC

p21 fw 5'- CAGCAGAGGAAGACCATGTGG

p21 rev 5'- GCCGTTTTTCGACCCTGAGAG

Id2 fw 5'- CTGACCACCCTCAACACG

Id2 rev 5'- CTTGTCCTCCTTGTAATGG