SUZ12 Is a Candidate Target of the Non-canonical WNT Pathway in the Progression of Chronic Myeloid Leukemia

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Polycomb proteins form multiprotein complexes that repress target genes by chromatin remodeling. In this work, we report that the *SUZ12* polycomb gene is over-expressed in bone marrow samples of patients at the blastic phase of chronic myeloid leukemia. We also found a direct interaction between polycomb group genes and the WNT signaling pathway in chronic myeloid leukemia transformation. Electrophoretic mobility shift assay (EMSA), Chromatin immunoprecipitation assay (ChIP), and mass spectrometry assays identified noncanonical WNT pathway members, such as WNT5A and WNT11, bound to the *SUZ12* promoter. Immunohistochemistry and immunofluorescence with WNT5A and WNT11 antibodies confirmed nuclear localization. Knockdown of *WNTs 1*, *5A*, and *11* with RNAi approaches showed that WNT members are capable of activating *SUZ12* transcription with varying promoter affinities. Finally, we suggest that SUZ12 is blocking cellular differentiation, as *SUZ12* knockdown release differentiation programs in chronic myeloid blastic phase (CML-BP) transformed cell line.

INTRODUCTION

Polycomb group proteins (PcG) are epigenetic regulators that operate as transcriptional repressors, silencing specific sets of genes by chromatin modification. Although primarily known for their role in maintaining cell phenotypes during the establishment of a body plan, several mammalian PcG members have been associated with the control of cell proliferation and with neoplastic development (Levine et al., 2004; Lund and van Lohuizen, 2004; Sparmann and Lohuizen, 2006).

Deregulation of PcG proteins has been observed in several cancer types, as is the case with the E2F/RB pathway that normally regulates the *PcG* gene family. Its frequent deregulation in neoplastic transformation probably leads to the abnormal expression of *PcG* genes in human tumors (Dahiya et al., 2001; Bracken et al., 2003).

Upregulation of the Enhancer of zeste (*EZH2*) gene has been significantly correlated with metastatic progression of prostate and breast cancers, and *BMI1* has been identified as a proto-oncogene cooperating with the transcriptional factor *MYC* in generating B and T cell lymphomas and in the progression of chronic myeloid leukemia (Lessard and Sauvageau, 2003; Sparmann and Lohuizen, 2006; Mohty et al., 2007). Another PcG protein, named Suppressor of Zeste 12 (*SUZ12*), is often upregulated in colon, breast, and liver tumors, but it has never been found to be altered in leukemias (Kirmiziz et al., 2003; Squazzo et al., 2006).

Interestingly, Kirmiziz et al. (2003), suggested a WNT/β-catenin-dependent regulation of *SUZ12* in solid tumors, like colon and liver. These authors indicated that recruitment of β-catenin to a TCF binding site at the *SUZ12* target promoter may be influenced by cell type, because β-catenin/TCF-mediated regulation did not occur in experiments with cell lineages derived from cervical cancer (HeLa cells) (Kirmiziz et al., 2003).

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder originating at the hematopoietic stem cell compartment, and its molecular signature is the *BCR-ABL1* gene

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rearrangement. Progression to blastic phase (BP) is characterized by loss of differentiation and resistance to therapy. Recently, an elegant study shed new light on the evolution of chronic myeloid leukemia, demonstrating that self-renewal mechanisms could be reactivated in committed hematopoietic progenitors by BCR-ABL1 interaction with the WNT signaling pathway (Jamienson et al., 2004).

WNT is a powerful signaling pathway that plays a crucial role in cell fate determination, survival, proliferation, and mobility in a variety of tissues. WNT abnormalities have been associated with a number of clinical conditions, mainly cancer. In CML-BP, several studies showed that β-catenin nuclear accumulation is indicative of transformation (Staal and Clevers, 2005; Khan and Bendall, 2006). This activation resulted from expression of the *BCR* gene, the *ABL1* fusion partner in the *BCR-ABL1* rearrangement, which was identified as a negative regulator of WNT signaling. *BCR* downregulates β-catenin/TCF-dependent transcription, resulting in decreased expression of *WNT* target genes like *MYC*. Conversely, the BCR-ABL1 oncogenic protein counteracts this inhibitory effect by tyrosine phosphorylation of the BCR protein, disrupting the BCR/β-catenin complex (Ress and Moelling, 2005).

Interestingly, we found for the first time that *SUZ12* is over-expressed in the bone marrow of patients with CML-BP. Our results suggests that this over-expression is not due to activation of the β-catenin/TCF complex. Using several approaches, including electrophoretic mobility shift assay (EMSA), chromatin immunoprecipitation (ChIP), and mass spectrometry, we identified proteins bound to the *SUZ12* promoter during CML evolution, and our data suggest that SUZ12 over-expression results from activation of a non-canonical WNT pathway. Moreover, we found that knockdown of *SUZ12* in the K562 cell line, suggests that SUZ12 may contribute to the undifferentiated phenotype of this phase.

MATERIALS AND METHODS

Cell and Bone Marrow Samples

All bone marrow samples were obtained from patients and donors admitted or registered at the Instituto Nacional de Câncer (Rio de Janeiro, Brazil), following the guidelines of the local Ethics Committee and the Helsinki declaration. Marrow aspirates were obtained from 20 healthy donors (mean age = 30; range = 20–37, male:female ratio = 9:11), 23 CML patients in chronic phase (mean age = 38; range = 31–48; male:female ratio = 13:10), and 22 CML patients in blastic phase (mean age = 34; range = 32–53; male:female ratio = 10:12).

Diagnoses and follow-ups were based on hematologic, cytogenetic, and molecular assays. Bone marrow mononuclear cells were isolated from 2-ml aspirates in a Ficoll-Hypaque density gradient. $CD34^+$ cells were purified from mononuclear cells of three healthy donors, three patients in chronic phase, and three patients in blastic phase with the Dynal CD34 progenitor cell selection system® (Dynal, Invitrogen). K562 were cultured in RPMI 1640 (Invitrogen), supplemented with 10% fetal calf serum.

RT-PCR Assays

For reverse transcriptase polymerase chain reaction assays (RT-PCR), total RNA was isolated with Trizol® (Invitrogen) from mononuclear bone marrow cells of healthy donors, patients, and cell cultures, and 1 μg of total RNA was transcribed and amplified with SuperScript One-step RT-PCR® (Invitrogen) with the following primers:

*SUZ*12VESP(s)5′-GCAGGCCAAAACGAACAAAAGC-3′

*SUZ*12VESP(a)5′-GTTTCGAGGGGAAGCA GATTCC-3′

GAPDH(s)5′-ACCACAGTCCATGCCATCAC-3′,

GAPDH(a)5-TCCACCACCCTGTTGCTGTA-3′

QRT-PCR Analysis

Quantitation of *SUZ12*, *WNT1*, *WNT5A*, and *WNT11* RNA transcripts was carried out by real-time PCR (QRT-PCR). Two micrograms of total RNA from mononuclear bone marrow cells, CD34+ purified bone marrow cells, and K562 cell line, were reverse transcribed with Superscript III Reverse transcriptase® (Invitrogen). cDNAs were mixed with SYBR Green PCR Master Mix® (Applied Biosystems) and specific primers. Real time PCR was performed in an ABI Prism 7000 thermocycler (Applied Biosystems),

with 40 cycles of 15 sec at 95°C and 2 m at 68°C. Expression levels were estimated in triplicate with specific and control primers. For each sample, the relative amounts of transcripts of the target gene and the internal control were estimated from a standard curve. Results were expressed in arbitrary units as the ratio of the target gene transcript/internal transcript (data represented by average ± SD of three measurements).

Electrophoretic Mobility Shift Assays (EMSA)

Ten micrograms of nuclear protein extracts were prepared as previously described (Binato et al., 2006) and incubated with 60,000 cpm of P^{32} labeled *SUZ12* promoter, 1 μg of poly (dI.dC)(dI.dC) (GE healthy care), 2 μl of binding buffer (50 mM HEPES—pH 7.4, 300 mM KCl, 5 mM EDTA, 5 mM DTT, 11.5% Ficoll) in a total volume of 20 μl, for 40 min at room temperature (25°C). Reactions were run in 4.5% polyacrylamide gels in 0.5X TBE for 90 min.

The design of double-stranded competitors (Kirmiziz et al., 2003) was based on the upstream *SUZ12* promoter. The following oligonucleotides were used as competitors: (i) oligonucleotides containing the TCF consensus binding site (TCF2), (ii) oligonucleotides containing a mutated site (TC2mt), and (iii) oligonucleotides containing only the TCF core binding site (cbs).

TCF2-5′AGCTTTGTGTCTATCG***TTCAAAG***CAAGACCTGGCCA3′

TCF2mt-5′AGCTTTGTGTCTATCG***GCCAGAG***CAAGACCTGGCCA3′

TCFcbs-5′GGTAAGA***TCAAAG***GG 3′

For supershift analysis of the *SUZ12* promoter, 1 μg of the anti-WNT1 (Zymed), anti-WNT5A (Santa Cruz), anti-WNT11 (Santa Cruz), and anti-βCATENIN (Sigma) antibodies were included in the first incubation. Oligonucleotides sequences based on *MDR1/ABCB1* promoter containing TCF binding site consensus were used as controls. Anti-SMAD1 antibody (Santa Cruz) was used as a nonrelated antibody.

MDR -5′GTAGGAATA***CAAAG***AATACT3′
MDRmt 5′GTAGGAATA***CAGAG***AATACT3′

Mass Spectrometry Identification of EMSA Shift Complexes

Shifted EMSA bands were excised from gels. Proteins were rehydrated overnight in 200 μl of elution buffer (3% Triton X-100, 20 mM HEPES—pH 7.6, 1 mM EDTA, 100 mM NaCl, 2 mM DTT, 0.1 mM PMSF, and protease inhibitors). Subsequently, sliced gel fragments were incubated at 37°C for 3 hr and centrifuged at 10,000 rpm for 15 min. Supernatants were precipitated with the 2D Cleanup kit (GE healthy care) following the manufacturer's protocol, and pellets were resuspended in SDS loading buffer (Ossipow et al., 1993). Suspensions were run in 7.5% SDS-polyacrylamide gels. Gels were stained with nondestructive silver staining (Shevchenko et al., 1996). Discernible protein bands were excised, ground, and processed for mass spectrometry.

Peptide mass fingerprinting (PMF) and MS/MS analyses were carried out with a Voyager-DE Pro MALDI-TOF and a MALDI-TOF-TOF 4700 (Applied Biosystems) mass spectrometers. Proteins were identified using an automated database (MASCOT; Matrix Science, London, United Kingdom) of all tandem mass spectra by comparisons with the Human International Protein Index sequence database (IPI, version 3.11).

Western Blotting and Immunoprecipitation

Total and nuclear protein extracts from healthy donors, K562 cells, and bone marrow samples from CML patients in chronic and blastic phases were prepared as previously reported (Binato et al., 2006).

Cell lysates were subsequently run in 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad, CA). Briefly, membranes were incubated with several primary antibodies and then with the appropriate secondary IgG conjugated with horseradish peroxidase (Santa Cruz, CA). Antibody binding was detected by enhanced chemiluminescence ECL Plus Western Blotting Detection Reagents® (GE healthy care, United Kingdom). Membranes were stripped between incubations with stripping buffer (10 mM Tris-HCl pH 2.3, 150 mM NaCl). The same primary antibodies were used for EMSA assays, including anti-SUZ12 (Abcam, USA). Anti-mouse, anti-goat, and anti-rabbit HRP (Santa Cruz, CA) were used as secondary antibodies. For immunoprecipitation assays, total, nuclear, and cytoplasmatic protein extracts were prepared as mentioned above. Protein extracts from cells untreated and treated with 10 μM LiCl were immunoprecipitated with 2.5 μg of anti-β-catenin (Sigma, USA) and with

anti-SUZ12 (Abcam, USA) antibodies following the Immunoprecipitation Starter Pack kit (GE) protocol. The immunoprecipitates were separated by SDS-PAGE followed by immunoblotting with the respective antibodies. Anti-CYCLIN D1 (Santa Cruz, USA) and Anti-HSP56 (Santa Cruz, USA) antibodies were used as controls for the purity of the nuclear and cytoplasmic fractions.

ChIP Assays on Native Chromatin

Chromatin from the K562 cell line was fractionated by incubation of purified nuclei with micrococcal nuclease (MNase) (Umlauf et al., 2003). Chromatin immunoprecipitation with WNT1, 5A, 11, and anti-β-catenin antibodies was performed as described Umlauf et al. (2003) protocol, and DNA extractions from bound fractions were performed following the Abcam (www.abcam.com) protocol. The immunoprecipitated DNA was amplified using the following SUZ12 promoter sequence primers:

*SUZ*12p(s)5′-TGTGCCAGAAGACTGAAATGGG-3′

*SUZ*12p(a)5′-TTGAAGCGATTCTCCTGCCTCA-3′

For a positive control of the β-catenin ChIP assay, the *MDR1/ABCB1* gene promoter was also amplified. Anti-SMAD1 (Santa Cruz, CA) was used as unrelated antibody.

*MDR/ABCB*1p(s)5′-CAACTCGTCAAAGGAATTAT-3′

*MDR/ABCB*1p(as)5′-TTGTACCTTTGATCAA CACC-3′

Immunohistochemistry, Immunofluorescence, and FACS Analysis

Immunohistochemical staining was performed in formalin-fixed, paraffin-embedded bone marrow slides from five CML patients in chronic phase and from six in blastic phase according to standard procedures. Heat-induced epitopes were retrieved with Tris buffer pH = 9.9 (Dako, Denmark) in a microwave processor. Tissue sections were subsequently incubated with anti-WNT5A and anti-WNT11 (1:100) overnight, and with anti-mouse IgG and peroxidase for 30 min at room temperature. Development was carried out with 3,3′-diaminobenzidine/H_2O_2 (Dako, Denmark) and hematoxylin counterstains. Slides were analyzed and photographed with a Nikon Eclipse E600 microscope. Immunofluorescence staining was carried out in other slides from the same patients, with exposure to primary antibodies followed by conjugation to the Alexa Fluor488 secondary antibody. Slides were counterstained with DAPI (Molecular Probes), analyzed, and photographed with a Zeiss AxioImager ApoTome microscope. For FACS analysis, antibodies that recognize cell surface myeloid-specific antigens *CD11B*-PE and *CD15*-FITC conjugated (Becton Dickinson) were used. Appropriated isotype-matched controls (Becton Dickinson) were used.

RNAi Knockdown and Transfection

All RNA oligonucleotides described in this study were synthesized and purified using high-performance liquid chromatography (HPLC) at Integrated DNA Technologies (Coralville, Iowa), and the duplex sequences are available upon request. RNAi knockdown and transfections were performed following the manufacturer's protocols of the TriFECTa Dicer-Substrate RNAi kit (Integrated DNA Technologies, Coralville, IA) and the CodeBreaker siRNA Transfection Reagent (Promega, USA). K562 cells (1×10^6 cells per well) were split in 24-well plates to 60% confluency in RPMI media 1 day prior to transfection. The TriFECTa kit contains control sequences for RNAi experiments which include a fluorescent-labeled transfection control duplex and a scrambled universal negative control RNA duplex that is absent in human, mouse, and rat genomes. Fluorescence microscopy and FACS monitored the transfection efficiency according to the manufacturer's recommendations. Only experiments in which transfection efficiencies were $\geq$90% were evaluated. RNA levels were measured 36 hr after transfection, and protein levels were measured 80 hr later. All duplexes used were evaluated at 10, 1, and 0.1 nM. All transfections were minimally performed in duplicate, and the data were averaged. Assays done in duplicate include "SD" in data reporting. Knockdown of *SUZ12*, *WNT1*, *WNT5A*, and *WNT11* was performed, and RNA, protein extraction, QRT-PCR, Western blot, and FACS analysis were done as described above.

RESULTS

Suz12 Is Upregulated in CML Blastic Crisis

We analyzed gene expression profiles from chronic and blastic phase samples from the same

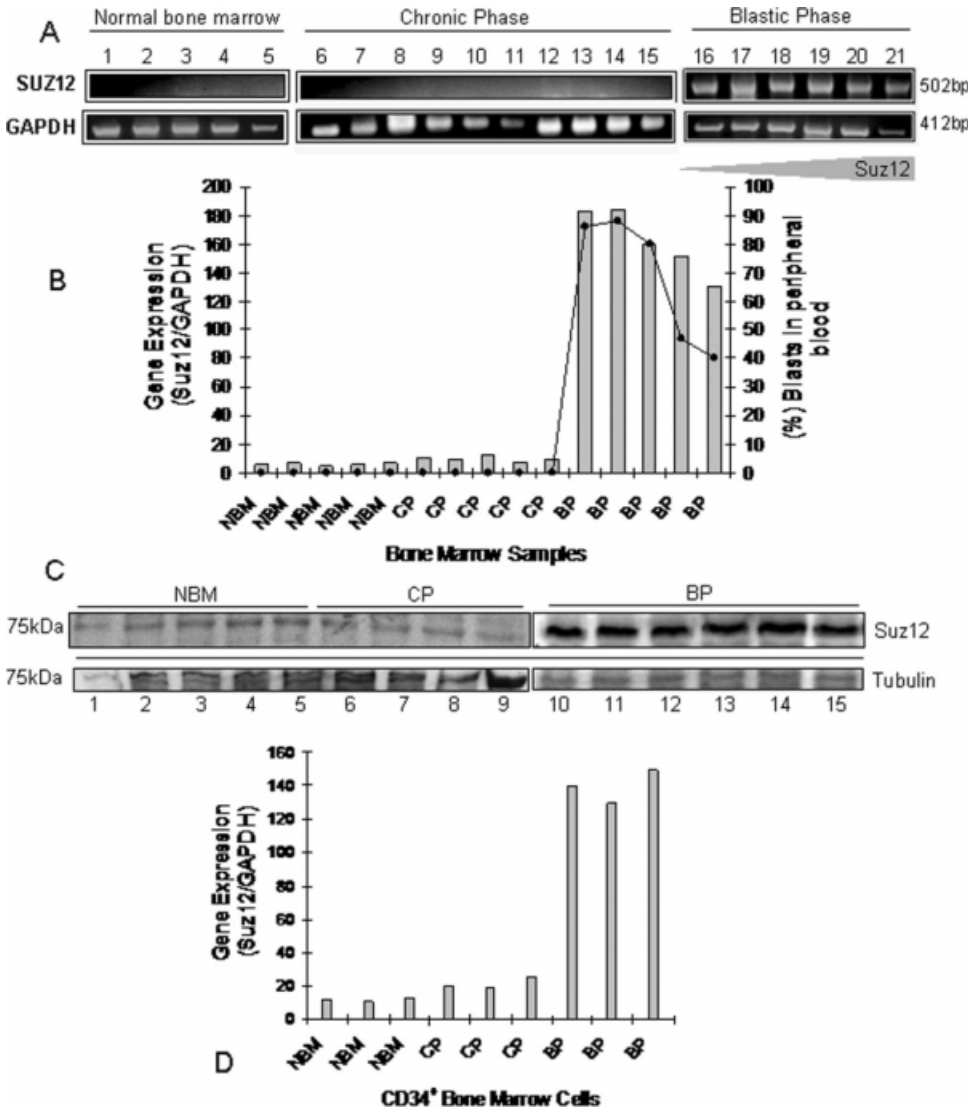

Figure 1. (A) RT-PCR amplification of SUZ12 VESP box from five normal controls, ten chronic phase patients and six blastic phase patients. (B) Real Time RT-PCR from: five normal bone marrow, five chronic phases, and five blastic phase samples. Suz12 gene expression was compared with the percentage of blasts in the peripheral blood. (C) Suz12 protein expression from five normal bone marrow samples, four chronic phase samples, and six blastic phase samples. (D) RealTime RT-PCR analysis of $CD34^+$ purified cells from three normal bone marrow, three chronic, and three blastic phases samples NBM: normal bone marrow; CP: chronic phase; BP: blastic phase.

patients using the differential display method (Liang et al.,1994; Liang and Pardee, 1995, 1998) and we identified *Suz12* gene as upregulated in the blast phase of CML (data not shown). This experiment was repeated twice using five patients. These patients were followed and RNA samples were extracted in chronic and in blastic phase of each patient. RNAs were transcribed and the cDNAs expression profiles were compared as described method (Liang et al., 1994; Liang and Pardee, 1995). Differentiated expressed genes were identified by sequencing and database search (BLAST engine). The most interesting gene overexpressed found in these analyses was *SUZ12*, a Polycomb gene family member.

To further investigate this upregulation in CML samples, we analyzed *SUZ12* expression in bone marrow mononuclear cells from five healthy donors, ten patients in chronic phase, and six in blastic phase (Fig. 1A). Amplification of the *SUZ12* VESP domain showed an increase in *SUZ12* expression in all blastic samples. Conversely, in donors and in chronic phase samples, *SUZ12* expression was undetected by conventional RT-PCR assays.

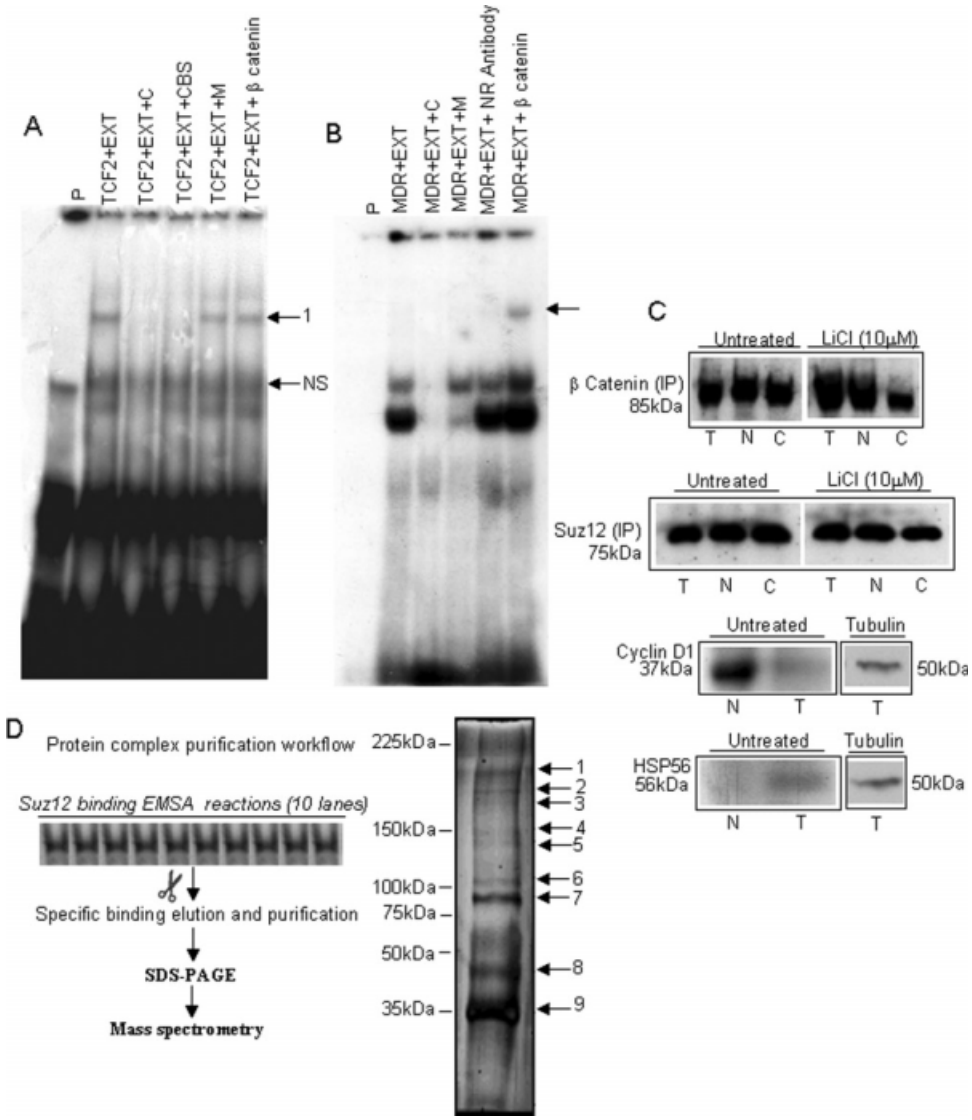

Figure 2. (A) Gel shift analysis of binding reactions using the Suz12 promoter sequence containing the TCF consensus binding site and K562 nuclear extracts. Lane indicated as "TCF2" represents binding reaction with K562 nuclear extracts. "+C" represents competition reaction with 200× excess fold of the unlabeled probe. "+CBS" represents competition reaction with the oligonucleotide containing only the TCF2 core binding site, "+M" represents competition reaction with 200× molar excess of a mutated version of the TCF2 oligonucleotide and "+β-catenin represents the supershift reaction. The arrow number 1 shows the specific binding and the arrow NS shows nonspecific binding. (B) Gel shift analysis of binding reactions using the MDR1/ABCB1 promoter sequence containing the TCF consensus binding site and K562 nuclear extracts. Lane indicated as "MDR" represents binding reaction with K562 nuclear extracts. "+C" represents competition reaction with 200× excess fold of the unlabeled probe. "+M" represents competition reaction with 200× molar excess of a mutated version of the MDR oligonucleotide, "+NR antibody" represents incubation with anti-smad1 antibody and "+β-Catenin" represents the supershift reaction. The arrow shows the supershift binding. (C) Immunoblotting with β-catenin and Suz12 antibodies in K562 cell extracts. Cyclin D1 and HSP56 were used as purity controls T: total protein extract, N: nuclear extract, and C: cytoplasmic extract. (D) Mass spectrometry and SDS-PAGE analysis of the Suz12 binding protein complex.

To quantify *SUZ12* differential expression between donors and the chronic and blastic phase samples, we used real-time PCR to amplify samples from five donors, five chronic phase, and five blastic phase patients (Fig. 1B). We found a 10-fold increase in *SUZ12* in blast phase. We also examined SUZ12 protein expression in five samples of normal bone marrow, four of chronic phase and six of blastic phase by Western blotting and we confirmed that this gene is upregulated in blastic transformation (Fig. 1C).

Since *SUZ12* transcript levels were associated with high blast cell counts in peripheral blood (Fig. 1B), we investigated *SUZ12* expression levels in $CD34^+$ cells isolated from bone marrow of normal donors and from patients in chronic and

blastic phases. These findings indicate that blastic transformation, rather than the accumulation of immature cells, accounted for the highest levels of SUZ12 expression (Fig. 1D).

β-Catenin Is not Recruited to the SUZ12 Promoter in CML Progression

In some solid tumors, the WNT/β-catenin-dependent regulation of *SUZ12* occurs by β-catenin recruitment to a specific TCF binding site on the *SUZ12* promoter (Kirmiziz et al., 2003; Kim et al., 2004).

To verify whether *SUZ12* upregulation in CML blast crisis resulted from β-catenin recruitment to the TCF consensus binding site, EMSA assays were performed with the *SUZ12* upstream promoter region and K562 nuclear extracts. These assays showed a strong, specific binding (Fig. 2A) whereas competitive assays using a 200-fold excess of TCF2 oligonucleotide (as the unlabeled competitor) deterred specific binding (Fig. 2A). Similar results were observed in competitive assays with a TCF2 core binding site oligonucleotide (TCFcbs) (Fig. 2A) or with an unlabeled, mutated oligonucleotide (TCF2mt). As shown in Figure 2A, mutations affecting the TCF consensus site of the competitor did not affect binding of the wild-type oligonucleotide, confirming its specificity in K562 nuclear extracts.

To confirm whether *SUZ12* regulation was mediated by the β-catenin/TCF complex in K562 cells, we searched for putative gel shifts associated with the β-catenin antibody in reactions with the TCF2 probe (supershift assay). Our findings show that the β-catenin/TCF complex did not alter the binding pattern with the *SUZ12* promoter in K562 cells (Fig. 2A). An in vivo confirmation of this result was done by ChIP assay (Fig. 3B). As positive control we included a β-catenin supershift assay, which occurs in TCF consensus binding sites, in *MDR/ABCB1* gene promoter. We used sequences from *MDR1/ABCB1* promoter as labeled oligonucleotides. Anti-SMAD1 (Santa Cruz, CA) was used as a nonrelated antibody (Fig. 2B).

Furthermore, to confirm that SUZ12 expression was not induced by or altered by high β-catenin levels or by β-catenin translocation to the nucleus, K562 cells were incubated with 10 μM LiCl for 24 hr, to mimic WNT signaling, as previously reported (Stambolic et al., 1996). Figure 2C shows that, even with the availability of nuclear β-catenin following LiCl treatment, SUZ12 protein expression remained the same as in untreated controls.

Despite the high levels of nuclear β-catenin reported in CML blastic phase (Jamienson et al., 2004) our supershift experiments with a β-catenin antibody did not detect any specific complex with the *SUZ12* promoter in CML cells. These results suggest that, in CML, *SUZ12* is not among the genes targeted by β-catenin.

SUZ12 Is a Putative Target of the WNT Noncanonical Signaling Pathway

To identify the specific EMSA protein complex bound to the *SUZ12* promoter in CML-BP cells, proteins in complex with the *SUZ12* promoter were analyzed by mass spectrometry using 10 replicates of specific shifted EMSA bands. The bands were then run on gels and excised for further analysis (Fig. 2A). SDS-PAGE separation (Fig. 2D) of eluted proteins from the sliced gels showed nine different proteins, which were identified by digestion and mass spectrometry. These included catenin P120 and WNT11 (Table 1).

To confirm the presence of WNT members bound to the *SUZ12*promoter, we performed supershift assays with antibodies against WNT1, WNT5A, and WNT11 proteins (Fig. 3A). Addition of anti-WNT signaling members resulted in an additional shifted band. Assays performed with anti-WNT5A and anti-WNT11, members of the noncanonical pathway, (Fig. 3A), bound more intensely to the *SUZ12* promoter than WNT1, a member of canonical pathway (Fig. 3A, lane TCF2+EXT+WNT1). Using an in vivo approach with chromatin immunoprecipitation experiments, we demonstrated that WNT family members definitely bind to the *SUZ12* promoter (Fig. 3B). We quantified by real-time quantitative PCR the bound and unbound fractions obtained in the ChIP assays as described (Umlauf et al., 2003) (Fig. 3C). We showed that, as expected, *MDR1/ABCB1* promoter was not enriched by WNT proteins but only by β-catenin protein. These results confirmed the supershift, which shows that WNT proteins can be enriched in *SUZ12* promoter.

Nuclear Localization of WNT Noncanonical Signaling Pathway Members in CML Bone Marrow

To confirm the nuclear localization of the noncanonical WNT pathway members in CML blasts, we analyzed nuclear expression of WNT11

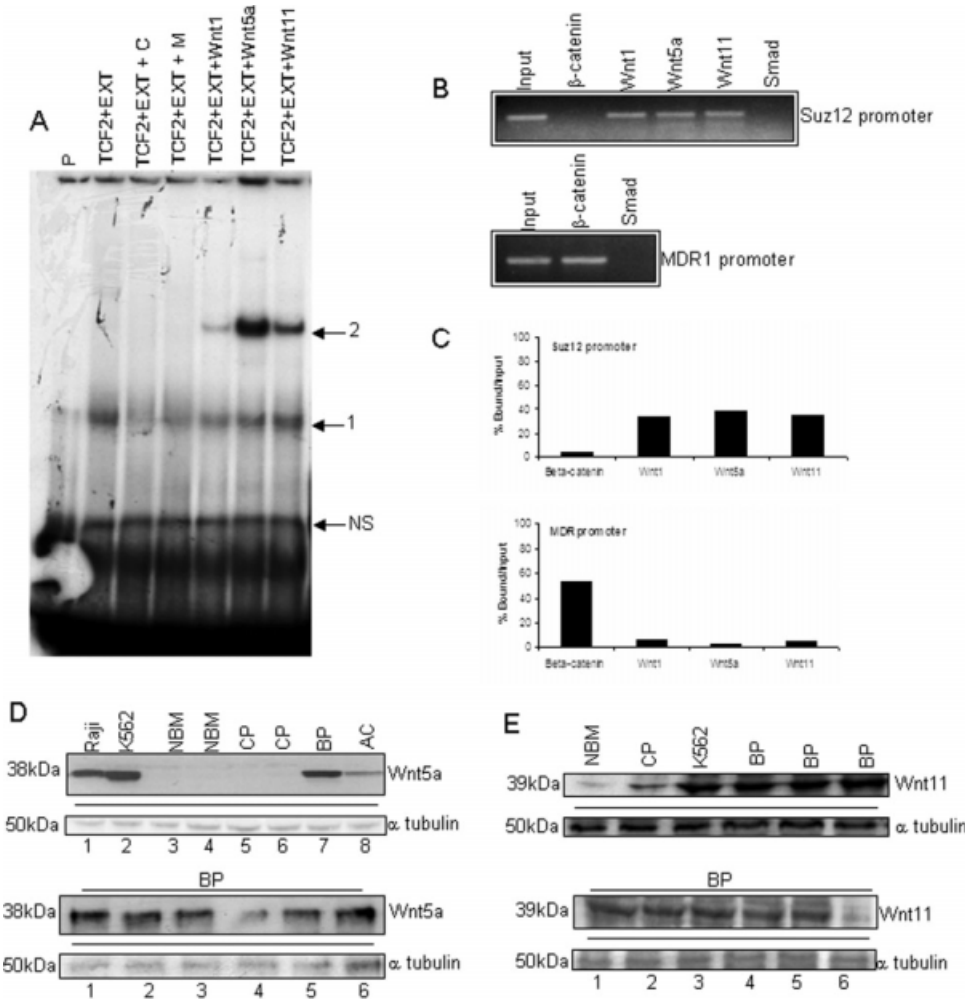

Figure 3. (A) Gel shift analysis of binding reactions using the Suz12 promoter sequence containing the TCF consensus binding site and K562 nuclear extracts. "TCF2" represents the binding reaction with K562 nuclear extracts. The competition reaction with 200× excess fold of unlabeled probe is represented by "+C." The competition reactions with 200× molar excess of mutated version of the TCF2 oligonucleotide are represented by "+M." The supershift assays using Wnt1, Wnt5, and Wnt11 antibodies are represented by "+Wnt1," "+Wnt5a," and "+Wnt11," respectively. The Arrow 1 shows the specific binding and the Arrow 2 shows the specific supershift binding. NS represents nonspecific binding. (B) Chromatin immunoprecipitation assay (ChIP) with β-catenin, Wnt1, Wnt5a, and Wnt11 antibodies showing the amplification of Suz12 and MDR1 promoter. Anti-smad1 was used as nonrelated antibody. (C) Chromatin immunoprecipitation assay (ChIP) quantification. In upper panel quantification of Suz12 promoter in: β-catenin, Wnt1, Wnt5a and Wnt11 immunoprecipitated fractions. In botton panel quantification of MDR1/ABCB1 promoter in: β-catenin, Wnt1, Wnt5a, and Wnt11 immunoprecipitated fractions. Bound and unbound fractions were quantified and used to calculate bound/input ratio. (D) Wnt5a protein expression in transformed cell lines (Lanes 1 and 2), normal controls (Lanes 3 and 4) and CML patients (Lanes 5–14). (E) Wnt11 protein expression in normal control (lanes 1), CML-CP (lane 2) and CML-BP patients (Lanes 3–12). NBM: normal bone marrow; CP: chronic phase; BP: blastic phase; and AC: accelerate phase.

and WNT5A proteins by Western blotting (Figs. 3D and 3E) in samples from twelve patients in blastic phase, three in chronic phase, one in accelerated phase, two transformed lineages (Raji and K562), and three normal bone marrow samples. Significant WNT5A and WNT11 expression was only observed in transformed cells and in accelerated and blastic CML phases.

Finally, by immunohistochemistry, we compared WNT5A and WNT11 expression in CML bone marrow from five patients in chronic and from six in blastic phase. WNT5A and WNT11 were significantly expressed in blast nuclei, especially in progenitor cells of paratrabecular localization (Fig. 4). We also confirmed the nuclear localization of WNT5A and WNT11 in different slides from the same patients by immunofluorescence (Fig. 5). Altogether, these results suggest that a noncanonical WNT signaling pathway is active in CML transformation.

WNT Proteins Are Required for Activation of the SUZ12 Promoter in CML Cells

To strengthen our observations, we performed functional analysis using RealTime-RT-

TABLE 1. Mass Spectrometry Identification of EMSA Shift Complex

Gel slice	ID	Accession	Protein name	Mows score	Coverage (%)	MW (Da)	MS	MS/MS
1	CHD1_HUMAN	O14646	Chromodomain-helicase-DNA-binding protein 1	2.345e+08	20.0	196.518	X	X
2	ITSN1_HUMAN	Q15811	Intersectin 1	3.700e+06	31.0	195.534	X	X
3	RPA1_HUMAN	O95602	DNA-directed RNA polymerase I largest subunit	2.596e+06	38.0	194.193	X	X
4	LAP2_HUMAN	Q96RT1	Erbin	3.678e+07	36.0	158.238	X	X
5	CND1_HUMAN	Q15021	Condensin subunit 1	1.113e+06	40.0	157.171	X	X
6	Q96SE1_HUMAN	Q96SE1	NGAP-like protein	2.395e+06	33.0	117.651	X	X
7	CTND1_HUMAN	O60716	Catenin p120	3.544e+07	42.0	108.170	X	X
8	WNT11_HUMAN	O96014	Wnt-11 protein	3.256e+06	48.0	39.180	X	X
9	T2EB_HUMAN	P29084	TFIIE-beta	3.496e+07	51.0	33.044	X	X

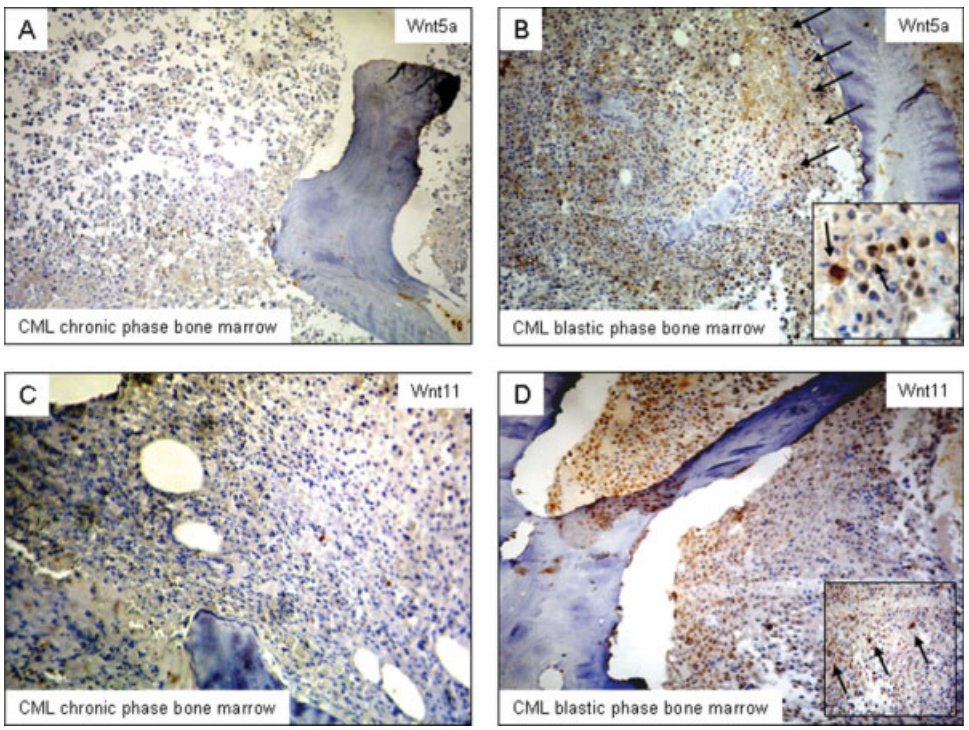

Figure 4. Immunohistochemistry staining of WNT5A (A) and (B) and WNT11 (C) and (D) in CML bone marrow slides. The arrows indicate the higher nuclear staining of WNT5A and WNT11 in bone marrow cells. Magnification ×400 fold.

PCR on *WNT1*, *WNT5A*, and *WNT11* knockdown in the K562 cell line. To understand if the WNT proteins are required for activation of the *SUZ12* promoter in CML transformed cells, we studied the effect of downregulating *WNT1*, *WNT5A*, and *WNT11* on *SUZ12* expression. Using an siRNA approach, a reduction of 75, 95, and 56% in *WNT1*, *WNT5A*, and *WNT11*, respectively, was achieved when compared with the mock knockdown cells or the scrambled control sequence (Fig. 6A). Thus, we used these samples to evaluate *SUZ12* expression. The knockdown cells of *WNT1*, *5A*, and *11* shows *SUZ12* expression reduction of 55, 90, and 72%, respectively (Fig. 6B).

SUZ12 Knockdown Induces Cellular Differentiation in CML Blastic Cells

We next investigated the biological contribution of SUZ12 to the progression toward blastic phase by examining its contribution to the undifferentiated status of CML-BP cells. We investigated whether *SUZ12* knockdown affects the global cell differentiation of the K562 cell line. Using siRNA, we decreased *SUZ12* expression in

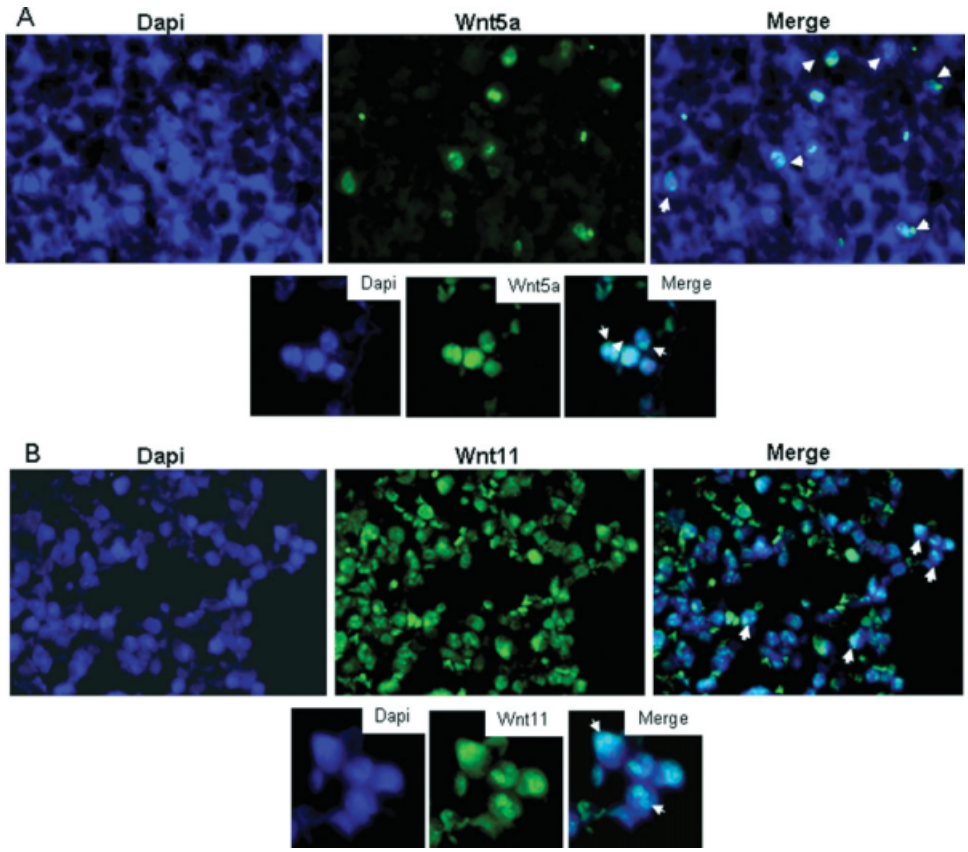

Figure 5. Immunofluorescence staining of WNT5A (A) and WNT11 (B) in CML bone marrow slides from a blastic phase patient. The arrows indicate the nuclear localization of WNT5A and WNT11 in bone marrow cells. Magnification ×400 fold.

K562 cells and quantified the levels of CD15 and CD11B by FACS analysis. These markers were used as indicators of maturation of the hematopoietic cells and also as granulocytic markers, as previously demonstrated (Lanotte et al., 1991). Results for CD11B were negative (data not show). The result shows that CD15 levels increased by 41.7% when *SUZ12* levels were decreased by siRNA, indicating that K562 cells are more prone to differentiation when *SUZ12* levels are decreased, or that reduced expression of *SUZ12* may release differentiation of the granulocytic program (Figs. 6C and 6D).

DISCUSSION

Polycomb proteins are likely to form multiprotein complexes that repress target genes by modifying their chromatin structure. In cancer, misexpression of several polycomb group proteins has been reported, although their role in cancer development is still poorly understood (Dahiya et al., 2001; Bracken et al., 2003; Cao and Zhang, 2004; Levine et al., 2004; Squazzo et al., 2006).

Particularly in CML, *BMI1* upregulation occurs in the advanced phase of the disease (Mohty et al., 2007). Mihara et al., (2007) suggested that it may be of help to maintain a more primitive cell stage by blocking normal differentiation programs and by inducing proliferation programs through different pathways, like through the methylation of *P15-INK4B* and *P16-INK4* or by deregulation of the E2F/RB1 pathway (Mihara et al., 2007).

Despite the fact that *BMI-1* deregulation occurs in CML and its expression is connected to several oncogenic processes, *BMI-1* seems to play a secondary role in CML transformation, due to its redundant role in the PcG maintenance complex it has been demonstrated that knockdown of *SUZ12* not only reverses histone modification, but it also induces DNA demethylation of *PML-RARALPHA* target genes (Merkerova et al., 2007; Villa et al., 2007). Moreover in acute myeloid leukemia, with the (t15; 17)-translocation, this

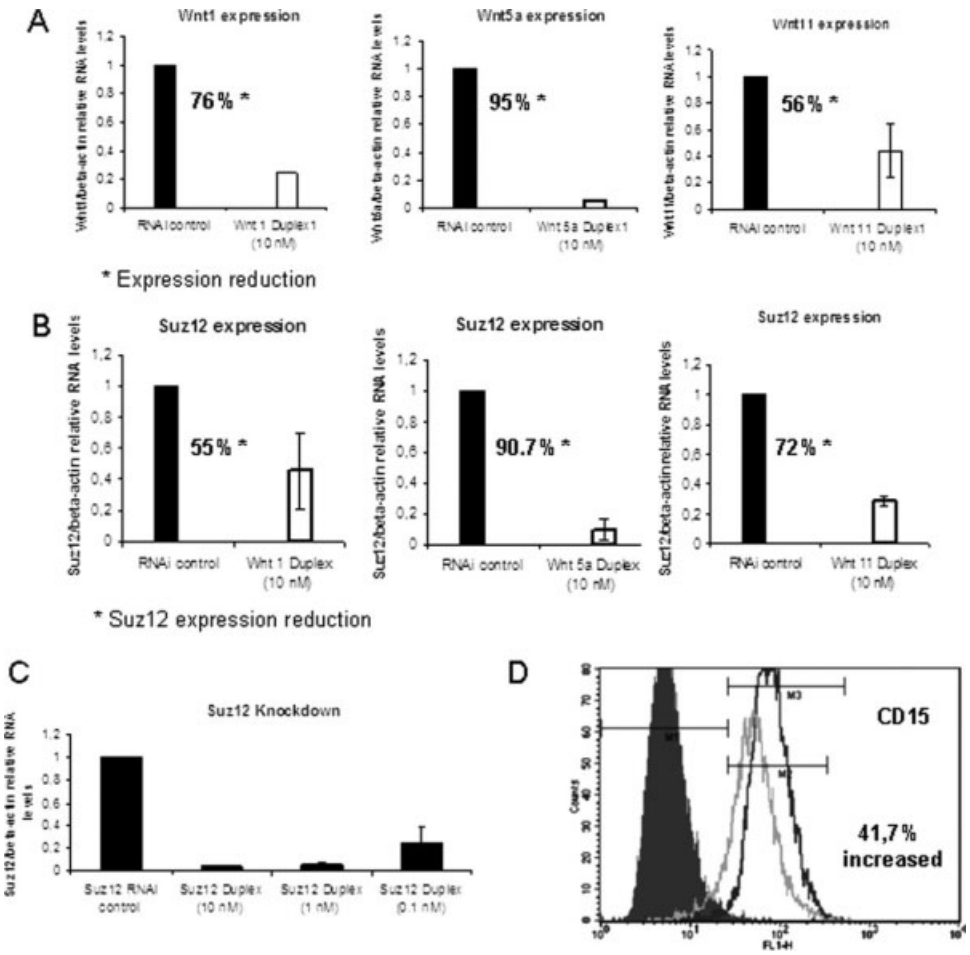

Figure 6. RNAi knockdown experiments. (A) Expression analysis of WNT1, WNT5A, and WNT11 K562 knockdown cells. (B) Expression analysis of SUZ12 gene in WNT1, 5A, and WNT11 knockdown cells. (C) Expression analysis of SUZ12 knockdown. (D) FACS analysis of SUZ12 knockdown cells. CD15 expression in K562 knockdown cells. M1 represents unlabeled K562 scrambled control. M2 represents K562 scrambled control labeled with CD15 and M3 represents K562 cells transfected with SUZ12 duplex (10 nM) after 80 hr-incubation.

modification results in reactivation and granulocytic differentiation (Villa et al., 2007).

Here, we describe for the first time that *SUZ12* is overexpressed in bone marrow cells of patients with CML-BP, suggesting that the blastic phase may be correlated with polycomb epigenetic transcriptional inhibition. The blastic phase might originate from more differentiated myeloid-restricted progenitor cells or from B-cell progenitors by acquisition of mutations that confer a self-renewal capacity and the suppression or disruption of further differentiation. Moreover, the overexpression of *SUZ12* in CML CD34+ cells, revealed by the QRT-PCR assay, highlights the transformation potential of this gene in CML stem cells, as CD34+ cells from normal donors and CML-CP do not over-express *SUZ12*.

Some reports have shown that the WNT pathway can activate the *SUZ12* promoter in vivo and in vitro in specific cell types like colon and liver tumor cells (Kirmiziz et al., 2003). In these cells, *SUZ12* is likely to represent a new β-catenin target whose expression increases by high levels of nuclear β-catenin. However, our results suggest that in CML this is not the case, because β-catenin did not seem to recognize the same *SUZ12* promoter consensus-binding site in EMSA, supershift, and ChIP assays.

Instead, our results suggest that *SUZ12* is regulated by a noncanonical WNT pathway in CML-BP. First, mass spectrometry analysis of proteins in shift complexes identified catenin P120 and WNT11, in addition to several other proteins. WNT11 and WNT5A are known to operate through a noncanonical pathway independently of β-catenin. Analyses by supershift, Western blotting, immunohistochemistry, immunofluorescence, and ChIP assays, suggest that WNT11, WNT5A, and WNT1 are present in blast nuclei. Moreover, WNT proteins appear to be part of

the protein complex bound to the *SUZ12* promoter at the TCF recognition region, although only WNT11 was detected in our MS/MS analysis, likely due to its higher concentration.

Characterized as secreted proteins, the nuclear localization of WNT members has already been reported (Struewing et al., 2006). Struewing et al. (2006), proposed that signals targeting WNT molecules to endoplasmic reticulum or mitochondria are located at their N-terminal region and that post-translational regulation may lead to loss of N-terminal sequences resulting in nuclear localization. Recently, it has been shown that mutations or deletions of the upstream initiation codons abrogate these translation inhibitory effects, demonstrating that WNT13 nuclear localization is controlled by upstream open reading frames (Tang et al., 2008).

Knockdown experiments of WNT proteins confirmed the downregulation of *SUZ12* expression and revealed the regulatory potential of WNT proteins in *SUZ12* expression. WNT5A and WNT11 had a stronger effect in this regulation, probably due to affinity characteristics. Moreover, flow cytometric analyses of *SUZ12* knockdown cells demonstrated an effect on granulocytic differentiation, highlighting the potential of SUZ12 to block the differentiation of CML cells.

Our results show that in CML-BP, PcGs are active during the course of the disease and may epigenetically inhibit genes involved in differentiation, through a noncanonical WNT signaling pathway, whereas the canonical WNT signaling pathway stimulates proliferation via β-catenin and BCR-ABL1-targeted genes. Future studies of specific receptor–ligand interactions in CML transformation will contribute to understanding the mechanistic interaction between both canonical and noncanonical WNT pathways in this disease.

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