**SALL4**, a novel oncogene, is constitutively expressed in human acute myeloid leukemia (AML) and induces AML in transgenic mice

Yupo Ma, Wei Cui, Jianchang Yang, Jun Qu, Chunhui Di, Hesham M. Amin, Raymond Lai, Jerome Ritz, Diane S. Krause, and Li Chai

**Introduction**

Myelodysplastic syndrome (MDS) is a hematologic disease marked by the accumulation of genomic abnormalities at the hematopoietic stem cell (HSC) level leading to pancytopenia, multilineage differentiation impairment, and bone marrow apoptosis.1 Mortality in this disease results from pancytopenia or transformation to acute myeloid leukemia (AML). AML is a hematologic cancer characterized by the accumulation of immature myeloid precursors in the bone marrow and peripheral blood. From the analysis of chromosomal translocation in bone marrow samples from AML patients, it is clear that transcription factors critical for hematopoiesis play an important role in leukemogenesis.2-5

The pathogenesis of AML is considered to involve multistep genetic alternations.6 Because only HSCs are considered to have the ability to self-renew, they are the best candidates for the accumulation of multistep, preleukemic genetic changes and transforming them into so-called leukemia stem cells (LSCs).7-9 Alternatively, downstream progenitors can acquire self-renewal capacity and give rise to leukemia. A good example is the Wnt/β-catenin signaling pathway, which has been associated with the self-renewal of normal HSCs and the granulocyte-macrophage progenitors (GMPs) of chronic myeloid leukemia (CML).10-15 LSCs are not targeted under current chemotherapy regimens and have been found to account for drug resistance and leukemia relapse.8,9 Hunting for genes or signaling pathways involved in leukemia self-renewal will promote the development of more effective leukemia treatments.

The **SALL** gene family, **SALL1**, **SALL2**, **SALL3**, and **SALL4**, was originally cloned on the basis of its DNA sequence homology to *Drosophila spalt* (**SALL**).6,16-19 In *Drosophila*, **SALL** is a homeotic gene essential for development of posterior head and anterior tail segments. It plays an important role in tracheal development,20 terminal differentiation of photoreceptors, and wing vein placement.21 In humans, the **SALL** gene family is involved in normal development, as well as tumorigenesis.19,22-27 **SALL** proteins belong to a group of C2H2 zinc finger transcription factors characterized by multiple finger domains distributed over the entire protein.28 During the tracheal development of *Drosophila*, **spalt** is an activated downstream target of Wingless, a Wnt ortholog.29 Of interest, Sato et al30 demonstrated that **SALL1** interacted with β-catenin by functioning as a coactivator, suggesting that the interaction between **SALL** and the Wnt/β-catenin pathway was bidirectional.

We report here on the identification of **SALL4** isoforms and their constitutive expression in all human AML we examined. The direct impact of **SALL4** expression in AML was tested in vivo. We show that constitutive expression of **SALL4** in mice is sufficient to induce MDS-like symptoms and transformation to AML that is transplantable. We also demonstrate that **SALL4** is able to bind β-catenin and activate the Wnt/β-catenin signaling pathway. **SALL4** and β-catenin share similar expression patterns at different phases of CML. A potential mechanism accounting for the oncogenic role of **SALL4** in LSCs is proposed.

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Materials and methods

Molecular cloning

Plasmid construction and DNA sequencing were performed in accordance with standard procedures. For cloning of SALL4 isoforms, polymerase chain reaction (PCR) primers were designed, based on the genomic clone RP5-1112F19 (GenBank accession no. AL034420). SALL4 isoforms were cloned with the use of the Marathon-Ready cDNA library derived from human fetal kidney (BD Biosciences Clontech, Palo Alto, CA), according to the supplier’s protocol. The amplified PCR products were cloned into a TA Cloning vector (Invitrogen, Carlsbad, CA), and the nucleotide sequences were determined by DNA sequencing.

Determination of SALL4 alternative splicing patterns in different tissues and Wnt/β-catenin downstream target gene expression (c-Myc and Cyclin D1)

Reverse transcription (RT)–PCR was used to evaluate mRNA expression patterns of SALL4 in adult tissues. A panel of 8 normalized first-strand cDNA preparations, derived from different adult tissues, was purchased from BD Biosciences Clontech. PCR amplification was performed in a 50-μL reaction volume containing 5 μL cDNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.2 mM dNTPs, and 1.25 U Taq DNA polymerase (PerkinElmer Life Sciences, Boston, MA). After an initial denaturation at 94°C for 10 minutes, amplification was performed for 30 cycles under the following conditions: 30-second denaturation at 94°C, 30-second annealing at 55°C, and 30-second extension at 72°C. The last cycle was followed by a final 7-minute extension at 72°C. Amplification of glyceralddehyde phosphate dehydrogenase (GAPDH) mRNA was used to control for template concentration loading. The primer pairs for GAPDH were as follows: forward primer, 5'-TTCGAGAACACGGCACTGAGTGGAAG-3'; and reverse primer, 5'-GTTCACATGACACCAGGGCCG-3'.

Antibody generation

The peptide MRSRRQAKPOHHIN of human SALL4 was chosen for its potential antigenicity (amino acids 1-13) and used to prepare an antipeptide antibody. This region is also identical to that of mouse SALL4 so that the generated antibody could be expected to cross-react with mouse SALL4. SALL4 antipeptide antibody was produced in rabbits in collaboration with Lampire Biologic Laboratories (Pipersville, PA).

Gel electrophoresis and Western blot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in SDS 10% wt/vol polyacrylamide slab gels according to Laemmli, and the proteins were then transferred to nitrocellulose membranes. Immunoblotting of rabbit immune serum with the SALL4 antipeptide antibody (1:100) was performed with an electrochemiluminescence detection system as described by the manufacturer (Amersham Biosciences, Piscataway, NJ).

Leukemia and normal tissues

Leukemia and normal samples, either in paraffins or frozen in dimethylsulfoxide (DMSO), were collected from the files of the University of Texas M. D. Anderson Cancer Center (Houston) and the Dana-Farber Cancer Institute (Boston, MA), between 1998 and 2004 under approved institutional review board protocols. The diagnosis of all tumors was based on morphologic and immunophenotypic criteria according to the French-American-British (FAB) Classification for Hematopoietic Neoplasms.

Real-time quantitative RT-PCR

We used the TaqMan 5’ nuclelease assay (Applied Biosystems, Foster City, CA) in these studies. Total RNA from purified CD34+ HSc/hematopoietic progenitor cells (HPCs) from normal bone marrow and peripheral blood, 15 AML samples, and 3 leukemia cell lines were isolated with the RNaseasy Mini Kit and digested with DNase I (Qiagen, Valencia, CA). RNA (1 μg) was reverse-transcribed in 20 μL with the use of Superscript II reverse transcriptase and a poly(dT)12-18 primer (Invitrogen). After the addition of 80 μL water and mixing, 5-μL aliquots were used for each TaqMan reaction. TaqMan primers and probes were designed with the use of Primer Express software version 1.5 (Applied Biosystems). Real-time PCR for SALL4 and GAPDH was performed with the TaqMan PCR core reagent kit (Applied Biosystems) and an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The PCR reaction mixture contained 3.5 mM MgCl2; 0.2 mM each of deoxyadenosine triphosphate (dATP), deoxyctydine triphosphate (dTTP), and deoxyguanosine triphosphate (dGTP); 0.4 mM deoxyuridine triphosphate (dUTP); 0.5 μM forward primer; 0.5 μM reverse primer; 0.1 μM TaqMan probe; 0.25 U uracil DNA glycosylase; and 0.625 U AmpliTaq Gold polymerase in 1 × TaqMan PCR buffer. cDNA (5 μL) was added to the PCR mix, and the final volume of the PCR reaction was 25 μL. All samples were run in duplicate. GAPDH was used as an endogenous control. Thermal cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 0.30 minutes and 60°C for 1 minute. Data were analyzed with the use of Sequence Detection System software version 1.6.3 (Applied Biosystems). Results were obtained as threshold cycle (Ct) values. The software determines a threshold line on the basis of the baseline fluorescent signal, and the data point that meets the threshold is given as the Ct value. The Ct value is inversely proportional to the starting number of template copies. All measurements were performed in duplicate. TaqMan sequences include the following: GAPDH forward primer (5'-GAAGGTGAAGGTCGGAGTC-3') and reverse primer (5'GATGATGTTGATGGATCTC-3'); SALL4 forward primer (5'-TGGCAAGAGGATGGATGCTT-3') and reverse primer (5'-ACATCTGGGCGTGGATATC-3'); and SALL4A forward primer (5'-ACATCTGGGCGTGGATGT-3') and reverse primer (5'-GACAGTTTGAAATGAC-3').

Design and construction of tissue arrays

Tissue arrays that included triplicate tumor cores from leukemia specimens were sectioned (5-μm thick). A manual tissue arrayer (Beecher Instruments, Silver Spring, MD) was used to construct the tissue arrays.

Immunohistochemistry

Immunohistochemical staining was performed according to standard techniques. Briefly, formalin-fixed, paraffin-embedded, 4-μm-thick tissue sections were deparaffinized and hydrated. Heat-induced epitopes were retrieved with a Tris buffer (pH 9.9; Dako, Carpinteria, CA) and a rapid microwave histoprocessor. After incubation at 100°C for 10 minutes, slides were washed in running tap water for 5 minutes and then with phosphate-buffered saline (PBS; pH 7.2) for 5 minutes. Tissue sections were then incubated with anti-SALL4 antibody (1:200) for 5 hours in a humidified chamber at room temperature. After 3 washes with PBS, tissue sections were incubated with anti–mouse immunoglobulin G and peroxidase for 30 minutes at room temperature. After 3 washes with PBS, tissue sections were incubated with 3,3’-diaminobenzidine/H2O2 (Dako) for color development; hematoxylin was used to counterstain the sections. Cells were considered to be positive for SALL4 when they showed definitive nuclear staining.
Image acquisition

Images were visualized using an Olympus BH-2 microscope (Olympus, Tokyo, Japan), equipped with one of the following: a Dplan4 4×/0.10 numeric aperture (NA) objective (Figures 2Bv, 4Bviii); an Splan10 10×/0.3 NA objective (Figures 4Bi,iv); an Splan 20×/0.46 NA objective (Figure 4Diii-vi and 5Ai,ii); an Splan40 40×/0.70 NA objective (Figures 2Bi-iv,vi, 4Bi,vi, and 4Bviii inset); or an Splan Apo60 60×/1.4 NA objective (Figures 4Ai-xvi, 4Bi,iv, 5Bi-iv; microscope immersion oil from Richard-Allan Scientific, Kalamazoo, MI). All micrographs were visualized using hematoxylin and eosin stain. All images including the gross-view pictures (Figures 4Biiv, 4Dii-ii) were taken at room temperature using an Olympus Q-color 5 camera (model 32-005SB-128) and were processed using Adobe Photoshop (Adobe Systems, San Jose, CA).

Generation of transgenic mice

SALL4B cDNA, corresponding to the entire coding region, was subcloned into a pCEP4 vector (IntroGene; now Crucell, Leiden, The Netherlands) to create the cytomegalovirus (CMV)/SALL4B construct for the transgenic experiments. Subsequent digestion with SalI, which does not cut within the SALL4B cDNA, released a linear fragment containing only the CMV promoter, the SALL4 cDNA coding region, the simian virus 40 (SV40) intron, and polyadenylation signal without additional vector sequences. Transgenic mice were generated via pronuclear injection performed in the transgenic mouse facility at Yale University. Identification of SALL4B founder mice and transmission of the transgene was determined by PCR analyses. Tissue expressions of transgene were confirmed by RT-PCR. The PCR primer sets used for the RT-PCR span the junction of the 5′ intron, and polyadenylation signal without additional vector sequences.

Hematologic analysis

Complete blood cell counts with automated differentials were determined with a Mascot Hemavet cell counter (CDC Technologies, Oxford, CT). For hematologic analysis in tissue samples, 1.5 × 104 bone marrow cells were plated in duplicate 1.25-mL methylcellulose cultures supplemented with recombinant mouse interleukin-3 (IL-3, 10 ng/mL), IL-6 (10 ng/mL), stem cell factor (SCF; 50 ng/mL), and erythropoietin (3 U/mL) (M3434; StemCell Technologies, Vancouver, BC). Colonies were recorded between days 7 and 14 of culture.(colony-forming unit–granulocyte [CFU-G], CFU–granulocyte-macrophage [CFU-GM]), CFU-macrophage [CFU-M], CFU–granulocyte-erythrocyte-megakaryocyte-macrophage [CFU-GEMM], and burst-forming unit–erythroid [BFU-E]). Peripheral blood, bone marrow smears, and clot were prepared for the assay according to the manufacturer’s instructions. To rule out the possibility that these 2 apparent splicing variants might result from artifacts, we compared both variant mRNA sequences with corresponding sequences of the human genome. SALL4A contained all exons (1-4) (Figure 1A), whereas SALL4B lacked the region corresponding to 3′ end of exon 2. Both exon-intron splice sites satisfied the G-T-A-G rule. Both splicing variants had the same translational reading frame, but SALL4B mRNA encoding a protein with internal deletion. SALL4A contained 8 zinc finger domains, whereas SALL4B had 3 zinc finger domains.

Expression pattern of the SALL4 isoforms in human tissues

The alternative splicing patterns of SALL4 were delineated by RT-PCR in a variety of human tissues. A fragment of the ubiquitous GAPDH gene cDNA was amplified as a control (Figure 1B). A 315-bp fragment representing the longer splice variant, SALL4A, was amplified in some tissues, achieving various expression levels. The SALL4B variant was present in every tissue at varying levels of expression.

Generation of SALL4 antibody and identification of SALL4 protein products

To identify SALL4 gene products and confirm the presence of SALL4 variants, we developed a polyclonal antibody against a synthetic peptide (amino acids 1-13) of SALL4. This region was chosen because it is common to both SALL4 variants. The
Constitutive expression of SALL4 mRNA in human primary AML and myeloid leukemia cell lines

Because another SALL gene family member SALL2 is involved in tumorigenesis, we examined SALL4 mRNA expression in AML. Expression of SALL4 was quantitatively investigated by real-time RT-PCR in bone marrow cells derived from AML samples (n = 15) and myeloid leukemia cell lines (n = 3) and compared with that of nonneoplastic hematopoietic cells from a purified CD34+ stem/progenitor pool (HSCs/HPCs purchased from Cambrex), normal bone marrow (n = 3), and normal peripheral blood (n = 3). With the use of isoform-specific primers (Figure 2A), we observed that during normal hematopoiesis, both SALL4 isoforms were downregulated. In contrast, both SALL4A and SALL4B mRNA levels were constitutively expressed in 60% of AML samples and in all 3 cell lines. In the remaining 40% of AML samples, either SALL4A or SALL4B was constitutively expressed. Compared with normal hematopoiesis cells, leukemia samples had a wide range of SALL4A or SALL4B expression levels. This is probably due to the fact that the leukemia samples had a variable range of leukemic blasts population (40%-90%).

Constitutive expression of SALL4 protein in human primary AML

To investigate whether the observed aberrant SALL4 expression was also present at the protein level, we examined 81 AML samples, ranging from AML subtypes M1 to M5 (FAB classification): M1 (n = 20), M2 (n = 27), M3 (n = 8), M4 (n = 16), M5 (n = 3), and AML nonspecified (n = 7); and several samples of normal bone marrow, thymus, and spleen, as well as normal CD34+ HSCs/HPCs.

Normal bone marrow, spleen, and thymus showed no detectable SALL4 protein expression, and CD34+ HSCs/HPCs exhibited positive but weaker SALL4 protein staining; however, much stronger SALL4 expression was detected in the nuclei of leukemic cells (Figure 2Bvi). All 81 AML samples showed aberrant SALL4 expression, which was consistent with SALL4 mRNA expression levels demonstrated by real-time RT-PCR (Figure 2A). The strongest staining was seen in AML-M1 and -M2. Our data suggested that SALL4 was present in CD34+ HSCs/HPCs and down-regulated in mature granulocytes and lymphocytes. As a result, the constitutive expression of SALL4 in leukemia may have prevented the leukemic blasts from differentiating and/or gaining properties that were normally seen in HSCs, probably by interacting with additional mutations since leukemogenesis is a multistep pathologic process.

Generation of transgenic mice constitutively expressing full-length human SALL4B

To directly test whether constitutive expression of SALL4 is sufficient to induce AML, we generated a SALL4 transgenic mouse model. The CMV promoter was fused to cDNA that encoded the 617 amino acids of human SALL4B (Figure 3A), which was chosen because it was expressed in every tissue previously examined (Figure 1Dii). The CMV promoter was previously used to ectopically express human genes in most murine organs. RT-PCR amplification was performed to examine the expression of wild-type (WT), full-length SALL4B in the transgenic mice. A SALL4B transcript was detected in a variety of tissues from the transgenic mice, including brain, kidney, liver, spleen, lymph nodes, peripheral blood, and c-kit–positive population in the bone marrow (Figure 3B). Abnormal gait and associated hydrocephalus 3 weeks after birth were observed in 20% of the transgenic mice from multiple lines; 60% had polycystic kidneys.
MDS-likes features and AML in SALL4B transgenic mice

Monitoring of hematologic abnormalities in a cohort of 16 transgenic mice from all 6 lines revealed that all mice had apparent MDS-like features at ages 6 to 8 months. Increased number of immature blasts and many atypical and dysplastic white cells, including hypersegmented neutrophils and pseudo-Pelger-Huet-like cells, were seen on peripheral blood smears (Figure 4A). Nucleated red blood cells and giant platelets were also present, as well as erythroid and megakaryocyte dysplastic features, such as binucleate erythroid precursors and hypolobulated megakaryocytes.

Eight (50%) of these 16 mice eventually progressed to acute leukemia (Table 1). Leukemic infiltration of many organs, including lungs, kidneys, liver, spleen, and lymph nodes, emphasized the aggressiveness of the disease (Figure 4B). Leukemia blast cells were CD34+ and considered to be myeloid in origin because they were positive for c-kit, Gr-1, Mac-1, and myeloperoxidase; they were negative for B-cell (B220 and CD19), T-cell (CD4, CD8, CD3, and CD5), megakaryocytic (CD41), and erythroid (Ter119) markers (Figure 4C).

SALL4B-induced AML was transplantable

Aggressive fatal AML with onset at approximately 6 weeks developed in immunodeficient NOD/SCID mice after serial transplantation of SALL4B-induced AML cells by subcutaneous injection. The transplanted disease was positive for c-Kit and characterized by dissemination to multiple organs, with marked splenomegaly and hepatomegaly (Figure 4D). SALL4B expression was detectable in transplanted leukemic cells (data not shown).
Ineffective hematopoiesis and excessive apoptosis in SALL4B transgenic mice

Investigation of hematologic abnormalities in younger SALL4B transgenic mice (2-6 months old) revealed that their peripheral blood showed minimal myelodysplastic features but statistically significant leukopenia and neutropenia, as well as mild anemia (Table 2). To determine whether the cause of cytopenia in these transgenic mice was related to ineffective hematopoiesis, we studied their bone marrow. Bone marrow samples showed increased cellularity and an increased myeloid population (Figure 5A), compared with those of WT controls (Gr-1/Mac-1 double-positive population in SALL4B transgenic mice: 67% ± 16% [n = 10] vs WT; 55.3% ± 4% [n = 11]; P = .048).

As excessive apoptosis plays a central role in ineffective hematopoiesis in human MDS, we next examined apoptosis in SALL4 transgenic mice in vivo and in vitro. Increased apoptosis was observed in SALL4B transgenic mice on both primary bone marrow (annexin V–positive, PI-negative population in transgenic mice: 11% ± 4.48% [n = 10] vs WT: 6.15% ± 4.98% [n = 7]; P = .03) and day-7 CFUs (annexin V–positive, PI-negative population in transgenic mice: 20.1% ± 6% [n = 10] vs WT: 10.9% ± 4% [n = 7]; P = .002) (Figure 5A-B). These findings...
may account for the fact that despite an increased myeloid population in bone marrow, these transgenic mice had statistically significant low neutrophil counts in the peripheral blood, secondary to an ongoing ineffective myelopoiesis in their bone marrow. An increased population of immature cells was also noted in SALL4B transgenic mice on both primary bone marrow (c-kit–positive population in transgenic mice: 10.2% ± 1.3%; [n = 14] vs WT; 6.5% ± 2.5% [n = 10]; P = .008) (Figure 5A) and day-7 CFUs (CD34⁺ population in SALL4B transgenic mice: 11% ± 2.2% [n = 8] vs WT: 6.3% ± 2.4% [n = 7]; P = .002) (Figure 5B). Similar numbers of total colonies were observed in SALL4A transgenic mice and WT controls (total colonies in SALL4A mice: 42 ± 29.5 [n = 10] vs WT: 39 ± 13.5 [n = 6]; P = .23). Statistically significant increased myeloid (CFU-GM in SALL4B transgenic mice: 53.6 ± 10.3 [n = 13] vs WT: 38.1 ± 3.1 [n = 8]; P = .002) and decreased erythroid (BFU-E in SALL4B transgenic mice: 7.8 ± 3.8 [n = 13] vs WT: 14.1 ± 2.7 [n = 8]; P = .001) colony populations (Figure 5Cii), however, were found in SALL4B transgenic mice compared with those of WT controls, as has been reported in human MDS patients and other MDS mouse models.31-33 These observations suggest that the defect in SALL4B transgenic mice lies at the stem cell/progenitor level affecting hematopoietic differentiation.

### Binding of SALL4A and SALL4B to β-catenin in vitro

We next explored the potential signaling pathway that SALL4 may affect in leukemogenesis. In Drosophila, Wnt signaling controls spalt (sal) expression during tracheal morphogenesis. SALL1, another member of the SALL gene family, can interact with β-catenin. The high-affinity site for this interaction is located at the C-terminal double zinc finger domain. This region of SALL1 was found to be almost exactly identical to that of SALL4 (data not shown). This finding prompted us to investigate whether SALL4 was also able to bind β-catenin. We generated expression constructs of SALL4A and SALL4B tagged with hemagglutinin (HA). As shown in Figure 6A, endogenous β-catenin was pulled down by HA-SALL4A and HA-SALL4B, but not by HA alone.

### Activation of the Wnt/β-catenin signaling pathway by both SALL4A and SALL4B

To investigate the functional effect of the interaction of the SALL4 isoforms with β-catenin, we used a luciferase reporter (TOPflash; Upstate USA) containing multiple copies of Wnt-responsive elements to determine the potential of SALL4A and SALL4B to activate the canonical Wnt signaling pathway. The FOPflash reporter plasmid was cotransfected with mutated Wnt-response elements was used as a negative control. This reporter construct has been shown to be efficiently stimulated by Wnt1 in a variety of cell lines.36-42 TOPflash or FOPflash reporter plasmid was transiently transfected in the HEK-293 cell line, in which both Wnt and its Wnt/β-catenin signal pathways were present. TOPflash reporter plasmid was also cotransfected with SALL4A or SALL4B. Significant activation of the Wnt/β-catenin signaling pathway by both SALL4A and SALL4B was indicated by increased luciferase activity in the TOPflash but not in the FOPflash reporter (Figure 6B).

### Up-regulation of Wnt/β-catenin downstream target genes in SALL4B transgenic mice

We then studied the effect of overexpression of SALL4 on β-catenin/Tcf-dependent gene expression in SALL4B transgenic mice. Many genes are regulated by Wnt/β-catenin signaling pathway. We chose to study c-Myc and Cyclin D1 since both genes are transactivated by β-catenin/Tcf complex and involved in

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**Table 1. Summary of MDS-like/AML in SALL4B transgenic mice**

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Sex</th>
<th>Founder</th>
<th>Age, mo</th>
<th>Phenotype</th>
<th>Outcome and organs involved by AML*</th>
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<tbody>
<tr>
<td>4</td>
<td>M</td>
<td>464</td>
<td>19</td>
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<td>Died of MDS</td>
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<td>M</td>
<td>504</td>
<td>19</td>
<td>MDS-like</td>
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<tr>
<td>86</td>
<td>F</td>
<td>504</td>
<td>18</td>
<td>AML</td>
<td>Killed; AML in BM, PB, liver, spleen, LNs</td>
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<tr>
<td>87</td>
<td>F</td>
<td>504</td>
<td>8</td>
<td>AML</td>
<td>Killed; AML in BM, PB, liver, spleen, LNs</td>
</tr>
<tr>
<td>506</td>
<td>M</td>
<td>506</td>
<td>19</td>
<td>MDS-like</td>
<td>Killed due to MDS</td>
</tr>
<tr>
<td>1336</td>
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<tr>
<td>2548</td>
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<td>506</td>
<td>14</td>
<td>AML</td>
<td>Killed; AML in BM, liver, spleen, LNs</td>
</tr>
<tr>
<td>507</td>
<td>F</td>
<td>507</td>
<td>24</td>
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</tr>
<tr>
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<td>M</td>
<td>507</td>
<td>22</td>
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<td>Killed due to MDS</td>
</tr>
<tr>
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<td>M</td>
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<td>8</td>
<td>AML</td>
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</tr>
<tr>
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<tr>
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<td>F</td>
<td>509</td>
<td>18</td>
<td>AML</td>
<td>Killed; AML in BM, PB, liver, spleen, LNs, lung</td>
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<tr>
<td>510</td>
<td>F</td>
<td>510</td>
<td>24</td>
<td>MDS-like</td>
<td>Killed due to MDS</td>
</tr>
</tbody>
</table>

*Mice were killed when noticed to be ill. AML is defined to have more than 20% c-kit-positive immature cells in the bone marrow or peripheral blood, with leukemia cells involving multiple organs.

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**Table 2. CBC from SALL4B transgenic mice and wild-type control**

<table>
<thead>
<tr>
<th>n</th>
<th>WBC count, × 10^9/L</th>
<th>Neutrophil count, × 10^9/L</th>
<th>Lymphocyte count, × 10^9/L</th>
<th>RBC count, × 10^12/L</th>
<th>Hb level, g/L</th>
<th>Hematocrit</th>
<th>PLT count, × 10^9/L</th>
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<tr>
<td>Transgenic mice</td>
<td>20</td>
<td>8.38 ± 1.76</td>
<td>0.93 ± 0.53</td>
<td>6.34 ± 2.31</td>
<td>8.85 ± 1.04</td>
<td>142.6 ± 15.2</td>
<td>.5052 ± .591</td>
</tr>
<tr>
<td>Control mice</td>
<td>18</td>
<td>11.59 ± 2.57</td>
<td>1.51 ± 0.43</td>
<td>9.04 ± 2.03</td>
<td>10.02 ± 0.92</td>
<td>156.6 ± 12.2</td>
<td>.5575 ± .481</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>—</td>
<td>.027</td>
<td>.048</td>
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<td>.038</td>
</tr>
</tbody>
</table>

Plus-minus values indicate SD. Hb indicates hemoglobin; PLT, platelet; —, not applicable.
various human cancers including leukemia and lymphoma.43 We found that in contrast to the wild-type controls, the mRNA expression of both genes was significantly up-regulated in preleukemia bone marrows and leukemic blasts from SALL4B transgenic mice (Figure 6C). These findings strengthen our hypothesis that SALL4B contributes to leukemogenesis, probably through activation of the Wnt/β-catenin signaling pathway.

Similar expression patterns of β-catenin and SALL4 at different phases of CML

Dysregulated Wnt/β-catenin signaling is known to be involved in the development of LSCs. The best evidence for β-catenin’s involvement in LSC self-renewal comes from the study of CML blast transformation. Jamieson et al11 demonstrated that Wnt signaling was activated in the blast phase of CML but not the chronic phase, concluding that dysregulated Wnt signaling, such as activation of β-catenin, could confer the property of self-renewal on the GMPs of CML and lead to their blastic transformation. Given the potential interaction between SALL4 and β-catenin and spalt’s position as a downstream target of Wnt signaling in Drosophila, we examined SALL4 protein expression in CMLs in different phases. SALL4 expression was present in blast-phase CML (n = 12, 75%) but not the chronic phase (n = 11 100%). In the accelerated phase (n = 6, 10%), in which blast counts are increased, immature blasts expressing SALL4 were observed with a background of nonstaining more mature myeloid cells (data not shown).

Discussion

Homeobox and homeotic genes play important roles in normal development. Some homeobox genes, such as Hox and Pax, also function as oncogenes or as tumor suppressors in tumorigenesis or leukemogenesis. The important role of SALL4, a homeotic gene
and a transcriptional factor, in human development was recognized because heterozygous SALL4 mutations lead to Duane-radial ray syndrome. SALL4’s oncogenic role in leukemogenesis is described here for the first time.

We identified the 2 SALL4 isoforms, SALL4A and SALL4B. During normal hematopoiesis, SALL4 isoforms are expressed in the CD34+ HSC/HPC population and rapidly turned off (SALL4B) or down-regulated (SALL4A) in normal human bone marrow and peripheral blood. In contrast, SALL4 was constitutively expressed in all AML samples (n = 81) that we examined, and failed to turn off in human primary AML and myeloid leukemia cell lines. To directly test the leukemogenic potential of constitutive expression of SALL4 in vivo, we generated SALL4B transgenic mice. The transgenic mice exhibited dysregulated hematopoiesis, much like that of human MDS, and AML that was transplantable. The MDS-like features in these SALL4B transgenic mice apparently did not require cooperating mutations and were observed as early as 2 months of age. The ineffective hematopoiesis observed in these mice was characterized, as it is in human MDS, by hypercellular bone marrow and paradoxic peripheral blood cytopenias (neutropenia and anemia) and dysplasia, which were probably secondary to the increased apoptosis noted in the bone marrow. The reason for the late onset of leukemia development in these transgenic mice may be the accumulation of additional genetic damage during the 8 or more months of replicative stress. Late onset of disease may also be a consequence of SALL4-induced genomic instability.

Our investigation of the potential mechanism of SALL4 involvement in leukemogenesis demonstrated that both SALL4A and SALL4B interacted with β-catenin, an essential component of the Wnt signaling pathway involving self-renewal of HSCs. In addition, both were able to activate the Wnt/β-catenin pathway in a reporter gene assay, consistent with SALL family function in Drosophila and humans. Furthermore, similar to the situation with β-catenin, SALL4 expression in CML varied at different phases of the disease: SALL4 expression was absent in the chronic phase, became detectable in the accelerated phase only in immature blasts, and was strongly positive in the blast phase. The downstream target genes of Wnt/β-catenin signaling pathway by both SALL4A and SALL4B. HEK-293 cells were transfected with 1 μg of either mock alone, or SALL4A or SALL4B plasmid, with or without Wnt1 (including Wnt1, and its coactivators: LRP6, MESD, and F5), and TOPflash (TOP) or FOPflash (FOP) reporter plasmid (Upstate USA, Chicago, IL). After 24 hours, luciferase activity was measured. SALL4A or SALL4B alone showed more potent activation of Wnt signaling pathway when compared with the positive control Wnt1. In addition, both SALL4 isoforms demonstrated a significantly synergistic activation of the Wnt signaling pathway with Wnt1. Data represent mean ± SD of 3 independent experiments. (C) Up-regulation of c-Myc and Cyclin D1 expression in SALL4B transgenic mice. RT-PCR analysis was performed on total bone marrow cells from 2 wild-type control mice (lanes 1-2), 2 preleukemic transgenic mice (lanes 3-4), and leukemic bone marrow cells from 2 leukemic transgenic SALL4B mice (lanes 5-6). Both c-Myc and Cyclin D1 expression were significantly up-regulated in SALL4B transgenic mice at both preleukemia MDS and leukemic stages. Beta actin was used as an internal standard.

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References

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