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Bmi-1 Regulation of INK4A-ARF Is a Downstream Requirement for Transformation of Hematopoietic Progenitors by E2a-Pbx1

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Summary

Loss-of-function alterations of *INK4A* are commonly observed in lymphoid malignancies, but are consistently absent in pre-B cell leukemias induced by the chimeric oncoprotein E2a-Pbx1 created by t(1;19) chromosomal translocations. We report here that experimental induction of E2a-Pbx1 enhances expression of *BMI-1*, a lymphoid oncogene whose product functions as a transcriptional repressor of the *INK4A-ARF* tumor suppressor locus. *Bmi-1*-deficient hematopoietic progenitors are resistant to transformation by E2a-Pbx1; however, the requirement for *Bmi-1* is alleviated in cells deficient for both *Bmi-1* and *INK4A-ARF*. Furthermore, the adverse effects of E2a-Pbx1 on pre-B cell survival and differentiation are partially bypassed by forced expression of p16^{Ink4a}. These results link E2a-Pbx1 with *Bmi-1* on an oncogenic pathway that is likely to play a role in the pathogenesis of human lymphoid leukemias through downregulation of the *INK4A-ARF* gene.

Introduction

Many acute B cell precursor leukemias contain inactivating deletions, mutations, or methylations of the *INK4A-ARF* gene (Drexler, 1998), which codes for tumor suppressor proteins that function on the Rb and p53 pathways (Chin et al., 1998). A notable exception, however, is the subset containing t(1;19) chromosomal translocations, which consistently lack alterations of *INK4A-ARF* (Ohnishi et al., 1996; Maloney et al., 1998). This translocation, present in approximately 20% of leukemias that display a pre-B cell phenotype, generates a prototypical chimeric transcription factor known as E2a-Pbx1, a fusion protein containing the strong transactivation domains of E2a and the DNA binding homeodomain of Pbx1 (Nourse et al., 1990; Kamps et al., 1990). The latter is a mammalian ortholog of *Drosophila extradenticle* and is required for fetal hematopoiesis, organogenesis, and skeletal patterning (Selleri et al., 2001; DiMartino et al., 2001; Kim et al., 2002). Pbx/exd proteins function biochemically as Hox DNA binding cofactors (Mann and Chan, 1996) and participate in various multiprotein complexes in transcriptional regulation (Mann and Affolter, 1998). The activated oncoprotein E2a-Pbx1, conversely, has been shown experimentally to transform several cell types in vitro and to induce lymphoblastic lymphomas in transgenic mice (Dedera et al., 1993; Kamps and Wright, 1994; Monica et al., 1994). In this capacity, E2a-Pbx1 functions as a rogue activator whose regulatory properties differ substantially from wild-type Pbx1 (Van Dijk et al., 1993; LeBrun and Cleary, 1994; Lu et al., 1994) but remain dependent on Hox DNA binding partners (Chang et al., 1995). The molecular properties of E2a-Pbx1 have been extensively characterized, and candidate target genes have been identified (Fu and Kamps, 1997; McWhirter et al., 1997, 1999). However, the subordinate transcriptional pathways that are perturbed by the aberrant activation of this transcription factor, and that contribute to the early events of leukemic transformation, are not yet known.

Here we report that E2a-Pbx1 initiates a transcriptional cascade that eventuates in the downregulation of *INK4a-ARF* expression via induction of the lymphoid oncoprotein Bmi-1. This has functional consequences for the growth, survival, and oncogenic transformation of hematopoietic precursors. The elucidation of this pathway also establishes a molecular basis for the negative correlation of *INK4a-ARF* mutation in t(1;19) leukemias and to our knowledge provides the first evidence of a role for Bmi-1 in human cancer pathogenesis.

Results

E2a-Pbx1 Induces Expression of Bmi-1

To ascertain the transcriptional programs that are subordinate to E2a-Pbx1, we employed gene expression profiling techniques using a microarray containing cDNAs for approximately 8000 different human genes (DeRisi et al., 1997; Iyer et al., 1999). Expression of these genes was evaluated in a human pre-B cell line (A2 clone of Reh) programmed to express E2a-Pbx1 inducibly under control of the metal response element (MRE) from the sheep metallothionine promoter (Smith et al., 1997). mRNA levels of approximately 40 genes (see Supplemental Data at http://www.molecule.com/cgi/content/full/12/2/393/DC1) were observed to increase by at least 2-fold at 8 hr following E2a-Pbx1 induction when compared to mRNA populations derived from a cell line stably transduced with vector alone (MT1). Most of the differences were quite modest (less than 3-fold). Of the previously reported candidate target genes for E2a-Pbx1 (Fu and Kamps, 1997; McWhirter et al., 1997, 1999), seven were present on the microarray but failed to show a 2-fold induction (see Supplemental Data at http://www.molecule.com/cgi/content/full/12/2/393/DC1).

Among the induced genes identified by our initial analysis, the protooncogene *BMI-1* (hybridization ratio of

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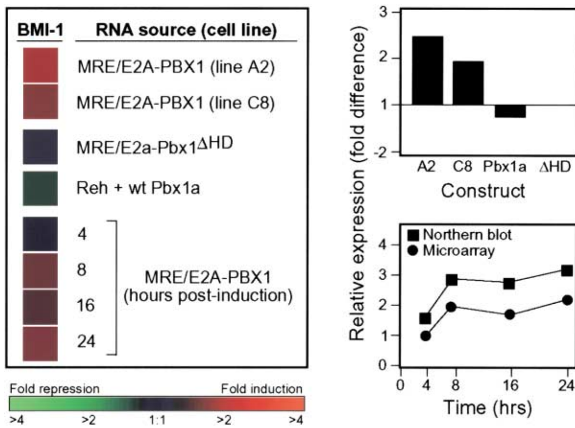

Figure 1. Microarray Analysis of *BMI-1* Expression

The expression of *BMI-1* is shown as a ratio of mRNA levels in various cell lines expressing exogenous full-length E2a-Pbx1 (A2 and C8), mutated E2a-Pbx1 (ΔHD), or wild-type Pbx1a compared to identically treated control cells (MT1 and Reh) harboring vector alone. Cell lines stably transfected with MRE expression vectors were cultured in the presence of 10 μM zinc sulfate for 8 hr unless otherwise indicated. The *BMI-1* expression ratio is represented by a color according to the color scale at the bottom. Conditions under which *BMI-1* mRNAs were more abundant compared to control are represented by red; less abundant *BMI-1* mRNA is represented by green. Black indicates no change in expression. Right hand panels depict quantitation of signals from microarray or Northern blot analyses.

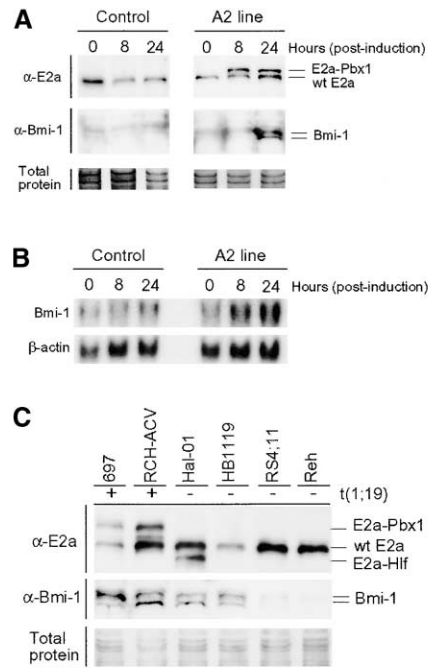

Figure 2. Enhanced Expression of Bmi-1 in Response to E2a-Pbx1

(A) Proteins were isolated from control MT1 (left panels) and A2 (right panels) cells following zinc sulfate induction for the times indicated at the tops of the gel lanes. Immunoblotting was performed using the primary antibodies indicated on the left. Migration positions of E2a-Pbx1, wt E2a, and Bmi-1 are indicated on the right.

(B) Total RNA was isolated from cells as described in (A) and analyzed by Northern blot analysis using probes specific for Bmi-1 (upper panel) or β-actin (lower panel) which served as a loading control.

(C) Proteins were isolated from human leukemia cell lines containing (+) or lacking (−) the t(1;19) chromosomal translocation as indicated above the gel lanes. Western blot analyses were performed using the primary antibodies indicated on the left. Migrations of wt and mutant E2a proteins are indicated on the right.

2.1×) was of particular interest because of its previous implication in lymphoid oncogenesis (Haupt et al., 1991; van Lohuizen et al., 1991a). We therefore investigated the kinetics and specificity of its expression using smaller cDNA microarrays, which were hybridized to cDNA probes prepared from independent E2a-Pbx1-expressing cell lines, and a variety of controls. In A2 cells, a time-dependent increase of *BMI-1* mRNA was observed from 4 to 24 hr following E2a-Pbx1 induction (Figure 1). Similar induction kinetics were observed by Northern blot analysis (Figures 1 and 2B). Independent demonstration that *BMI-1* is transcriptionally subordinate to E2a-Pbx1 was obtained by similarly analyzing a second subclone of Reh cells (C8) containing the MRE/E2A-PBX1 construct (Figure 1). In contrast, no perturbations of *BMI-1* expression were observed following induced expression of a mutant E2a-Pbx1 incapable of binding DNA due to deletion of its homeodomain (Figure 1). Furthermore, expression of exogenous wild-type Pbx1a in the Reh pre-B cell line resulted in reduced levels of *BMI-1* RNA compared to steady-state levels of expression in this line (Figure 1). The observed reduction is consistent with transcriptional repressor properties for wt Pbx1a (Asahara et al., 1999) in contrast to the strong activator properties displayed by E2a-Pbx1 (Van Dijk et al., 1993; LeBrun and Cleary, 1994; Lu et al., 1994).

Western blot analysis of protein extracts prepared from A2 cells showed low, but detectable, levels of Bmi-1 prior to induction and modestly elevated levels of Bmi-1 at 24 hr after induction of E2a-Pbx1 (Figure 2A). Consistent with a regulatory role for E2a-Pbx1 upstream of Bmi-1, leukemia cell lines harboring t(1;19) chromosomal translocations demonstrated Bmi-1 expression by Northern blot (data not shown) and Western blot analyses (Figure 2C). Although expression of Bmi-1 was not exclusively associated with t(1;19) cell lines, they displayed higher steady-state levels when compared with ALL cell lines carrying other chromosomal translocations (Figure 2C). Furthermore, RT-PCR analysis of RNA from clinical samples of progenitor B cell leukemias also showed that t(1;19)-positive cases consistently expressed Bmi-1 transcripts (data not shown).

To ascertain whether Bmi-1 may be a direct transcriptional target of E2a-Pbx1, transient transfection assays were performed using reporter genes containing the mouse or human Bmi-1 promoter and upstream regions. No induction of the reporter genes was observed following coexpression with E2a-Pbx1b in various cell lines (data not shown), suggesting that Bmi-1 is not directly regulated by E2a-Pbx1 under these conditions.

E2a-Pbx1 Postpones Replicative Senescence of Human Diploid Fibroblasts

Bmi-1 is a member of the mammalian Polycomb-group of transcriptional repressors (van Lohuizen et al., 1991b). It serves as a negative regulator of the *INK4A-ARF* locus (Jacobs et al., 1999a), which encodes the tumor sup-

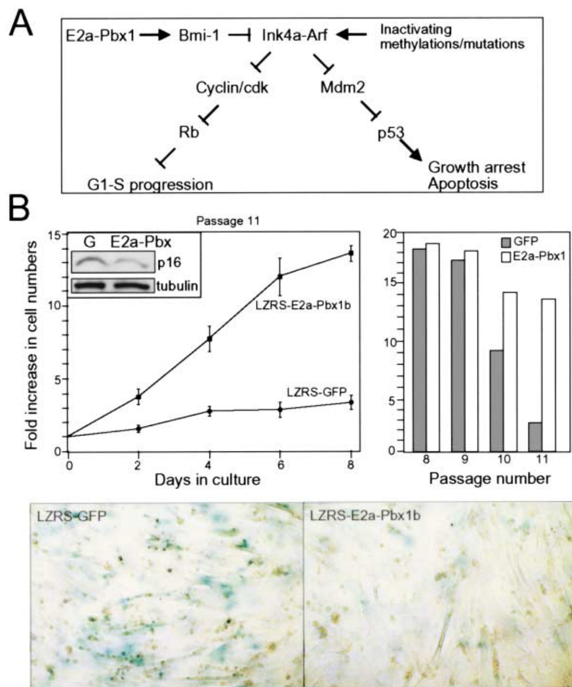

Figure 3. Delay of Replicative Senescence in Human Diploid Fibroblasts Expressing E2a-Pbx1

(A) Schematic representation of the *INK4A-ARF* gene at the nexus of the Rb and p53 pathways and its relationship to Bmi-1 and E2a-Pbx1.

(B) Growth curves are shown at passage 11 for human diploid fibroblasts infected at passage 5 (one passage = two PDL) with control (LZRS-GFP) or E2a-Pbx1-expressing retroviruses. Fold increase in cell numbers at day 8 for passages 8–11 are shown in the bar graph on right. Western blots are shown of cell lysates from control (GFP) and LZRS-E2a-Pbx1-injected cells at passage 11. β-galactosidase (pH 6) activity is shown for passage 11 fibroblasts.

pressor proteins p16^{Ink4a} and p14^{Arf} (Quelle et al., 1995) (Figure 3A). The *INK4A-ARF* locus plays a major role in the regulation of cellular lifespan (Noble et al., 1996; Alcorta et al., 1996; Carnero et al., 2000). One or both protein products (p16/arf) of this locus have been implicated in the induction of replicative senescence (Haber, 1997). Consistent with the regulatory relationship between *INK4A-ARF* and Bmi-1, MEFs that are deficient for Bmi-1 undergo premature senescence, whereas overexpression of Bmi-1 allows for murine fibroblast immortalization and postpones senescence in human fibroblasts (Jacobs et al., 1999a).

Therefore, we evaluated whether E2a-Pbx1 would also influence the replicative life span of fibroblasts given its role as an upstream regulator of Bmi-1 expression. Human diploid fibroblasts (HDFs) that expressed E2a-Pbx1 proliferated at an enhanced rate and displayed increased cell densities compared to control cells (data not shown). At passage 11, control cells entered replicative senescence as evidenced by cytoplasmic enlargement, flattening of the cells, apparent growth arrest, and expression of senescence-associated β-galactosidase (Figure 3B). E2a-Pbx1-expressing HDFs, in contrast, showed limited changes in morphology, grew steadily to confluence, and maintained downregulated p16^{Ink4a} protein levels (Figure 3B). HDFs expressing E2a-Pbx1 were not fully immortalized, but displayed a delayed entry into senescence and arrested after approximately three more population doublings (PDL) than their normal counterparts. These effects are similar to those induced by Bmi-1 overexpression in HDFs and suggest a pathway linking E2a-Pbx1 and p16 that has important physiologic consequences for the replicative state of cells.

Bypass of E2a-Pbx1-Associated Phenotypes by Forced Expression of p16^{Ink4a}

Given the role of p16^{Ink4a} in growth control (Serrano et al., 1996; Sherr, 1998) and apoptosis (Wang and Walsh, 1996), we evaluated whether its forced expression might abrogate some of the adverse effects of E2a-Pbx1 on the survival (Smith et al., 1997) and differentiation of pre-B cells. A2 cells were stably transduced with a conditional allele (Urashima et al., 1997) of p16^{Ink4a} (p16^{ts}) and evaluated for their survival in response to E2a-Pbx1 induction. Apoptosis was measured by flow cytometric analysis of cell surface annexin-V. In the absence of exogenous p16, E2a-Pbx1 induced apoptosis in approximately 50% of A2 cells, a level substantially above that observed in the control population lacking E2a-Pbx1 (Figure 4A). However, levels of apoptosis were substantially reduced in A2 cells containing the p16^{ts} transgene when incubated in identical (permissive for p16^{ts}) conditions. There was little deviation in the levels of apoptosis between the A2 parental cells and A2 cells containing the temperature-sensitive mutant of p16 when the assay was conducted in nonpermissive conditions (data not shown). Therefore, cells maintaining constitutive p16^{Ink4a} activity were able to circumvent a physiologic response that normally occurs following forced expression of E2a-Pbx1.

In addition to its effects on apoptosis, forced expression of p16^{Ink4a} also modulated the phenotype of cells that expressed endogenous E2a-Pbx1. RCH-ACV cells, which harbor a t(1;19) chromosomal translocation, were stably transfected with p16^{ts}. Incubation at the permissive temperature was associated with downregulation of CD19 (Figure 4B), a cell surface antigen that is normally downregulated with B cell differentiation (Stamenkovic and Seed, 1988). A similar shift in phenotype of RCH-ACV cells (Figure 4C) was observed following transient forced expression of a dominant-negative mutant of E2a-Pbx1 (E2a-Pbx1^{DN}) containing a nonfunctional E2a transactivation domain (Quong et al., 1993) (data not shown). In addition to its effects on cellular phenotype, E2a-Pbx1^{DN} also increased p16^{Ink4a} expression 3-fold above basal levels in RCH-ACV cells (Figure 4C). These observations established a direct relationship between E2a-Pbx1 and *INK4a* in cells harboring the t(1;19) translocation and provide further evidence that negative regulation of the *INK4A* gene may be a significant component of the oncogenic pathway initiated by E2a-Pbx1 in B lineage progenitors.

Requirement for *Bmi-1* in Transformation by E2a-Pbx1

A serial myeloid replating assay (Lavau et al., 1997) was employed to determine the potential requirement for Bmi-1 in experimental transformation of hematopoietic progenitors by E2a-Pbx1 (Kamps and Wright, 1994).

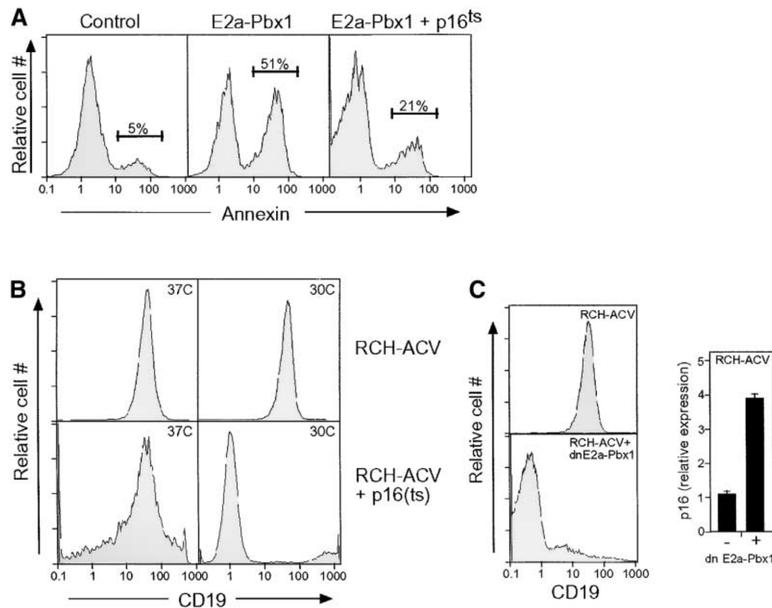

Figure 4. Forced Expression of p16^{Ink4a} Partially Bypasses the Adverse Effects of E2a-Pbx1 on Cell Survival and Differentiation

(A) Apoptosis was measured by flow cytometric analysis of cell surface annexin V expression by MT1 control, A2 cells, or A2(+p16^{ts}) cells incubated at the permissive temperature for p16 activity. Numbers indicate percentages of total cells undergoing apoptosis (brackets) with elevated levels of surface annexin V expression.

(B) Cell surface CD19 expression was measured by flow cytometric analysis of RCH-ACV cells following 7 days incubation at the nonpermissive (37°C) or permissive (30°C) temperature for p16^{ts}.

(C) Cell surface CD19 expression was measured by flow cytometric analysis of RCH-ACV cells in the presence (lower panel) or absence (upper panel) of a transiently expressed dominant-negative mutant of E2a-Pbx1. Data are shown for the subpopulation of cells electronically gated for high level coexpression of GFP. Bar graph indicates levels of p16^{Ink4a} expression determined by Western blot analysis of RCH-ACV cells FACS purified based on high level coexpression of GFP following retroviral transduction of E2a-Pbx1^{DN}. Data were corrected against levels of a control (α-tubulin) protein and represent the mean and standard deviation from two independent experiments.

Progenitors (c-kit$^+$) purified from the bone marrow of wt or *Bmi-1*$^{-/-}$ mice were transduced with MSCV retroviruses encoding E2a-Pbx1, or the related chimeric oncoprotein E2a-Hlf (Hunger et al., 1992) as a control. Plating of transduced cells under selective conditions showed similar numbers, size, and morphology of myeloid colonies after 7 days in primary methylcellulose cultures (Figure 5A and data not shown). To assess progenitor self-renewal, cells were harvested and serially replated for three subsequent rounds of methylcellulose culture. As expected, progenitors transduced with vector alone yielded no colonies due to exhaustion of their self-renewal potential. Wild-type cells transduced by either of the *E2A* fusion genes, however, gave rise to numerous colonies that displayed blast-like or CFU-GM morphology (Figure 5B). *Bmi-1*$^{-/-}$ progenitors transduced with E2a-Hlf also yielded substantial numbers of blast-like colonies in the third and fourth round cultures (Figure 5A). In contrast, Bmi1-deficient progenitors transduced with E2a-Pbx1 predominantly formed diffuse clusters of differentiated myeloid cells (Figures 5A and 5B). Only rare colonies were observed and these were typically smaller and surrounded by a "halo" of more differentiated myeloid cells indicating that the ability of E2a-Pbx1 to enhance progenitor self-renewal was substantially abrogated in the absence of Bmi-1. Wild-type cells transduced by either gene readily adapted to growth in liquid medium supplemented with interleukin 3 (IL-3), whereas only the Bmi-1-deficient cells expressing E2a-Hlf yielded similar immortalized cell lines. Therefore, under these in vitro conditions, Bmi-1 is necessary for immortalization of myeloid progenitors by E2a-Pbx1, but not by the related leukemia oncoprotein E2a-Hlf.

Western blot analysis showed that p16^{Ink4a} was reduced in wt progenitors expressing E2a-Pbx1 compared to those expressing E2a-Hlf (Figure 5C). p19^{Arf} expression was absent in wt progenitors expressing E2a-Pbx1 but present in *Bmi-1*$^{-/-}$ progenitors (Figure 5C), consistent with a Bmi-1 requirement for *INK4A-ARF* downregulation. By comparison, p19^{Arf} was expressed at high levels in both wt and *Bmi-1*$^{-/-}$ progenitors immortalized by E2a-Hlf, demonstrating that its oncogenic effects were independent of ARF levels. These data provide additional evidence that Bmi-1 plays a critical and biologically relevant role in linking E2a-Pbx1 with the *INK4A-ARF* locus.

Loss of *INK4A-ARF* Alleviates the Requirement for *Bmi-1* in Hematopoietic Transformation by E2a-Pbx1

We hypothesized that if the requirement for *Bmi-1* in E2a-Pbx1-associated transformation was primarily mediated through its repressive effects on the *INK4A-ARF* locus, it should be possible to bypass the need for *Bmi-1* by using myeloid progenitors that were deficient for both *Bmi-1* and *INK4A-ARF*. Indeed, previous studies have demonstrated that the in vitro and in vivo growth deficiencies associated with *Bmi-1* nullizygosity were rescued by inactivation of the *INK4A-ARF* locus (Jacobs et al., 1999a). To test our hypothesis, myeloid transformation assays were initiated with bone marrow progenitors harvested from donor mice that were compound deficient for both the *Bmi-1* and *INK4A-ARF* genes. Transduction of *Bmi-1*$^{-/-}$*INK4A-ARF*$^{-/-}$ progenitors with either E2a-Pbx1 or E2a-Hlf resulted in enhanced replating potentials as evidenced by numerous blast-type colonies through the fourth round of methylcellulose culture (Figures 5A and 5B) comparable to results obtained with wild-type cells transduced by either *E2A* gene. Colonies harvested from methylcellulose cultures were readily adapted to growth in liquid culture supplemented with IL-3, and could be passaged indefinitely under these conditions. Thus, the resistance of *Bmi-1*-deficient cells to transformation by E2a-Pbx1 was suppressed by the

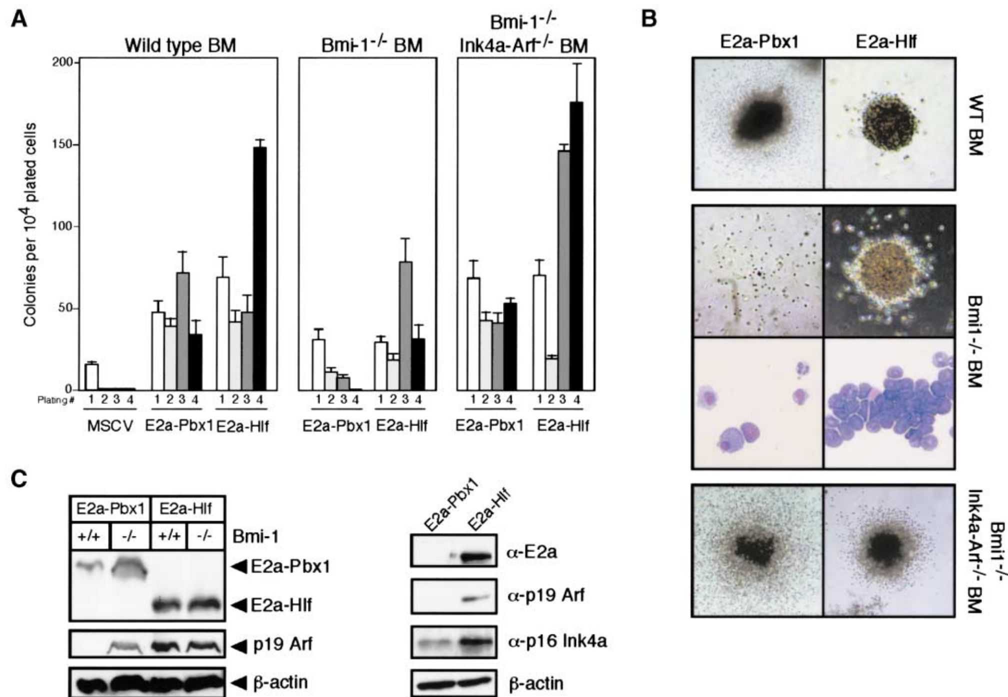

Figure 5. Bmi-1 Is Required for Immortalization of Hematopoietic Progenitors by E2a-Pbx1

(A) Bar graph indicates numbers of blast-like progenitor colonies generated in methylcellulose cultures of bone marrow cells transduced with E2a-Pbx1 or E2a-Hlf. Genotypes of donor progenitors used to initiate the assays are indicated at the tops of the panels. Data represent the mean plus standard deviations for three experiments performed in triplicate.

(B) Morphologies are shown for typical compact, blast-type colonies formed in methylcellulose by wt bone marrow cells transduced with E2a-Pbx1 or E2a-Hlf, respectively. Similar compact colonies were observed for *Bmi1*-deficient bone marrow cells transduced with E2a-Hlf. In contrast, *Bmi1*-deficient cells transduced with E2a-Pbx1 predominantly formed diffuse clusters of more differentiated myeloid cells compared to the predominantly undifferentiated cells in E2a-Hlf colonies. However, cells deficient for both *Bmi-1* and *INK4A-ARF* formed blast-type colonies when transduced by either *E2A* oncogene.

(C) Western blot analyses were performed on cells harvested from third passage methylcellulose cultures initiated with wild-type (+/+) or *Bmi-1*-deficient (−/−) hematopoietic progenitors. Identities of transfected genes are indicated at the top; primary antibodies are indicated on the right of each panel.

absence of *INK4A-ARF* gene products. These results strongly suggest that the requirement for *Bmi-1* in E2a-Pbx1-mediated transformation of hematopoietic progenitors is primarily mediated through Bmi-1-repressive effects on the *INK4A-ARF* locus.

Discussion

An Essential Oncogenic Pathway Links E2a-Pbx1 with *INK4A-ARF* in Human Leukemias

BMI-1 is normally expressed at elevated levels in early hematopoietic progenitors and downregulated with their progressive differentiation (Lessard et al., 1998). Our studies suggest that this programmed reduction may be abrogated in a subset of human leukemias that express E2a-Pbx1. These leukemias are arrested at the pre-B cell stage of differentiation and consistently express Bmi-1. In a pre-B cell line that does not express endogenous E2a-Pbx1, conditional expression of exogenous E2a-Pbx1 leads to increased levels of Bmi-1. Although Bmi-1 does not appear to be a direct target of E2a-Pbx1, our studies provide evidence that it is downstream of this chimeric transcriptional protein in human pre-B cell leukemias. This is surprising given that E2a-Pbx1 functions as a DNA binding partner for Hox proteins, which genetic studies implicate to be downstream of Bmi-1. One possibility is that E2a-Pbx1 triggers a feedback loop to increase Bmi-1 levels as a consequence of altered Hox/E2a-Pbx1 transcriptional activity.

The in vivo roles of Bmi-1 in murine tumorigenesis and hematopoietic development are critically dependent on its ability to repress the *INK4A-ARF* locus (Jacobs et al., 1999a, 1999b) that encodes tumor suppressor proteins with regulatory functions on the Rb and p53 pathways (Figure 3A). Consistent with this relationship, elevated levels of Bmi-1 in t(1;19) E2a-Pbx1-expressing human pre-B cells or forced expression of E2a-Pbx1 in murine hematopoietic progenitors is associated with reduced levels of endogenous $p16^{Ink4a}$ and $p14^{Arf}$ expression. Furthermore, transient expression of dominant-negative E2a-Pbx1 in pre-B cells results in elevated levels of $p16^{Ink4a}$, whereas forced expression of $p16^{Ink4a}$ abrogates some of the adverse effects of E2a-Pbx1 on pre-B cell survival and differentiation. These observations provide evidence of a pathway linking E2a-Pbx1 with the *INK4A-ARF* tumor suppressor locus in B cell progenitors.

Loss-of-function *INK4A* alterations are commonly observed in human lymphoid malignancies (Drexler, 1998); however, they are curiously absent in pre-B cell leuke-

mias that express E2a-Pbx1 (Ohnishi et al., 1996; Maloney et al., 1998). A pathway placing *INK4A-ARF* downstream of E2a-Pbx1 provides a molecular basis for this exception since it would reduce selection for genetic or epigenetic inactivation of *INK4A*. Taken together, these observations provide strong evidence that the observed relationship between *Bmi-1* and E2a-Pbx1 has significant consequences for pre-B cell growth and differentiation with relevance for leukemogenesis. Indeed, signaling through *Bmi-1* appears to be an important step in leukemogenesis initiated by E2a-Pbx1, since murine hematopoietic progenitors deficient for *Bmi-1* cannot be immortalized by E2a-Pbx1 under our in vitro culture conditions. The inability of *Bmi-1*-deficient cells to undergo E2a-Pbx1-mediated transformation, however, was rescued in progenitors that were compound deficient for both *Bmi-1* and *INK4A-ARF*, further confirming the existence of a Bmi-1-dependent pathway linking E2a-Pbx1 with the *INK4A-ARF* locus. Although E2a-Pbx1 is likely to perturb multiple transcriptional targets and growth-regulatory genes, our studies implicate this chimeric Hox DNA binding partner with disruptions in major tumor suppressor pathways by way of *Bmi-1* in hematopoietic progenitors with likely consequences for the pathology of t(1;19)-associated leukemias.

Experimental Procedures

Cell Lines
Cell lines that inducibly express E2a-Pbx1 (A2 and C8) or a homeodomain deletion mutant (ΔHD) under control of the sheep metallothionine promoter have been reported previously (Smith et al., 1997). Human fetal lung fibroblasts were kindly provided by I. Verma and were propagated by serial replating by splitting 1:4 (one passage = two PDLs) as soon as cultures became confluent.

Microarray
Cells were cultured at a density of 1×10^6 per ml in complete RPMI medium containing $ZnSO_4$ (100 μM). At the indicated time points, cells were harvested by low-speed centrifugation and mRNA was isolated using FastTrack reagents (Invitrogen) according to the manufacturer's protocol. Cy5-labeled cDNA was synthesized from mRNA isolated from A2 or other cells conditionally expressing Pbx1 constructs. Reference probes consisted of Cy3-labeled cDNAs prepared from mRNA isolated from MT1 cells treated identically to the test cells. Microarray hybridizations were conducted as described previously (DeRisi et al., 1997) using a 10k microarray (Iyer et al., 1999) or custom-printed 1k arrays. Fluorescence was measured by confocal laser scanning microscopy. The data were analyzed by cluster analysis (Eisen et al., 1998) to identify differences in gene expression resulting from E2a-Pbx1 induction.

Western Blot Analysis
Proteins isolated from whole-cell lysates were resolved through 10% or 14% SDS-PAGE gels as previously described (Smith et al., 1997). Primary antibodies were directed against E2a, Bmi-1 (Cohen et al., 1996), $p16^{Ink4a}$ (Neomarkers), $p14^{Arf}$ (Santa Cruz Biotech), p21 (Pharmingen), and Cdk2 (Neomarkers). Secondary antibodies consisted of horseradish preoxidase conjugated goat anti-rabbit or anti-mouse IgG (Accurate Antibodies) (1:5000 dilution). Immune complexes were detected by enhanced chemiluminescence (Amersham). Quantification of Western blot data was performed using NIH Image v1.62 (NIH) software.

Retroviral Transductions
For retroviral transductions of lymphoid cells with $p16^{ts}$ (Urashima et al., 1997) or E2a-$Pbx1b^{DN}$, the respective cDNAs were cloned into the polylinker site of LZRSpBMN-IRES-GFP (Kinsell and Nolan, 1996). Retroviral stocks were prepared by transient transfection of ecotropic packaging cell line Phoenix-E, amphotropic packaging cell line 293/ampho (provided by I. Verma), or pseudotyping with the VSVG envelope using Phoenix-GP cells (provided by G. Nolan) as previously described (Yee et al., 1994). The ecotropic retroviral receptor was introduced into RCH-ACV cells by amphotropic retroviral transduction. Lymphoid cells were transduced by spinoculation (1 hr at 1500 rpm), then diluted 1:2 in RPMI-PSG medium and cultured overnight at 37°C. Transduced cells were enriched by FACS sorting based on GFP expression. For retroviral transductions of myeloid progenitor cells, the cDNAs for E2a-Pbx1b and E2a-Hlf were cloned by blunt end ligation into the HpaI site of MSCV-puromycin.

Growth and Senescence Assays
Human embryonic lung fibroblasts were transduced with recombinant retroviruses at passage 5. Growth rates and expression of senescence-associated β-galactosidase were determined as described previously (Serrano et al., 1997).

Apoptosis Assays
The ability of $p16^{Ink4a}$ to prevent E2a-Pbx1-induced apoptosis was evaluated in A2 cells stably transduced with a temperature-sensitive p16 construct (Urashima et al., 1997) and compared with the survival of parent A2 cells and the MT1 control cell line. In brief, all cell cultures (3×10^5 cells/ml in RPMI containing 5% FBS) were incubated at 30°C ($p16^{ts}$ permissive temperature) for 24 hr to activate p16. $ZnSO_4$ was added (final concentration of 100 μM) and incubation was continued at 37°C (seminonpermissive) for 8 hr to allow for inducible expression of E2A-Pbx1. Cultures were placed back at 30°C (permissive temperature) for 18 hr and then processed for determination of cell surface annexin-V expression and PI content by flow cytometry (FACStar).

Flow Cytometric Analysis
To evaluate the effects of p16 on surface antigen expression, transduced cells (RCH-ACV) were seeded at 5×10^5 cells/ml and cultured at 30°C for the indicated number of days. Washed cells were then incubated with anti-CD19 (Pharmingen) and propidium iodide (2 μg/ml) and analyzed by FACS (FACstar). Data were analyzed by gating on FITC-positive (GFP) and PI-negative cells.

Hematopoietic Progenitor Immortalization Assays
Transduction of murine bone marrow cells was performed essentially as described previously (Lavau et al., 1997) with minor modifications. Bone marrow cells were harvested from femurs of wt, *Bmi1*-deficient, or *Bmi-1/INK4A-ARF*-deficient 4- to 6-week-old C57BL/6 mice. Progenitors were enriched by pretreatment of donor mice with 5-fluorouracil or purified by immunomagnetic absorption for c-Kit expression using an autoMACS separation machine (Miltenyi Biotec Inc., Auburn, CA). Retroviral supernatants were produced by transient transfection of ϕNX packaging cells with MSCV constructs (Pear et al., 1993). BM cells were infected with recombinant virus by spinoculation at 2500 × g for 2 hr at 32°C. Transduced cells were plated in 0.9% methylcellulose (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 20 ng/ml SCF and 10 ng/ml each of IL-3, IL-6, and GM-CSF (R&D Systems, Minneapolis, MN), and 1 mg/ml G418 (or 1 μg/ml puromycin). After 7–10 days, colonies were counted, pooled, and single-cell suspensions of 10^4 cells were replated in methylcellulose cultures without chemical selection. For liquid cultures, cells were maintained in RPMI 1640 (GIBCO-BRL, Grand Island, NY) supplemented with L-glutamine, penicillin/streptomycin, 20% FCS, and 20% WEHI-conditioned medium.

Transient Transfection Assays
Bmi-1 reporter gene constructs contained the mouse (5 kb HindIII-NsiI fragment containing exon 1 in pGL-3-basic) or human (1.5 kb fragment in pTAL) Bmi-1 promoters. Cos-7, Phoenix, 293, or U2-OS cells were cotransfected with reporter and expression (pCMV-E2a/Pbx1b) constructs using standard conditions. 24 or 48 hr after transfection, luciferase and β-galactosidase activities were analyzed using commercially prepared reagents. Luciferase activities were normalized based on β-galactosidase levels.

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References

Alcorta, D.A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., and Barrett, J.C. (1996). Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Proc. Natl. Acad. Sci. USA *93*, 13742–13747.

Asahara, H., Dutta, S., Kao, H.Y., Evans, R.M., and Montminy, M. (1999). Pbx-Hox heterodimers recruit coactivator-corepressor complexes in an isoform-specific manner. Mol. Cell. Biol. *19*, 8219–8225.

Carnero, A., Hudson, J.D., Price, C.M., and Beach, D.H. (2000). p16INK4A and p19ARF act in overlapping pathways in cellular immortalization. Nat. Cell Biol. *2*, 148–155.

Chang, C.-P., Shen, W.-F., Rozenfeld, S., Lawrence, H.J., Largman, C., and Cleary, M.L. (1995). Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. Genes Dev. *9*, 663–674.

Chin, L., Pomerantz, J., and DePinho, R.A. (1998). The INK4a/ARF tumor suppressor: one gene-two products-two pathways. Trends Biochem. Sci. *8*, 291–296.

Cohen, K.J., Hanna, J.S., Prescott, J.E., and Dang, C.V. (1996). Transformation by the Bmi-1 oncoprotein correlates with its subnuclear localization but not its transcriptional suppression activity. Mol. Cell. Biol. *16*, 5527–5535.

Dedera, D.A., Waller, E.K., LeBrun, D.P., Sen-Majumdar, A., Stevens, M.E., Barsh, G.S., and Cleary, M.L. (1993). Chimeric homeobox gene E2A–PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. Cell *74*, 833–843.

DeRisi, J.L., Iyer, V.R., and Brown, P.O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. Science *278*, 680–686.

DiMartino, J.F., Selleri, L., Traver, D., Firpo, M.T., Rhee, J., Warnke, R., O'Gorman, S., Weissman, I.L., and Cleary, M.L. (2001). The Hox cofactor and proto-oncogene Pbx1 is required for maintenance of definitive hematopoiesis in the fetal liver. Blood *98*, 618–626.

Drexler, H.G. (1998). Review of alterations of the cyclin-dependent kinase inhibitor INK4 family genes p15, p16, p18 and p19 in human leukemia-lymphoma cells. Leukemia *12*, 845–859.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA *95*, 14863–14868.

Fu, X., and Kamps, M.P. (1997). E2a-Pbx1 induces aberrant expression of tissue-specific and developmentally regulated genes when expressed in NIH 3T3 fibroblasts. Mol. Cell. Biol. *17*, 1503–1512.

Haber, D.A. (1997). Splicing into senescence: the curious case of p16 and p19ARF. Cell *91*, 555–558.

Haupt, Y., Alexander, W.S., Barri, G., Klinken, S.P., and Adams, J.M. (1991). Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in Eμ-myc transgenic mice. Cell *65*, 753–763.

Hunger, S.P., Ohyashiki, K., Toyama, K., and Cleary, M.L. (1992). Hlf, a novel hepatic bZIP protein, shows altered DNA binding properties following fusion to E2A in t(17;19) acute lymphoblastic leukemia. Genes Dev. *6*, 1608–1620.

Iyer, V.R., Eisen, M.B., Ross, D.T., Schuler, G., Moore, T., Lee, J.C.F., Trent, J.M., Staudt, L.M., Hudson, J., Jr., Boguski, M.S., et al. (1999). The transcriptional program in the response of human fibroblasts to serum. Science *283*, 83–87.

Jacobs, J.J.L., Kieboom, K., Marino, S., DePinho, R.A., and van Lohuizen, M. (1999a). The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature *397*, 164–168.

Jacobs, J.J.L., Scheijen, B., Voncken, J.-W., Kieboom, K., Berns, A., and van Lohuizen, J. (1999b). Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. Genes Dev. *13*, 2678–2690.

Kamps, M.P., and Wright, D.D. (1994). Oncoprotein E2A-Pbx1 immortalizes a myeloid progenitor in primary marrow cultures without abrogating its factor-dependence. Oncogene *11*, 3159–3166.

Kamps, M.P., Murre, C.M., Sun, X.-H., and Baltimore, D. (1990). A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. Cell *60*, 547–555.

Kim, S.K., Selleri, L., Lee, J.S., Zhang, A.Y., Gu, X., Jacobs, Y., and Cleary, M.L. (2002). *Pbx1* inactivation disrupts pancreas development and in *Ipf1*-deficient mice promotes diabetes mellitus. Nat. Genet. *30*, 430–435.

Kinsell, T.M., and Nolan, G.P. (1996). Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. Hum. Gene Ther. *7*, 1405–1413.

Lavau, C., Szilvassy, S.J., Slany, R., and Cleary, M.L. (1997). Immortalization and leukemic transformation of a myelomonocytic precursor by retrovirally transduced HRX-ENL. EMBO J. *16*, 4226–4237.

LeBrun, D.P., and Cleary, M.L. (1994). Fusion with E2A alters the transcriptional properties of the homeodomain protein PBX1 in t(1;19) leukemias. Oncogene *9*, 1641–1647.

Lessard, J., Baban, S., and Sauvageau, G. (1998). Stage-specific expression of Polycomb Group genes in human bone marrow cells. Blood *91*, 1216–1224.

Lu, Q., Wright, D.D., and Kamps, M.P. (1994). Fusion with E2A converts the Pbx1 homeodomain protein into a constitutive transcriptional activator in human leukemias carrying the t(1;19) translocation. Mol. Cell. Biol. *14*, 3938–3948.

Maloney, K.W., McGavran, L., Odom, L.F., and Hunger, S.P. (1998). Different patterns of homozygous p16^{INK4A} and p15^{INK4B} deletions in childhood acute lymphoblastic leukemias containing distinct E2A translocations. Leukemia *12*, 1417–1421.

Mann, R., and Affolter, M. (1998). Hox proteins meet more partners. Curr. Opin. Genet. Dev. *8*, 423–429.

Mann, R.S., and Chan, S.-K. (1996). Extra specificity from extradenticle: the partnership between HOX and PBX/EXD homeodomain proteins. Trends Genet. *12*, 258–262.

McWhirter, J.R., Goulding, M., Weiner, J.A., Chun, J., and Murre, C. (1997). A novel fibroblast growth factor gene expressed in the developing nervous system is a downstream target of the chimeric homeodomain oncoprotein E2A-Pbx1. Development *124*, 3221–3232.

McWhirter, J.R., Neuteboom, S.T., Wancewicz, E.V., Monia, B.P., Downing, J.R., and Murre, C. (1999). Oncogenic homeodomain transcription factor E2A-Pbx1 activates a novel WNT gene in pre-B acute lymphoblastoid leukemia. Proc. Natl. Acad. Sci. USA *96*, 11464–11469.

Monica, K., LeBrun, D.P., Dedera, D.A., Brown, R., and Cleary, M.L. (1994). Transformation properties of the E2a-Pbx1 chimeric oncoprotein: fusion with E2a is essential but the Pbx1 homeodomain is dispensible. Mol. Cell. Biol. *14*, 8304–8313.

Noble, J.R., Rogan, E.M., Neumann, A.A., Maclean, K., Bryan, T.M., and Reddel, R.R. (1996). Association of extended in vitro proliferative potential with loss of p16INK4 expression. Oncogene *13*, 1259–1268.

Nourse, J., Mellentin, J.D., Galili, N., Wilkinson, J., Stanbridge, E.,

Smith, S.D., and Cleary, M.L. (1990). Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell *60*, 535–546.

Ohnishi, H., Hanada, R., Horibe, K., Hongo, T., Kawamura, M., Naritaka, S., Bessho, F., Yanagisawa, M., Nobori, T., Yamamori, S., et al. (1996). Homozygous deletions of p16/MTS1 and p15/MTS2 genes are frequent in t(1;19)-negative but not in t(1;19)-positive B precursor acute lymphoblastic leukemia in childhood. Leukemia *10*, 1104–1110.

Pear, W.S., Nolan, G.P., Scott, M.L., and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA *90*, 8392–8396.

Quelle, D.E., Zindy, F., Ashmun, R.A., and Sherr, C.J. (1995). Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell *83*, 993–1000.

Quong, M.W., Massari, M.E., Zwart, R., and Murre, C. (1993). A new transcriptional-activation motif restricted to a class of helix-loop-helix proteins is functionally conserved in both yeast and mammalian cells. Mol. Cell. Biol. *13*, 792–800.

Selleri, L., Depew, M.J., Jacobs, Y., Chanda, S.K., Tsang, K.Y., Cheah, K.S.E., Rubenstein, J.L.R., O'Gorman, S., and Cleary, M.L. (2001). Requirement for *Pbx1* in skeletal patterning and programming chondrocyte proliferation and differentiation. Development *128*, 3543–3557.

Serrano, M., Lee, H.-W., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R.A. (1996). Role or the INK4a locus in tumor suppression and cell mortality. Cell *85*, 27–36.

Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R.A. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593–602.

Sherr, C.J. (1998). Tumor surveillance via the ARF-p53 pathway. Genes Dev. *12*, 2984–2991.

Smith, K.S., Jacobs, Y., Chang, C.-P., and Cleary, M.L. (1997). Chimeric oncoprotein E2a-Pbx1 induces apoptosis of hematopoietic cells by a p53-independent mechanism that is suppressed by Bcl-2. Oncogene *14*, 2917–2926.

Stamenkovic, I., and Seed, B. (1988). CD19, the earliest differentiation antigen of the Bcell lineage, bears three extracellular immunoglobulin-like domains and an Epstein-Barr virus-related cytoplasmic tail. J. Exp. Med. *168*, 1205–1210.

Urashima, M., DeCaprio, J.A., Chauhan, D., Teoh, G., Ogata, A., Treon, S.P., Hoshi, Y., and Anderson, K.C. (1997). $p16^{INK4A}$ promotes differentiation and inhibits apoptosis of JKB acute lymphoblastic leukemia cells. Blood *90*, 4106–4115.

Van Dijk, M.A., Voorhoeve, P.M., and Murre, C. (1993). Pbx1 is converted into a transcriptional activator upon acquiring the N-terminal region of E2A in pre-B-cell acute lymphoblastoid leukemia. Proc. Natl. Acad. Sci. USA *90*, 6061–6065.

van Lohuizen, M.S., Verbeek, S., Schiejen, B., Wientjens, E., van der Gulden, H., and Berns, A. (1991a). Identification of cooperating oncogenes in Eμ-myc transgenic mice by provirus tagging. Cell *65*, 737–752.

van Lohuizen, M., Frasch, M., Wientjens, E., and Berns, A. (1991b). Sequence similarity between the mammalian Bmi-1 proto-oncogene and the Drosophila regulatory genes Psc and Su(z)2. Nature *353*, 353–355.

Wang, J., and Walsh, K. (1996). Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation. Science *273*, 359–363.

Yee, J.K., Friedmann, T., and Burns, J.C. (1994). Generation of high-titer pseudotyped retroviral vectors with very broad host range. Methods Cell Biol. *43*, 99–112.